Construction of Recombinants Between Molecular Clones of Murine Retrovirus MCF 247 and Akv: Determinant of an In Vitro Host Range Property That Maps in the Long Terminal Repeat

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The leukemogenic mink cell focus-forming (MCF) retroviruses such as MCF 247 have biological properties distinct from those of their ecotropic progenitors. Nucleotide sequences encoding portions of gp70, Prp15E, and the long terminal repeat differ between the two types of viruses. To investigate the role of each of these genetic elements in determining the biological properties of MCF viruses, we prepared infectious molecular clones of MCF 247 and generated a set of recombinants between these clones and a molecular clone of Akv, the ecotropic parent of MCF 247. Each molecular clone of MCF 247 was distinct. All the recombinants between Akv and MCF 247 yielded infectious virus upon transfection. Most interestingly, recombinants which contain the long terminal repeat of MCF 247 were found to have an in vitro host range property that has been correlated with high oncogenic activity and thymotropism of certain MCF isolates; namely, they plated with higher efficiency on SC-1 cells than on NFS mouse embryo cells. Nononcogenic MCF isolates showed a slight preference for NFS cells, whereas Akv virus plated with approximately equal efficiency on the two cell types.

Mink cell focus-forming (MCF) virus MCF 247 is the prototype of a class of recombinant C-type viruses isolated from thymic neoplasms of inbred mice (5, 8, 11, 13, 18, 29, 30). The biological characteristics of MCF 247, which was isolated from an AKR mouse, are distinct from those of its ecotropic progenitor, Akv. MCF 247 is dual tropic, leukemogenic, thymotropic, does not form XC plaques (XC⁻) (35), and induces foci on mink cells (MCF⁺). In contrast, Akv is ecotropic, nonleukemogenic, XC⁺, and MCF⁻ (5, 8, 18, 29, 34). In addition, a host range property distinguishes the two viruses. It has been noted in studies of the tissue culture growth of MCF viruses from different sources that the class I, oncogenic, thymus-derived, thymotropic isolates from AKR, such as MCF 247, plate more efficiently in SC-1 cells than in NFS mouse embryo cells, SC-1 cells being about 10-fold more sensitive (34). In contrast, nonleukemogenic class II MCF viruses derived from nonthymic lymphomas plate approximately equally well on these cell types or with slightly greater efficiency in NFS cells (34). Although in general SC-1 cells are somewhat more sensitive than NFS cells to infection by freshly isolated N-tropic, ecotropic viruses, the progenitor of the molecularly cloned Akv used in this study was tissue culture adapted, and thus the efficiency of plating in SC-1 versus NFS cells is more nearly equal (17).

Nucleotide sequences encoding at least three viral genetic elements have been shown to distinguish leukemogenic MCF viruses and their ecotropic progenitors (7, 20, 23, 26, 27, 29, 30). These include sequences encoding the amino terminus of the viral envelope glycoprotein (gp70), the carboxy terminus of Prp15E (precursor of virion p15E which anchors gp70 to the virus surface), and the U3 region of the

long terminal repeat (LTR). We were interested in determining the contribution of these three genetic segments to the distinctive biological properties of MCF viruses. To approach this problem, we have molecularly cloned the prototype MCF virus, MCF 247, and have constructed recombinants in vitro between DNA clones of MCF 247 and Akv. Restriction endonuclease sites common to the cloned viruses (5, 6, 28) allowed us to separate the distinctive regions of the three genetic elements, gp70, Prp15E, and the LTR, and to rejoin them in different combinations. Here, we describe biochemical and biological properties of three infectious molecular clones of MCF 247 and report that all the cloned DNAs of recombinants between MCF 247 and Akv were biologically functional upon transfection. As expected, the ability of MCF 247 to form foci on mink cells and the ability of Akv to form XC plaques were found to segregate with gp70 coding sequences. Unexpectedly, another tissue culture host range property, the plating efficiency on SC-1 versus NFS cells, did not segregate with gp70 but rather with LTR sequences.

