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Nucleotide Sequence of the 3′ End of MCF 247 Murine Leukemia Virus

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We isolated DNA clones of MCF 247, a leukemogenic, recombinant type C virus obtained from the thymus of an AKR mouse. We determined the nucleotide sequence of the viral long terminal repeat (LTR) and the 3′ end of *env*, and we compared the sequences to corresponding sequences of the genome of Akv virus, the putative ecotropic parent of MCF 247. By analogy with Moloney leukemia virus, we identified the amino terminus of Prp15E, the C-terminal proteolytic cleavage product of *env* and precursor to mature virion p15E. In MCF 247 the presumptive Prp15E is encoded by a 603-nucleotide open reading frame. The majority of this sequence is identical to that of Akv. However, a recombination event near the 3′ end of the Prp15E-coding region introduces nonecotropic sequences into MCF 247, and these extend to the 3′ end through the U3 portion of the LTR. The U3 regions of Akv and MCF 247 are about 83% homologous. The R and U5 regions of the LTR of MCF 247 and Akv are identical. Large RNase T_1-resistant oligonucleotides analyzed previously in numerous ecotropic and MCF viral genomes were located within the Akv and MCF 247 DNA sequences. The resulting precise T_1 oligonucleotide maps of the 3′ ends of MCF viral genomes reveal that the biologically defined, leukemogenic class I MCFs isolated from thymic neoplasms of inbred mice all share the sequence pattern seen in MCF 247, a representative of this group; they possess recombinant Prp15E genes and derive U3 from their nonecotropic parents.

Mink cell focus-forming (MCF) type C viruses that differ in oncogenicity can be distinguished from one another and from their nononcogenic ecotropic progenitors by RNase T_1-resistant oligonucleotides located in U3 and the 3′ portion of *env* (4, 16, 23, 24, 33). To determine the precise structural differences in this region of the genome between leukemogenic MCF 247 and its non-leukemogenic ecotropic progenitor, Akv virus, we determined the nucleotide sequence of the 3′ 1.3 kilobases of a DNA clone of MCF 247. This region includes sequences encoding the long terminal repeat (LTR), the primer binding site for plus-strand DNA synthesis, Prp15E, the C-terminal proteolytic cleavage product of *env* and precursor to mature virion p15E (9, 19, 27, 38), and the C terminus of gp70. The LTR of Akv had been sequenced previously by Van Beveren et al. (36), and Lenz et al. (21) and V. Corbin and W. Herr (unpublished results) have sequenced the *env* gene of Akv. The DNA sequences reveal the precise location of large RNase T_1-resistant oligonucleotides analyzed previously in viral RNA fingerprints (23, 24, 31, 32). Together, the T_1 oligonucleotide data and the DNA sequences reveal that the amino-terminal half of p15E and the C terminus of Prp15E, as well as U3, distinguish biologically distinct MCF and ecotropic viruses of inbred mice. Previous biological and biochemical studies have emphasized differences in gp70 between these viruses (7, 16). Together, the studies indicate that particular sequences of gp70, p15E, and the LTR may all be important in determining the leukemogenic phenotype of MCF type C viruses of inbred mice.

MATERIALS AND METHODS

Molecular cloning of MCF 247 virus. To obtain circular DNA intermediates of MCF 247, *Mus dunii* cells were infected with MCF 247 virus obtained from chronically infected cells (2). Eighteen hours after infection, DNA was extracted by the Hirt procedure (18) as described previously (30). To enrich for genomic-length molecules, DNA from the Hirt supernatant was cleaved with *Hin*dIII and electrophoresed on a 1%

