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Identification of a DNA Fragment from a Molecularly Cloned Mink Cell Focus-Inducing Murine Leukemia Virus Specific for Xenotropic Virus-Related Sequences

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Molecular clones of closed circular DNA molecules of a mink cell focus-inducing murine leukemia virus (MCF-13 MuLV) were generated. Closed circular DNA molecules isolated from a Hirt extraction of recently infected NIH/3T3 cells were inserted at their unique *Eco*RI site into λgtWES.λB. Restriction endonuclease analysis of inserts of two clones indicated that they represented intact MCF-13 MuLV genomes. One viral insert contained two large terminal repeat sequences, and the other contained only one. A 300-base-pair DNA fragment located in the envelope region of the MCF-13 MuLV genome was determined to be related to xenotropic MuLV sequences.

A class of recombinant murine retroviruses that has both ecotropic and xenotropic murine leukemia virus (MuLV) characteristics has recently been described (9). These recombinant dual-tropic MuLVs have been implicated in the development of lymphocytic leukemia in high-leukemic strains of mice, such as AKR. They are first expressed by these mice just before the onset of leukemogenesis and usually not before (9). Extra MuLV proviral sequences have been detected in leukemic but not normal tissue DNAs (3, 13, 16). We recently demonstrated that the AKR ecotropic MuLV did not account for these extra proviruses (18). Our analysis of the AKR ecotropic proviruses was facilitated by use of a molecular clone of the AKR MuLV genome developed by Lowy et al. (11). Since availability of a molecular clone of a recombinant dual-tropic MuLV would also be critical in analysis of the extra proviruses in DNA from leukemic tissues, molecular cloning of a mink cell focus-inducing (MCF-13) MuLV, a dual-tropic retrovirus isolated from an AKR mouse (6), was undertaken. Details of the molecular cloning of covalently closed circular MCF-13 MuLV DNA molecules into the bacteriophage vector λgtWES.λB and analysis of two isolated clones are included in this report. Furthermore, identification of a 300-base-pair (bp) *Sma*I-*Eco*RI DNA fragment of the MCF-13 MuLV cloned DNA which recognizes MCF-13 and xenotropic MuLV sequences but not AKR ecotropic MuLV DNA is included.

Digestion with various restriction endonucleases of Hirt-extracted linear DNA from NIH/3T3 cells recently infected with a cloned stock of MCF-13 MuLV (5×10^6 to 5×10^7 infectious units per ml) indicated that *Eco*RI made a single cut in the viral genome (data not shown; 4). This unique *Eco*RI site made it feasible to clone the MCF-13 MuLV DNA in λgtWES.λB. Hirt-extracted covalently closed circular DNA was isolated by equilibrium centrifugation on ethidium bromide-CsCl gradients (19) and digested with *Eco*RI.

The *Eco*RI DNA arms of λgtWES.λB were separated from the internal fragment by electrophoresis through a 0.7% agarose gel, extracted from the agarose by electroelution (18), and treated with bacterial alkaline phosphatase at 67°C for 30 min. A 0.5-μg amount of *Eco*RI-digested MCF-13 MuLV closed circular DNA molecules was then ligated to 2 μg of the isolated λ arms in 10 mM Tris-hydrochloride (pH 7.5)–10 mM $MgCl_2$–6 mM KCl–1 mM dithiothreitol–1 mM ATP–0.1 mg of bovine serum albumin per ml–1 mM spermidine–2 U of T4 ligase at 14°C for 16 h. Ligated DNA molecules were in vitro packaged by Blattner et al. (2) into infectious λ particles which were spread without amplification onto 15-cm agar plates. About 6×10^4 plaques were screened for positive recombinants by the filter hybridization procedure of Benton and Davis (1), using MCF-13 MuLV [^{32}P]cDNA as a probe. ^{32}P-labeled cDNA probes were prepared by exogenous reverse transcriptase reactions, using purified 38S viral RNA (17). By this technique five stable recombinant clones were isolated after two cycles of plaque purification.

*Eco*RI digestion of DNAs isolated from the five stable recombinant clones revealed four differently sized viral inserts. Cloned DNA fragments were digested with *Eco*RI, electropho-

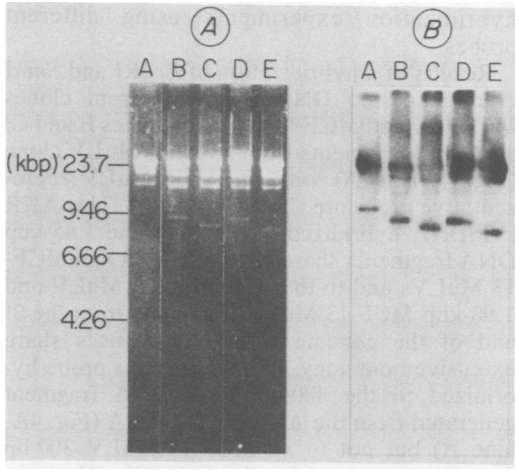

FIG. 1. *Eco*RI digestion of DNAs from λgtWES.λB clones of MCF-13 MuLV. DNAs (10 μg) were digested with *Eco*RI for λ MCF-13 clones 1 (lane A), 5 (lane B), 7 (lane C), 15 (lane D), and 17 (lane E). DNAs were electrophoresed through a 1% agarose horizontal slab gel for 16 h at 2 V/cm in low-salt E buffer (18). DNAs in (A) were stained with 0.5 μg of ethidium bromide per ml. (B) DNAs from the same gel transferred to nitrocellulose filter and hybridized with MCF-13 MuLV [^{32}P]cDNA. Sizes in kilobase pairs of marker DNA fragments (*Hin*dIII-digested λ DNA) are indicated.