MATERIALS AND METHODS

Molecular cloning of MCF 247. A Hirt extract (21) of *Mus dunii* cells infected with MCF 247 virus was prepared by S. Chattopadhyay as described previously (32). MCF 247 viral DNA obtained from the Hirt extract was digested with *Hind*III or *Pst*I, phenol extracted, ethanol precipitated, and suspended at a concentration of 1 μg/μl. The DNA was cloned with either a plasmid vector (pBR322) as described previously (25) or bacteriophage λ vector NM 788. A total of 25 molecular clones which contained sequences homologous to MCF 247 were identified. Of these, 15 had an insert of ca. 8 to 9 kilobases (kb). The inserts were cleaved with *Kpn*I, *Sma*I, *Bam*HI, and *Pvu*II. Nine of the fifteen clones had restriction enzymes sites consistent with those mapped (5, 6) for DNA intermediates of MCF 247 virus. Inserts from these nine clones were isolated, ligated, and transfected (14) into
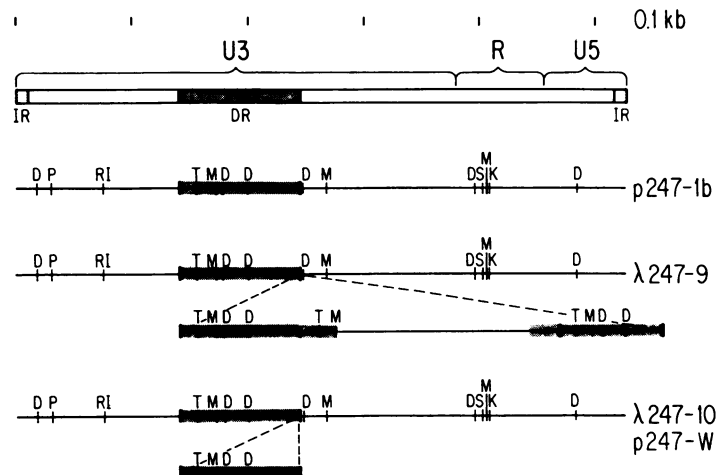
FIG. 1. Restriction endonuclease map of the LTRs of four molecular clones of MCF 247. The top line represents a retroviral LTR with one copy of the sequence that is often present as a direct repeat (DR). The direct repeat region is shaded. IR, Inverted repeat sequence. The lines below are the restriction endonuclease maps of the LTRs of three other molecular clones of MCF 247 where cleavage sites of DdeI (D), PstI (P), EcoRI (RI), TaqI (T), MspI (M), SmaI (S), and KpnI (K) are shown. Extra copies of the direct repeats are shown as insertions flanked by dotted lines.

MCT or SC-1 cells (17). Three of them, λ247-9, λ247-10, and p247-W, were infectious as monitored by reverse transcriptase activity in supernatant fluids of the transfected culture. The DNA inserts of λ247-9 and λ247-10 were cloned into plasmid pBR322. The subclones, designated pλ247-10 and pλ247-9, retained biological activity in a transfection assay.

Preparation of restriction fragments and molecular cloning of site-specific recombinants. From 50 to 500 μg of each of pAKV-623 (28), p247-W, λ247-9, and pλ247-10 DNA was cleaved with restriction endonucleases HindIII, XhoI, XbaI, or PstI and electrophoresed on 0.5, 1.0, or 2.0% agarose gels. The fragments were electroeluted from the gel and purified by DEAE-Sephacel chromatography and phenol extraction. After ethanol precipitation, each fragment was suspended at a concentration of 0.1 to 0.5 μg/μl. The identity of the fragments was confirmed by digestion with one or more restriction enzymes. To be sure that fragments could be ligated, each was ligated to the fragments that eventually would flank it in the recombinant clones and visualized on an analytical gel.

Recombinants (see Fig. 3) were cloned using HindIII bacteriophage λ arms (recombinants 10, 5, 12, and 1), PstI-digested pBR322 (recombinants 7, 8, 11) or HindIII-digested pBR322 (recombinant 16) as vectors. Equimolar quantities of ends of the vector and each fragment necessary to compose the recombinant were ligated. The ligated molecules were packaged in vitro or used to transform bacterial

strains RR1 (9) or DH1 (16). Plaques or bacterial colonies which contained recombinant molecules were identified by screening with a ^{32}P-labeled, nick-translated, internal fragment of the recombinant as a probe (15).

DNA from molecular clones of each recombinant which contained an insert of the correct length was further analyzed by digestion with restriction endonucleases XhoI, XbaI, PstI, KpnI, and EcoRI. MCF 247 contains an EcoRI site in gp70 and in the LTR, whereas AKV does not (6). Digestion of the DNAs with EcoRI was used as a diagnostic to confirm the identity of the gp70 and LTR fragments.