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agarose gel. DNA migrating between 6.7 and 9.5 kilobases was isolated (2). A 2- to 20-fold excess of enriched DNA was ligated to *Hin*dIII-cleaved pBR322 at a DNA concentration of 25 to 50 μg/ml. After ligation, 100 to 500 ng of DNA was used to transform the RR-1 strain of *Escherichia coli* (5). Colonies that grew on agar plates containing ampicillin were screened for viral DNA sequences by filter hybridization (14) with ^{32}P-labeled cDNA prepared from 70S MCF 247 RNA as a probe (2). Single colonies which hybridized to cDNA were amplified in liquid culture, and plasmid DNA was isolated (3). Plasmid DNA was analyzed by restriction endonuclease cleavage with *Kpn*I, *Sma*I, and *Eco*RI. Plasmid inserts which contained restriction sites similar to those of MCF 247 (1) were purified from pBR322 after *Hin*dIII digestion of plasmid DNA. From 1 to 5 μg of each insert was ligated and transfected by the calcium phosphate precipitation method (12) onto NIH 3T3, MCT, or SC-1 cells (15). Cells were passaged for 2 to 6 weeks, and then culture fluids were assayed for reverse transcriptase activity.

Sequence determination. The pMCF247-1b DNA clone was cleaved with restriction endonucleases (from New England Biolabs or Bethesda Research Laboratories), and fragments were labeled at their 5′ ends with [γ-^{32}P]ATP (from The Radiochemical Centre, Amersham, England) and polynucleotide kinase (from Boehringer Mannheim Corp.) after elution from agarose preparative gels. Fragments were recleaved, and their sequence was determined by using the chemical modification procedures of Maxam and Gilbert (25). Reactions were run on 15, 8, or 6% polyacrylamide–8 M urea gels, 0.4 mm thick. Sequences were compiled and analyzed with the computer programs of Gingeras et al. (11).

RESULTS

Characterization of molecular clones of MCF 247 virus. Molecular clones of MCF 247 were prepared by cleavage of circular viral DNA at the unique *Hin*dIII site and insertion into pBR322 or other cloning vectors as described above. At the time the DNA sequencing studies were initiated, eight molecular clones had been obtained in this way. Of these, six had restriction patterns similar to those determined by Chattopadhyay et al. (1, 2) for proviral DNA of MCF 247-infected cells, yet none was infectious when transfected into appropriate mouse cells.

To determine whether the 3′ end of one of these clones was biologically functional, we generated a recombinant in vitro between an MCF clone, designated pMCF247-1b, and an infectious DNA clone of integrated Akv murine leukemia virus (MuLV), AKR MuLV 623 (22). The MCF DNA was digested with *Xba*I and *Hin*dIII, and the fragment encoding the 3′ end of the viral genome was isolated and ligated to an *Eco*RI-*Xba*I fragment containing the 5′ remainder of the genome from AKR MuLV 623 (Fig. 1). When transfected, this DNA was infectious, yielding ecotropic, XC plaque-forming virus, whose RNase T_1 fingerprint was consistent with its genomic structure and with previously determined T_1 oligonucleotide maps (data not shown; 32). Therefore, we were confident of the biological integrity of pMCF 247-1b in the region from *Xba*I to the 3′ end of the MCF 247 genome, and we proceeded to sequence this DNA.

Recently, Holland (unpublished data) obtained infectious DNA clones of MCF 247, and the sequence of the region from *Xba*I to the 5′ end of the p15E-coding region, presented below, was determined again with one of these clones. One nucleotide difference (out of 173 bases) was found between the two clones within this region.

DNA sequencing. All DNA sequences were determined by the Maxam and Gilbert procedure (25). The strategy for generating appropriate fragments for sequencing the 3′ end of the MCF 247 DNA clone is shown in Fig. 2. The nucleotide sequences obtained are shown in Fig. 3 and 4, where the Prp15E- and LTR-coding regions of MCF 247 are compared with the corresponding sequences of Akv obtained by Van Beveren et al. (36) (LTR), Lenz et al. (21) (Prp15E), and V. Corbin and W. Herr (Prp15E; unpublished results).