resed through a 1% agarose gel, and stained with ethidium bromide (Fig. 1). The viral DNA insert of clone MCF 13-1 was not visible by ethidium bromide staining of this agarose gel but was detected by Southern blot analysis (15; Fig. 1, lane A). The sizes of the virus-specific DNA fragments detected by hybridization to MCF-13 MuLV [^{32}P]cDNA were 9.7, 8.9, 8.3, 8.9, and 8.1 kilobase pairs (kbp) (Fig. 1; lanes A through E, respectively).

Since one of the objectives of this study was to identify specific viral probes for analysis of tissue DNAs, two clones containing viral inserts of orthodox MuLV DNA sizes, i.e., 8.3 and 8.9 kbp (8), were selected for further analysis. Phage particles were propagated in *Escherichia coli* strain LE392 grown in NZCY amine broth at 37°C, and DNA was extracted from them by the procedure of Maniatis et al. (12). DNAs were double-digested with the restriction endonucleases *Eco*RI and *Xho*I, *Sal*I, or *Sma*I. These were enzymes whose cleavage sites for MCF-13 MuLV we had previously mapped, using Hirt-extracted DNA (data not shown). While this work was in progress, a detailed restriction endonuclease map of MCF-13 MuLV was similarly derived by Chattopadhyay et al. (4). Virus-specific DNA fragments were detected by Southern transfer to a nitrocellulose filter and hybridization with MCF-13 MuLV [^{32}P]cDNA. The MCF-13 MuLV DNA fragments whose sizes were predicted by the restriction endonuclease map (unpublished data; 4) were observed for the different enzyme digestions (Fig. 2). Digestions with *Eco*RI and *Xho*I generated a 2.2-kbp virus-specific fragment which was shared by both clones. However, a 6.1- and a 6.7-kbp fragment were generated from clones MCF 13-7 and MCF 13-15, respectively (Fig. 2, lanes A and B). Similarly, *Eco*RI and *Sal*I digestions yielded a 2.6-kbp fragment from both clones, but a 5.7- and a 6.3-kbp fragment from clones MCF 13-7 and MCF 13-15, respectively (Fig. 2, lanes C and D). The two clones also yielded identical *Eco*RI and *Sma*I viral DNA fragments (4.65, 1.9, 1.5, 0.25 kbp), except for an extra 0.6-kbp fragment generated from MCF 13-15 (Fig. 2, lanes E and F).

The DNA fragments, which included the large terminal repeat (LTR) region generated by double digestion with *Eco*RI and *Xho*I or *Sal*I, differed by 0.6 kbp between the two clones (Fig. 2, lanes A through D). This difference would

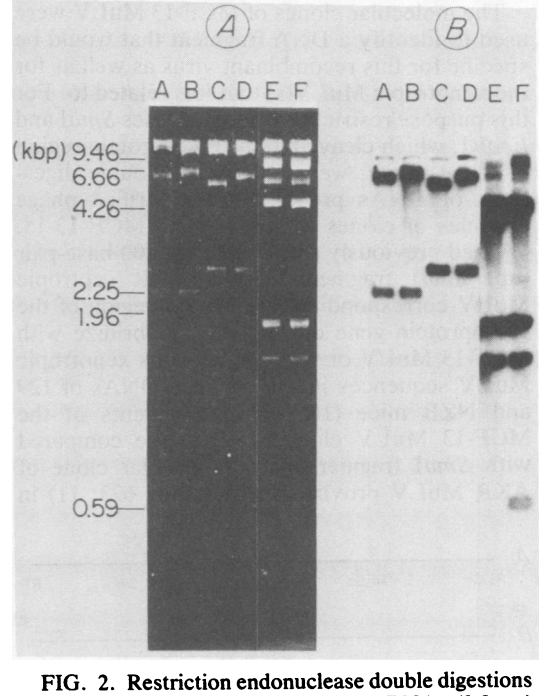

FIG. 2. Restriction endonuclease double digestions of DNAs from two MCF-13 clones. DNAs (0.3 μg) from clones MCF 13-7 or MCF 13-15 were sequentially digested with *Eco*RI and *Xho*I, *Sal*I, or *Sma*I. Digested DNAs were electrophoresed through a 1% agarose gel and analyzed by the Southern blot technique. *Eco*RI and *Xho*I digestions of clone 7 (lane A) and clone 15 (lane B). *Eco*RI and *Sal*I digestions of clone 7 (lane C) and clone 15 (lane D). *Eco*RI and *Sma*I digestions of clone 7 (lane E), and clone 15 (lane F). *Hin*dIII-digested λ DNA marker fragments are indicated to the left of the lanes.