Transfection. Infectivity of the molecular clones was tested by transfection of DNA onto NIH 3T3, MCT, or SC-1 (17) cells by a slight modification of the calcium phosphate precipitation technique described previously (14). After transfection, cells were passaged either 1:5 or 1:10 for 10 to 30 days. After every other passage, the cells were treated with Polybrene (8 μg/ml) for 24 h. Each culture was assayed for reverse transcriptase activity in a 24-h harvest (1).

RNase T$_1$ fingerprints. Preparation of ^{32}P-labeled viral RNA, its digestion with RNase T$_1$, and separation of the resulting oligonucleotides by two-dimensional gel electrophoresis was performed as described previously (12).

Viruses and virus assays. Viruses recovered as a result of transfection procedures were propagated in SC-1 mouse cells (17). The tissue culture host range phenotype of each recombinant virus was determined by titrations in NFS

TABLE 1. Biological properties of clones

Virus	Tissue culture assays			Leukemogenicity assays[a]		
	XC	MCF	SC-1/NFS[b]	No. of mice (dead/injected)	% Incidence	Mean latent period
MCF 247	−	+	1.1 ± .12	29/29	100	96 ± 25
p247 - W	−	+	1.3	7/8	88	90 ± 18
λ247 - 9	−	+	1.4 ± 1.2	10/12	83	105 ± 22
λ247 - 10	−	+	1.2 ± .35	13/13	100	108 ± 36
pAKV - 623	+	−	0.2 ± .1	1/17	5.8	145

[a] AKR/N or AKR/J mice (0 to 2 days old) were injected in the region of the thymus with 0.02 ml of undiluted tissue culture virus. Latent periods are expressed in days as the mean ± the standard error of the mean. Tumors were almost exclusively thymic lymphomas. Mice were observed for 180 days.

[b] Difference in titer (log$_{10}$ PFU [XC] or FFU [MCF]) obtained in SC-1 cells and NFS mouse embryo cells; mean ± the standard error of the mean.
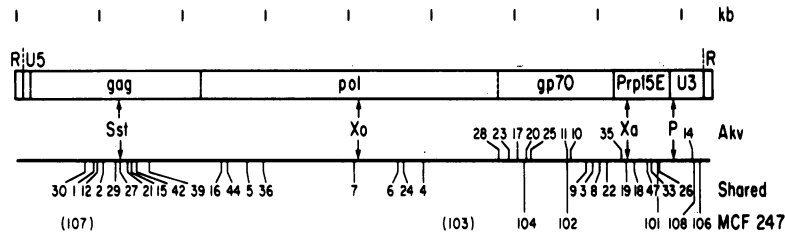
FIG. 2. Schematic representation of the genomic location of large RNase T_1-resistant oligonucleotides of Akv and MCF 247. The first line represents the viral RNA of MCF 247 or Akv. The genes were located by nucleotide sequence studies (15, 17, 21, 22). Placement of restriction sites for SstII (Sst), XhoI (Xo), XbaI (Xa), PstI (P), and the location of the RNase T_1 oligonucleotides unique to Akv or MCF 247 or shared between them was determined by examining the DNA sequences (20, 24, 26, 27) and by T_1 oligonucleotide maps (29, 30, 33). The placement of MCF 247 oligonucleotides 107 and 103 is based on RNase T_1 fingerprints of the recombinants constructed in this study.

embryo SC-1 cells or both and mink lung (ATCC CCL 64) cells. XC plaque assays for ecotropic viruses were performed as previously described (35). MCF viruses were assayed by observation of cytopathic foci in mink lung cells; infection of mouse cells was determined by the UV-mink procedure (19).

RESULTS

Biochemical and biological heterogeneity of molecular clones of MCF 247. We molecularly cloned MCF 247 from circular viral DNA intermediates present in a Hirt supernatant 18 h after virus infection of M. dunii cells (32). A majority of clones (16 of 25) had deletions, insertions, or other changes detectable by restriction enzyme mapping. Among nine clones without obvious alterations, only three yielded infectious virus after transfection.