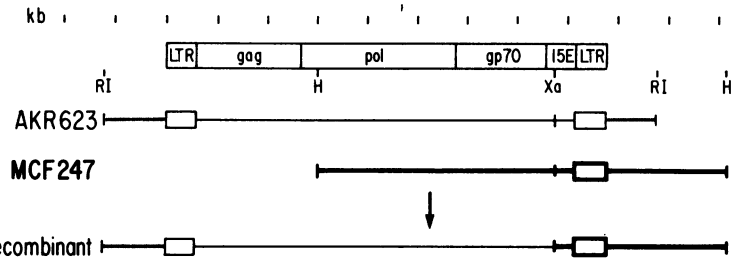

FIG. 1. Schematic representation of DNA clones of MCF 247 and Akv and a recombinant between them constructed in vitro. AKR 623 was cloned from integrated Akv provirus present in infected NIH/3T3 cells after *Eco*RI cleavage of the cellular DNA. This viral clone therefore contains mouse cellular sequences (22). The molecular clone of MCF 247 was obtained by *Hin*dIII cleavage of a circular molecule and is permuted as shown. Restriction enzyme sites used to construct the recombinant between Akv and MCF 247 are indicated at the top of the figure. Abbreviations: RI, *Eco*RI; H, *Hin*dIII; Xa, *Xba*I.

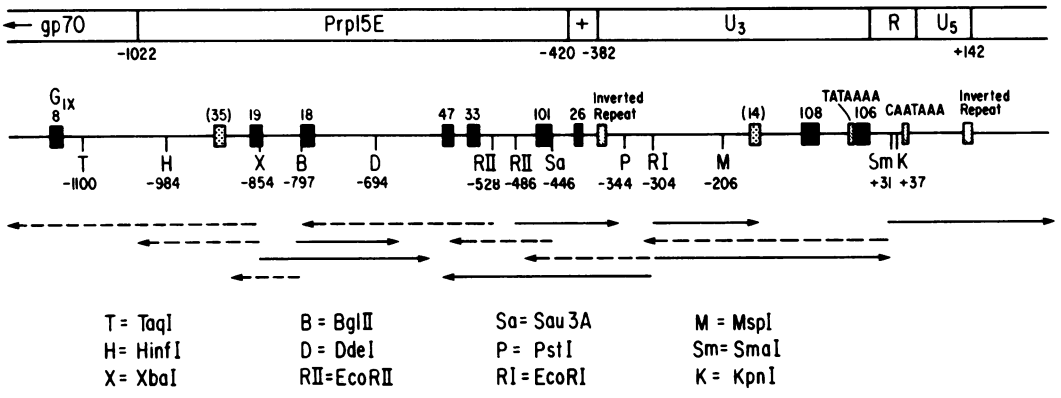

FIG. 2. Strategy for sequencing DNA encoding the 3′ end of the MCF 247 genome. Arrows indicate regions of the DNA that were sequenced from the restriction site corresponding to the tail of the arrow. Symbols: (←---) regions sequenced from minus-strand DNA; (→) regions sequenced from plus-strand DNA; (+) origin of plus-strand DNA synthesis; (■) T_1 oligonucleotides analyzed previously in fingerprints of MCF 247 genomic RNA; (□) positions of two T_1 oligonucleotides that are present in Akv, but not in MCF 247. The boundaries of Prp15E and the U3, R, and U5 regions were identified as described in the text. The numbers above the T_1 oligonucleotides are those given in published fingerprints (23, 31, 32). G_{1X}, T_1 oligonucleotide 8 has been shown to be genetically linked to the presence of the gp70 antigen G_{1X}. The first base pair in R is considered as 1.