The three infectious clones, λ247-9, pλ247-10, and p247-W, were further characterized by detailed restriction endonuclease analysis, and the viruses they yielded upon transfection were characterized biologically. Restriction enzyme analysis revealed that the LTRs of the three clones differ, whereas their gag, pol, and env regions were indistinguishable. Figure 1 shows a restriction map of the LTRs of λ247-9, λ247-10, and p247-W and also of a fourth molecular clone p247-1b. Clone p247-1b does not yield infectious virus upon transfection, but its LTR was previously shown to be biologically functional since it can substitute for the LTR of an infectious clone of Akv virus (26). Since the LTR of p247-1b has been sequenced (26), it was used as the reference (Fig. 1). The restriction map shows that the major difference in the LTRs of the infectious clones is in the region of the direct repeat. The LTR of p247-1b has one copy of a sequence 105 nucleotides long which becomes a direct repeat in λ247-10 and p247-W. λ247-9 has three complete copies of this sequence and a portion of a fourth, but in addition, the direct repeat sequences are interrupted by a sequence 175 nucleotides long which does not contain the restriction sites characteristic of the direct repeat. Restriction enzyme analysis, nucleotide sequencing and Southern blot analysis indicate that this 175-nucleotide-long sequence is not homologous to other sequences in the viral genome but is homologous to sequences present in AKR genomic DNA. These sequences are lost during replication of virus obtained from clone 247-9 after transfection (Y. Li and C. A. Holland, unpublished data).

We analyzed the biological properties of the viruses produced after transfection of λ247-9, pλ247-10, and p247-W in tests which distinguish MCF 247 from Akv virus (Table 1). The viruses produced after transfection of all three cloned DNAs resembled MCF 247. One biological property was unique to pλ247-10. Virus produced by transfection of this

clone consistently induced in mink lung cells focal alterations which were less sharply defined than the foci induced by λ247-9, p247-W or MCF 247 viruses.

Since the biochemical and biological studies described above indicate that different molecular clones of MCF 247 are distinct, we have used all three clones and, in some cases, the 3' end of p247-1b as parental molecules in constructing site-specific recombinants.

Construction, molecular cloning, and preliminary biological characterization of site-specific recombinants. Four restriction endonuclease sites, SstII, XhoI, XbaI, and PstI shared by Akv and MCF 247 viral DNAs (5, 6, 23, 26) allowed us to separate fragments that encode the portions of gp70, Prp15E, and most of the region of the LTR (U3) known to distinguish the genomes of the two viruses (26, 29, 30) (Fig. 2 and 3). It must be noted that these fragments carry additional sequences, and differences between Akv and MCF 247 are found outside the regions of gp70, Prp15E, and U3. For
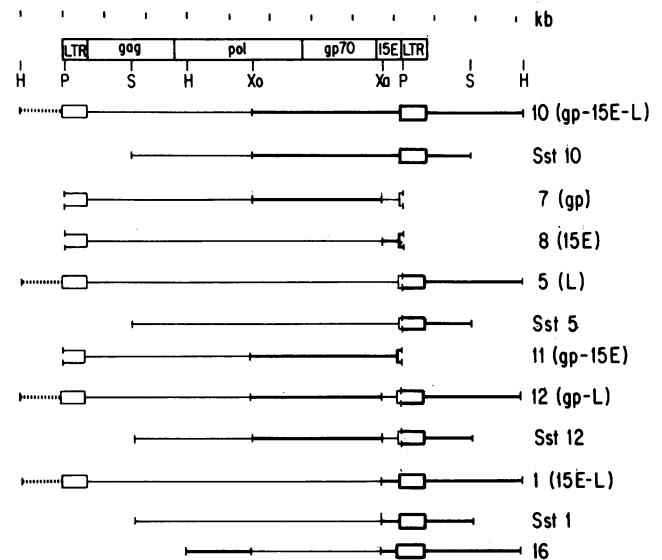

FIG. 3. Structure of recombinant virus genomes. Restriction endonuclease sites for HindIII (H), PstI (P), SstII (S), XhoI (Xo), and XbaI (Xa) used to construct recombinants are shown relative to the genome of a murine retrovirus except for the one HindIII site to the left of the genome which indicates a site in AKR cellular DNA. The portion of the recombinant derived from Akv (thin line) or MCF 247 (heavy line) is shown. The dashed lines are AKR genomic sequences present in the clone, pAKV 623. The recombinant numbers and the genes from MCF 247 which they contain (gp70 [gp]; Prp15E [15E]; LTR [L]) are shown to the right.

example, the 3.2-kb $XhoI$-to-$XbaI$ fragment contains the gp70 gene, but it also contains 1.4 kb of sequences that code for the carboxy-terminal portion of pol. We sequenced 0.2 kb at the 3' end of the MCF 247 pol gene and found ca. 85% homology with Akv (C. A. Holland, J. Wozney, and N. Hopkins, unpublished data). Similarly, the fragment used to exchange the LTR sequences contains a portion of the gag gene, and MCF 247 has a large T_1-resistant oligonucleotide (number 107) in this region that is not present in Akv (33).

One would expect, and RNase T_1 fingerprints confirm in most cases (see Fig. 4), that recombinants 10, 12, 1, and 5 in Fig. 3 would derive their gag genes from Akv and their U3 regions from MCF 247. However, to generate recombinants

with only one gag and one U3 gene, we digested recombinants 10, 12, 5, and 1 with $SstII$ and isolated the 8.6-kb genome. As expected, these recombinants yielded viruses whose genomes harbored the MCF-specific T_1 oligonucleotide 107 (data not shown).

Using each of the three infectious molecular clones of MCF 247, we prepared two independent isolates of each of the recombinants shown in Fig. 3. All of the recombinants produced infectious viruses when the cloned viral DNAs were transfected onto NIH 3T3 or SC-1 cells. The genomic structures of viruses obtained by transfection of site-specific recombinants were verified by RNase T_1 fingerprinting. With one exception, the fingerprints of all recombinants (Fig. 4)
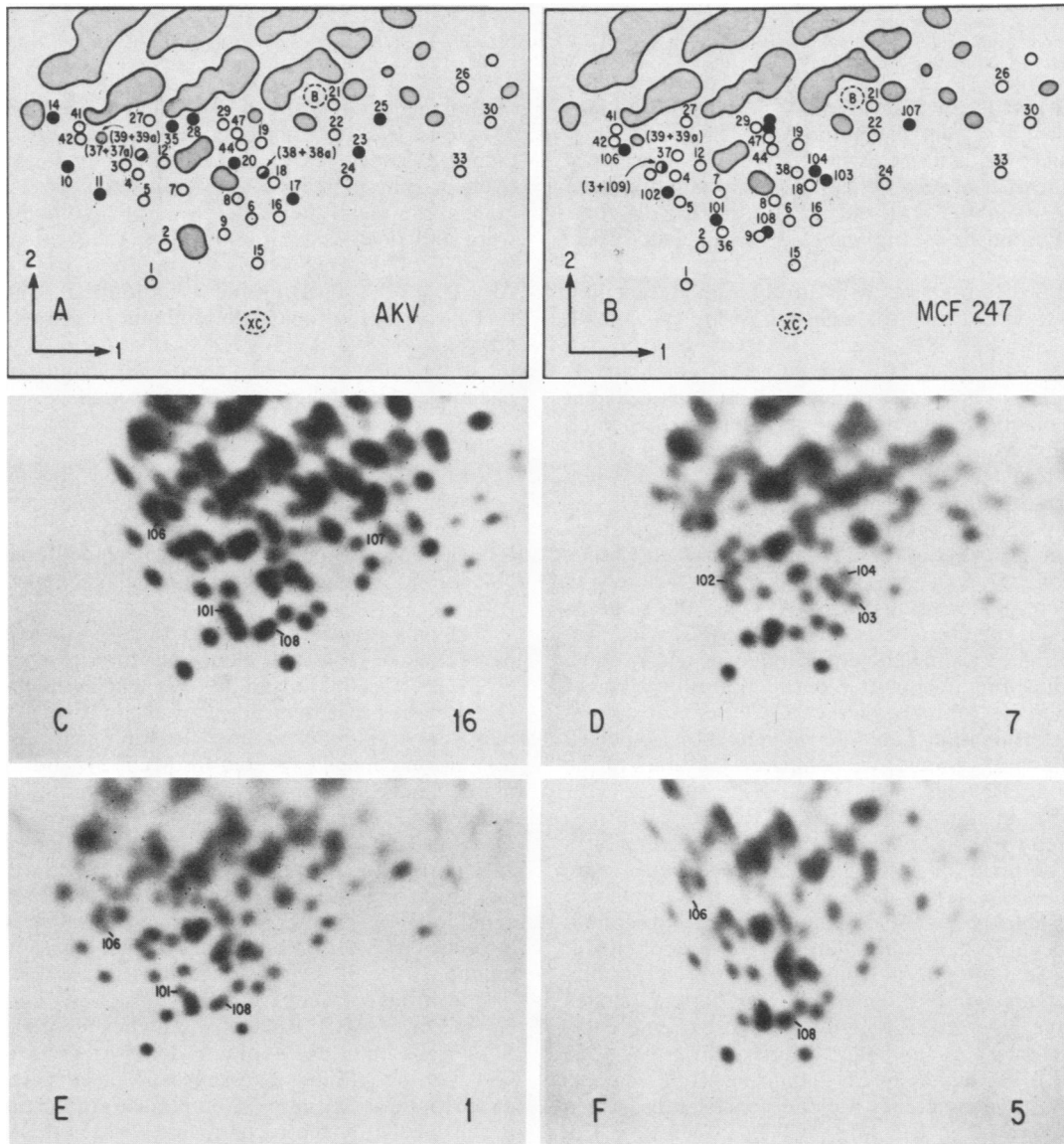