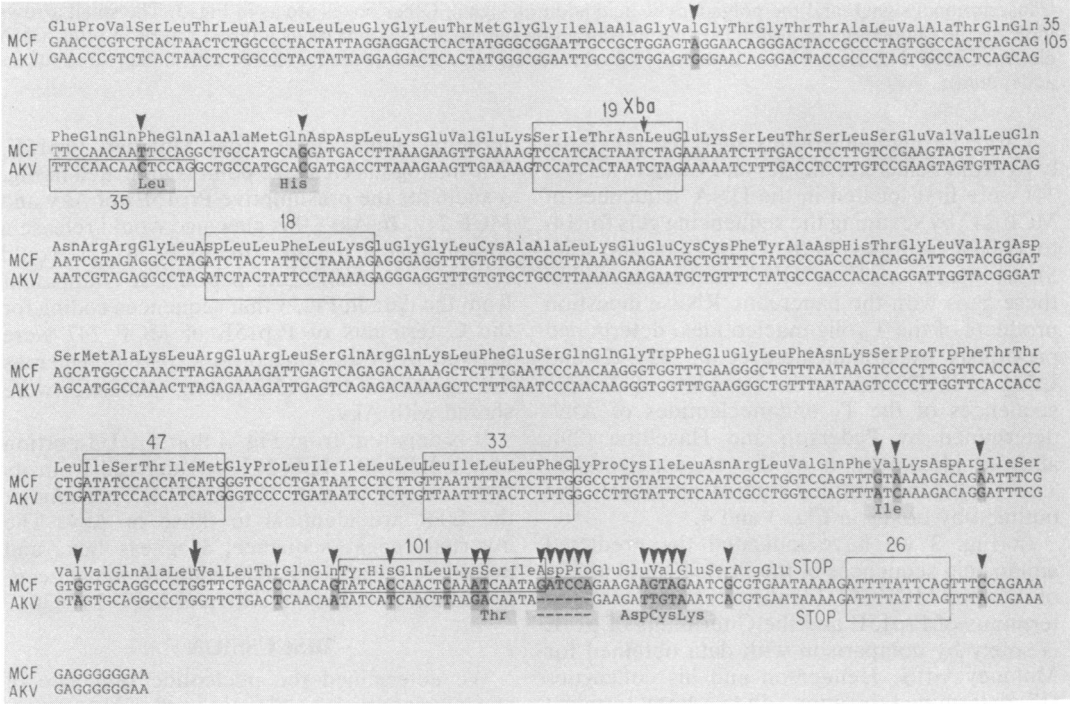

FIG. 3. Comparison of nucleotide seqences of DNA clones corresponding to the 3′ ends of Akv and MCF 247 viral genomes. Nucleotide sequences encoding Prp15E and a region between Prp15E and the LTR believed to contain the origin of plus-strand DNA synthesis. The amino terminus of Prp15E was identified by comparison with data for Moloney leukemia virus as described in the text. Open boxes enclose sequences corresponding to large RNase T_1-resistant oligonucleotides analyzed previously in Akv and MCF 247 fingerprints (23, 32). Nucleotide differences between the viruses are accentuated by gray boxes and arrow heads above the sequences. Amino acids of Prp15E that would differ between the two viruses are indicated within gray boxes below the Akv sequence. Dotted lines indicate that the Prp15E of Akv is two amino acids shorter than that of MCF 247. The large arrowhead above the sequences indicates the putative cleavage site for generating mature p15E (see text). The last nucleotide in the figure is the first preceding the LTR.