FIG. 4. RNase T_1 fingerprints of viruses obtained by transfection of recombinants. (A) and (B) are schematic diagrams of T_1 fingerprints of Akv and MCF 247, respectively (29, 30, 33). Oligonucleotides that are unique to each virus are shown as closed circles (●), whereas those that are shared are shown as open circles (○). The direction of electrophoresis and the locations of the bromphenol blue (B) and xylenecyanol FF (XC) dyes are shown. (C), (D), (E), and (F) show fingerprints of the viruses obtained after transfection of recombinants 16, 7, 1, and 5. MCF-specific oligonucleotides are numbered. Fingerprints of recombinants 1 and 5 (E and F) contain oligonucleotides 108 and 106 which are in the U3 portion of the MCF 247 LTR and lack oligonucleotide 14 in the Akv LTR. The $SstII$ inserts of recombinants 1 and 5 yielded viruses after transfection whose fingerprints were identical to those shown in (E) and (F) except that they contained MCF oligonucleotide 107 located in the gag gene.

TABLE 2. Relative efficiency of infection of SC-1 and NFS embryo cell cultures by Akv-MCF247 recombinant viruses.

Recombinant	Genome[a]				No. of tests	No. of clones tested	SC-1/NFS (mean ± SEM)[b]
	gag-pol	gp70	Prp15E	LTR			
10		+	+	+	4	4	1.1 ± 0.31[c]
16	+		+	+	10	6	1.4 ± 0.35
12		+		+	4	3	1.1 ± 0.10
1			+	+	14	7	0.9 ± 0.37
5				+	15	7	0.9 ± 0.48
11		+	+		6	4	0.32 ± 0.24
7		+			6	6	0.17 ± 0.13
8			+		5	4	0.12 ± 0.11

[a] +, Portion of genome derived from MCF 247.

[b] SC-1/NFS is the difference in titer ($\log_{10}$ PFU [XC] or focus-forming units [MCF]) obtained in SC-1 cells and in NFS mouse embryo cells.

[c] Comparisons of the mean titers for recombinants 10, 1, 5, and 11 by Student's t test modified for small samples, gave the following values: recombinant 10 versus 1, $P > 0.1$; recombinant 10 versus 5, $P > 0.1$; recombinant 10 versus 11, $P < 0.01$ and $P > 0.002$; recombinant 1 versus 11, $P < 0.01$ and $P > 0.002$; and recombinant 5 versus 11, $P < 0.02$ and $P > 0.01$.

contained the MCF- or Akv-specific T_1 oligonucleotides predicted from Fig. 2. The exception was a clone of recombinant 10 which yielded virus whose RNase T_1 fingerprint was identical to that of recombinant 11 viruses: it appeared to have the env of MCF 247, but a U3 derived from Akv. This aberrant clone of recombinant 10 was excluded from further studies.