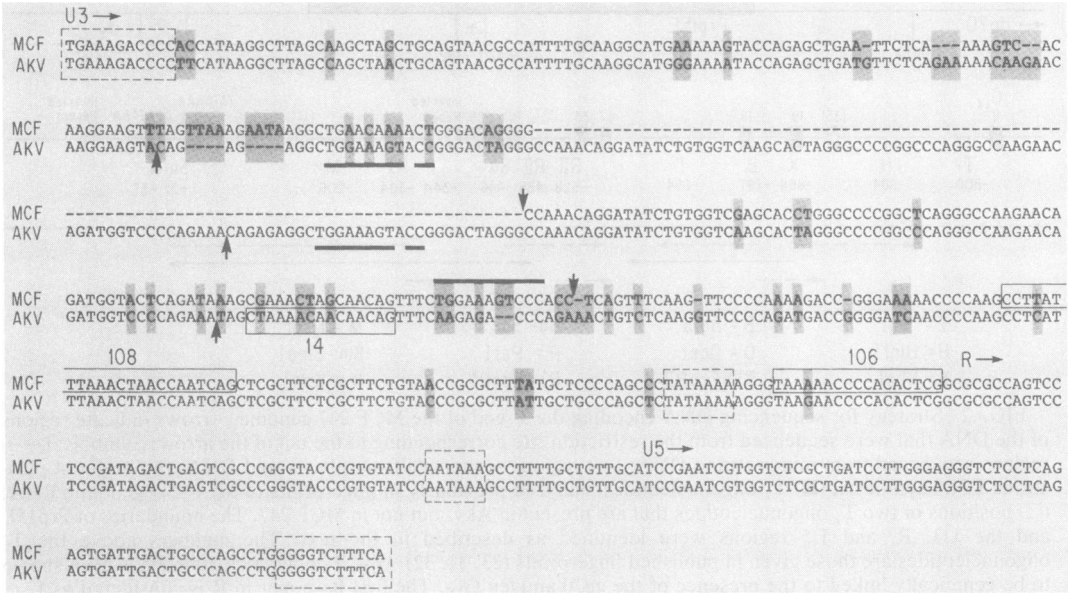

FIG. 4. Nucleotide sequences of the LTRs of Akv versus MCF 247. The boundaries of U3, R, and U5 were determined as described in the text. Dotted line boxes enclose the inverted repeats of the LTR, a putative transcriptional signal, and the polyadenylic acid addition signal. Other boxes are as in Fig. 3. The small arrows indicate the boundaries of a direct repeat in Akv and a sequence that appears as a direct repeat in other DNA clones of MCF 247. Heavy black lines within direct repeats underline a sequence shared with simian virus 40 (see addendum).

Large T_1 oligonucleotides analyzed previously by Rommelaere et al. (32) and Lung et al. (23, 24) were first located in the DNA sequences of MCF 247 by scanning the sequencing gels for 14- to 35-base-long gaps in the G (or C) lane(s) (Fig. 5). Comparison of the DNA sequences within these gaps with the pancreatic RNase digestion products of the T_1 oligonucleotides, determined previously by Rommelaere et al. (31, 32) and Lung et al. (23), or to the complete nucleotide sequences of the T_1 oligonucleotides of Akv, determined by Pederson and Haseltine (29), allowed identification of the oligonucleotides. The T_1 oligonucleotides studied previously are outlined by boxes in Fig. 3 and 4.

In Fig. 3 we have indicated the predicted amino acid sequences of the Prp15Es and p15Es of Akv and MCF 247. Identification of the amino terminus of Prp15E and the C terminus of p15E is solely by comparison with data obtained for Moloney virus. Henderson and his colleagues (17) determined the amino and carboxy terminal residues of p15E of Moloney virus, and Sutcliff et al. (35) and Shinnick et al. (34) determined the nucleotide sequence of a DNA clone of Moloney virus, allowing placement of the Prp15E-coding portion of *env*. A comparison of our data with that for Moloney virus Prp15E is shown in Fig. 6. In Moloney virus, Prp15E is proteolytically cleaved to yield mature virion p15E (13, 34). This cleavage involves the removal of the 16 C-terminal amino acids from Moloney Prp15E. The analogous cleavage point is indicated in Fig. 3 and 6 for the presumptive Prp15Es of Akv and MCF 247. In Akv, this cleavage would release a 19-amino-acid peptide; in MCF 247, it would release a 21-amino-acid peptide. It is apparent from the data in Fig. 3 that sequences coding for the C terminus of Prp15E of MCF 247 were derived from its nonecotropic parent, whereas the majority of the p15E-coding sequences were shared with Akv.

It is apparent from Fig. 4 that the U3 portion of the LTR of MCF 247 was derived from its nonecotropic parent. The R and U5 portions of the LTR are identical to those of Akv. The inverted repeat sequence, Hogness box, and putative polyadenylic acid addition signal occupy similar positions in Akv and MCF 247 (36).