As expected, recombinants with an MCF gp70 (recombinants 10, 7, 11, and 12) had the ability to form foci on mink cells but did not make XC plaques, whereas the recombinants which contained an Akv-derived gp70 (recombinants 8, 5, and 16) did not grow on or make foci on mink cells but did make XC plaques. When titers were determined on mink cells, all recombinants deriving gp70 sequences from pλ247-10 produced foci that had an aberrant morphology when compared with those produced by λ247-9 or p247-W.

Determinant of host range maps in the LTR. Viruses produced after transfection of any of the three infectious clones of MCF 247 consistently plated more efficiently in SC-1 cells than in NFS mouse embryo cells, the same as other oncogenic, thymotropic isolates from AKR mice (Table 1). In contrast, the efficiency of plating was nearly equal for virus produced by transfection of the molecular clone of Akv (Table 1). To determine whether a role in tissue culture phenotype determination could be assigned to a specific segment of the viral genome, we compared the titers of the recombinant virus clones in SC-1 and NFS embryo cells. Only those recombinants containing LTR sequences from MCF 247 plated with high efficiency on SC-1 relative to NFS cells, and those recombinants whose LTR sequences were derived from Akv plated with an efficiency very similar to that of Akv (Table 2). It should be noted that with recombinant viruses 1 and 5, less consistent results were obtained. Although repeated tests of individual clones were reproducible within about twofold, independently derived clones frequently gave variant results. In the most extreme case, titers of one clone of recombinant 1 derived from pλ247-10 were 0.8 to 1.0 log higher in SC-1 than in NFS, whereas another pλ247-10 recombinant 1 clone consistently gave a very small difference in titer (0.1 ± 0.14 in 4 tests). The results with the second, aberrant clone were confirmed with a biologically cloned stock (SC-1/NFS = 0.1) and an Sst recombinant (SC-1/NFS = 0.2); the data for this clone were excluded in calculating the data given in Table 2. The basis for the variability of recombinant 1 and 5 clones is unknown. The variability does not appear to be related to the particular parental clone of MCF from which the recombinant virus was derived, since there was no consistency in pattern with

recombinants constructed from each of the 3 parental MCF 247 clones (i.e., sets of clones derived from each parent gave comparable degrees of variation) (data not shown). However, in spite of the variability between some independent clones, the mean titer differences of recombinants 1 and 5 were statistically similar to those of other recombinants with an MCF 247 LTR ($P > 0.1$) and different from those with an Akv LTR ($P < 0.01$) (Table 2, footnote c). Thus, the LTR seems to be the primary determinant of the relative plating efficiency on SC-1 versus NFS cells. It should be noted that the data do not exclude a possible contribution to this phenotype from other viral genes as well.

DISCUSSION

With the goal of identifying viral genes that determine the oncogenicity and other distinctive phenotypes of murine leukemia viruses of inbred mice, we molecularly cloned MCF 247 and prepared in vitro recombinants between clones of this virus and its nonleukemogenic progenitor, Akv.

Many investigators have found that a variable, often high percentage of molecular clones of retroviruses are defective (2, 24, 25, 31, 36, 37), and this was true in the present study. Of 25 clones that hybridized to an MCF 247 cDNA probe that were prepared from one Hirt supernatant, 9 had restriction enzyme maps expected for MCF 247, but only three of these yielded infectious virus. Furthermore, one of these three carries a mutation that affects the morphology of foci on mink cells, whereas the other two clones differ in the structure of their direct repeats within U3, with one clone having an insertion in this region of what appears to be cellular DNA. Thus, even among the three viable DNA clones of MCF 247 that we obtained, each molecule was unique.

One of the correlates of high oncogenicity and thymotropism of class I MCF isolates is a higher plating efficiency on SC-1 cells than NFS mouse embryo cells (34); it has, however, been difficult to suggest a clear biological significance for this association. Comparisons of the titers achieved in these cells by the recombinant viruses described in this report indicate that sequences contained in the LTR are the major determinant of this phenotype, although we have noted that the data do not rule out a possible contribution to the phenotype from other viral genes as well. The finding that a determinant of an in vitro host range property resides in the LTR is almost certainly related to the recent findings from our laboratory (3, 4) and others (10) that the LTR can determine tissue tropism and disease specificity in vivo,

probably via transcriptional enhancers in the U3 region of the LTR. However, as described in a separate report, the LTR is only one determinant of the complex phenotype of leukemogenicity of MCF 247 virus in AKR mice.

ACKNOWLEDGMENTS

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