DISCUSSION

We determined the nucleotide sequence of approximately 1.3 kilobases of DNA corresponding to the 3′ terminus of the MCF 247 viral genome. By analogy with Moloney leukemia virus (13, 17, 34), we identified sequences encoding the amino terminus of Prp15E, a protein which in MCF 247 is specified by a 603-nucleotide open reading frame. The termination codon at the end of this sequence is followed by 34 bases presumably containing the origin of plus-strand DNA synthesis (10, 26) and then by

the LTR, defined in MCF 247 by 11-base-long inverted repeats at its termini. The particular clone of MCF 247 whose complete 3' end sequence was determined possessed an LTR 524 base pairs long. This LTR did not possess an internal direct repeat sequence as has been seen in LTRs of several other retroviruses (6, 20, 34, 35). However, the LTR of another MCF DNA clone, whose sequence was partially determined (data not shown), did contain two copies of a 105-base-long direct repeat sequence. Several aspects of the sequences, of their comparison to corresponding sequences of Akv and Moloney viruses, and their use in identifying genomic hallmarks of biologically distinct MCF viruses are of note.

Prp15E sequences. The presumptive Prp15Es of MCF 247 and Akv are 201 and 199 amino acids long, respectively, and the nucleotide sequences encoding them are 96% homologous. Moloney Prp15E is 196 amino acids long (34). Although the nucleotide sequence of Moloney Prp15E is only about 77% homologous to that of MCF 247, the predicted amino acid sequences of their respective proteins are 90% homologous. Figure 6 shows a comparison of the predicted amino acid sequences of the Prp15Es of the three viruses. As noted by Lerner and his colleagues (34), the C terminus of Prp15E, a region that is proteolytically removed to yield mature virion p15E, is variable among different murine type C viruses.

Lenz et al. (21) have presented a model for the placement of the *env* precursor, and its Prp15E portion in particular, relative to the membrane. In these models amino acids 134 through 164 of Prp15E extend through the membrane, and the remaining C-terminal tail is cytoplasmic. The rules employed to develop these models would apply with similar results to the predicted MCF 247 Prp15E amino acid sequence.

It is apparent from Fig. 3 that the majority of the Prp15E-coding region of MCF 247 is shared with Akv virus, although sequences encoding the C terminus, commencing at nucleotide 507, must be derived from the nonecotropic parent of MCF 247. Because the nonecotropic parents of MCF viruses have not been isolated, we could not, from these data alone, have been certain that the majority of the p15E of MCF 247 was derived from Akv. However, Chattopadhyay et al. (1, 2) found that the *Xba*I cleavage site in the Prp15E gene (Fig. 3), a site shared by Akv and MCF 247, is found only in the Akv endogenous virus of AKR mice. Thus, this region of MCF 247 must be ecotropic in origin, and it seems reasonable to suppose that the majority of the Prp15E gene of MCF 247 was inherited from Akv. There are three nucleotide differences between Akv and MCF 247 in the amino terminal-coding portion of the Prp15E gene. One of these leads to the displacement of an Akv T_1 oligonucleotide (35 in Fig. 3) in fingerprints of MCF 247 (32). These three base changes may simply be point mutations; alternatively, they could have been introduced by recombination. In the latter case, since sequences encoding the C terminus of gp70 of Akv and MCF 247 are identical (unpublished results) and since the *Xba*I site in MCF 247 is derived from Akv, two crossover events would probably be needed to introduce the three nucleotide changes.

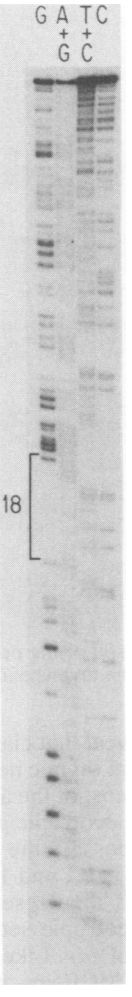

FIG. 5. Sequencing gel showing a region of DNA encoding Prp15E that contains the large T_1-resistant oligonucleotide 18.

A major motivation for our studies was to localize ecotropic and MCF-specific T_1 oligonucleotides whose presence or absence in MCF genomic T_1 fingerprints had been found by Lung et al. (23, 24) to be characteristic of biologically distinct groups of MCFs (4, 33). Figures 3 and 4, in conjunction with the previously determined

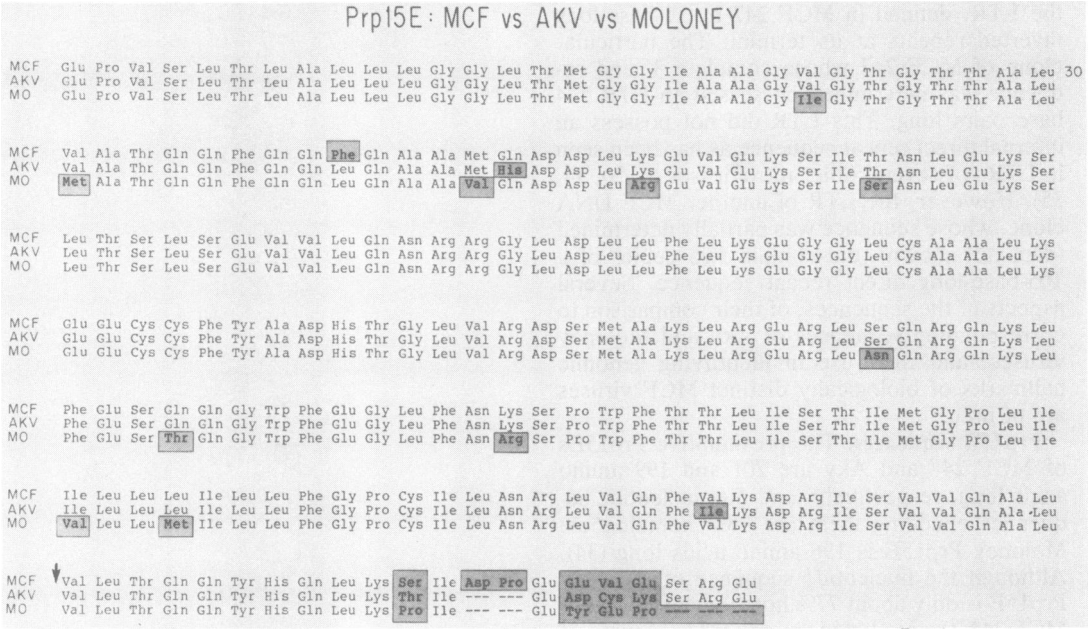

FIG. 6. Comparison of the predicted amino acid sequences of the Prp15Es of Akv, MCF 247, and Moloney leukemia viruses. The arrowhead indicates the position of proteolytic cleavage to generate mature p15.

T_1 fingerprints, reveal that class I, leukemogenic MCFs isolated from thymic neoplasms can probably derive all or most of the amino-terminal half of p15E from their ecotropic parent since almost all of the viruses possess the three ecotropic T_1 oligonucleotides 35, 19, and 18 in Fig. 3. About half of the viruses then resemble MCF 247 in continuing with ecotropic sequences almost up to the C terminus of *env*. Like MCF 247, however, all possess the MCF-specific T_1 oligonucleotide 101 at the very end of this region. The other half of the leukemogenic class I MCFs apparently undergo recombination in the middle of the p15E gene and thus lose the two ecotropic T_1 oligonucleotides 47 and 33 (Fig. 3) and acquire oligonucleotide 101. This general pattern is summarized schematically in Fig. 7. Interestingly, oligonucleotide analysis of a group of MCFs isolated from thymus of AKR mice by O'Donnell et al. (28) suggests that if the recombination event in the middle of Prp15E that generates some class I MCFs occurs too far to the 5′ side, so that oligonucleotide 18 is lost, then the resulting MCF viruses are non-leukemogenic or only weakly so in the AKR acceleration assay (M. L. Lung, P. O'Donnell, and N. Hopkins, unpublished results). In other words, ecotropic T_1 oligonucleotide 18 in Prp15E-coding sequences appears to be genetically linked to the highly leukemogenic phenotype of class I MCFs.

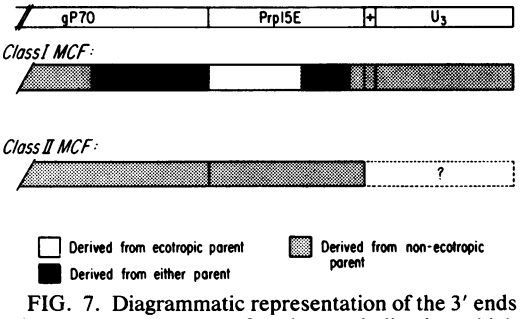

FIG. 7. Diagrammatic representation of the 3′ ends of class I and class II MCF viruses, indicating which regions are derived from the ecotropic and nonecotropic parents. The conclusion that the patterns shown are representative of viruses belonging to the two classes of MCFs is based upon the assignment of T_1 oligonucleotides to particular genetic elements, determined from DNA sequencing, and on the T_1 oligonucleotide maps obtained by Lung et al. (23, 24).

Class II MCFs isolated from nonthymic neoplasms lack all of the ecotropic T_1 oligonucleotides present in Prp15E and thus may derive this entire portion of *env* from their nonecotropic parents (4, 23). These viruses all possess the MCF-specific T_1 oligonucleotide 101 encoding a C-terminal region of Prp15E.

The termination codon at the end of Prp15E is followed by 34 nucleotides that are highly conserved between MCF 247, Akv, and Moloney viruses and which may encode the origin of plus-strand DNA synthesis (10, 26, 34).

LTR. As noted above, the MCF DNA clone we sequenced possessed only one copy of a potentially direct repeat sequence. Identification

of this sequence was made by partially determining the nucleotide sequence of another MCF 247 clone, clone p247-13, possessing a direct repeat. Khan et al. (20) also sequenced an MCF LTR containing a direct repeat. It is apparent that the direct repeats of Akv and MCF are staggered relative to one another (see arrows in Fig. 4).

All class I, thymic MCFs whose genomic T_1 fingerprints have been examined lack the ecotropic T_1 oligonucleotide 14 in U3 (23, 24). Like MCF 247, all of the highly oncogenic isolates also acquire oligonucleotides 106 and 108 in U3, whereas one non-leukemogenic isolate did not. However, isolates which possess the pattern oligonucleotide 14 absent, 106 and 108 present are not necessarily leukemogenic, since as noted above, loss of ecotropic oligonucleotide 18 in the p15E gene is associated with low oncogenicity. Class II MCFs, all of which are non-leukemogenic upon injection (4, 33), share oligonucleotide 14 in U3 with their ecotropic parents, and none possesses oligonucleotide 108 (23, 24). These observations are probably insufficient to determine the parental origin of the U3s of class II MCFs, although they are consistent with the possibility that they are inherited from the ecotropic parents.

Conclusion. These studies, in conjunction with T_1 fingerprinting, have suggested that particular nucleotide and amino acid sequences of Prp15E and U3 contribute to the oncogenicity of MCF type C viruses of inbred mice. The role of gp70, p15E, and the LTR in determining this complex viral phenotype remain unclear.

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ADDENDUM

We noted above that when the LTR sequences of Akv and MCF 247 are aligned as shown in Fig. 4, their direct repeat sequences within U3 are staggered relative to one another. Recently, Laimons et al. (Proc. Natl. Acad. Sci. U.S.A., in press) obtained evidence that a sequence critical for activator (enhancer) function in simian virus 40 is $TGG^{TTT}_{AAA}G$, which occurs within the sequence TGGAAAGTCCC in simian virus 40 and is also found in other viruses and suspected enhancer regions. An identical 11-base sequence is found in the direct repeat of the MCF 247 LTR, and 10 bases of the 11-base sequence are present in the Akv direct repeat. However, in Akv the sequence appears 10 nucleotides from the 5' end of the direct repeat, whereas in MCF 247 it appears four bases from the 3' end of the direct repeat. Thus it is apparent (Fig. 4) that the staggered positions of the direct repeats of Akv and MCF 247 ensure that in both viruses the direct repeat includes the potentially critical sequence noted by Laimons et al.

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