correspond to the presence of a single LTR for clone MCF 13-7 and two tandemly joined LTRs for clone MCF 13-15. This conclusion was supported by *Eco*RI and *Sma*I double digestion where a 0.6-kbp fragment present for clone MCF 13-15 (Fig. 2, lane F) was missing from clone MCF 13-7 (Fig. 2, lane E). This 0.6-kbp fragment presumably corresponds to an LTR segment and demonstrates the presence of a *Sma*I cleavage site in this region of the MCF-13 MuLV DNA. The results from the restriction endonuclease analysis of clone MCF 13-7 and MCF 13-15 DNAs indicated that these clones contained MCF-13 MuLV DNA with orthodox enzyme cleavage sites when compared with the restriction map derived from Hirt DNA (unpublished data; 4). A restriction endonuclease cleavage map of clone MCF 13-15 is presented in Fig. 3. The map of clone MCF 13-7 is similar except that only one LTR is present. Because these viral DNA inserts were cloned at the internal *Eco*RI site of MCF-13 MuLV (6.9 kbp from the 5′ end), they represent permuted viral genomes with respect to the LTR sequences (Fig. 3).

The molecular clones of MCF-13 MuLV were used to identify a DNA fragment that would be specific for this recombinant virus as well as for the xenotropic MuLV(s) that it is related to. For this purpose restriction endonucleases *Sma*I and *Eco*RI, which cleaved in the glycoprotein region of the genome, were chosen for double digestions of DNAs prepared from purified phage particles of clones MCF 13-7 and MCF 13-15. We had previously shown that the 400-base-pair (bp) *Sma*I fragment of the AKR ecotropic MuLV corresponding to a similar region of the glycoprotein gene did not cross-hybridize with MCF-13 MuLV or with endogenous xenotropic MuLV sequences in the genomic DNAs of 129 and NZB mice (18). DNA fragments of the MCF-13 MuLV cloned DNA were compared with *Sma*I fragments of a molecular clone of AKR MuLV provirus DNA (clone 623; 11) in hybridization experiments using different probes.

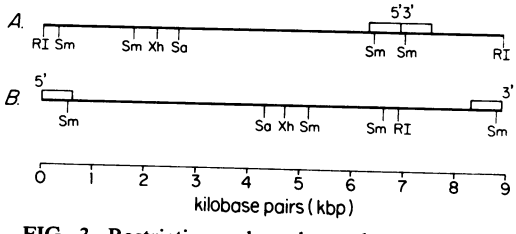

FIG. 3. Restriction endonuclease cleavage maps of MCF-13 MuLV. (A) Map of clone MCF 13-15. (B) Hirt-extracted linear viral DNA (4). Numbers refer to kilobase pairs, and the orientation is 5′ to 3′ with respect to the viral genome. The boxes indicate LTR regions. The cleavage sites are indicated for the restriction enzymes *Sma*I (Sm), *Sal*I (Sa), *Xho*I (Xh), and *Eco*RI (RI).

Results of a hybridization of *Eco*RI and *Sma*I double-digested DNA fragments from clones MCF 13-7 and MCF 13-15 (Fig. 4, lanes B and C) and *Sma*I fragments from an AKR MuLV clone (Fig. 4, lane A) with an AKR MuLV representative probe are shown in Fig. 4A. This AKR [^{32}P]DNA hybridized to the 4.65- and 1.45-kbp DNA fragments shared by both AKR and MCF-13 MuLVs and to the 1.7-kbp AKR MuLV and 1.90-kbp MCF-13 MuLV fragments from the 3′ end of the genome where the viruses share extensive homology. In addition, this probe hybridized to the 400-bp *Sma*I DNA fragment generated from the AKR MuLV DNA (Fig. 4A, lane A) but not to an MCF-13 MuLV 300-bp fragment generated by *Sma*I and *Eco*RI cleavages at 6.6- and 6.9-kbp from the 5′ end (Fig. 4A, lanes B and C).

An MCF-13 MuLV [^{32}P]cDNA probe hybrid-

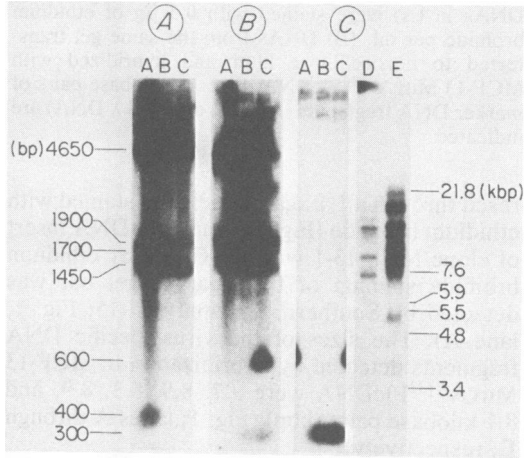

FIG. 4. Hybridization of DNAs from molecular clones of AKR or MCF-13 MuLVs and 129 or NZB mouse liver DNAs. Digested cloned DNAs in lanes A through C were electrophoresed through a 1.4% agarose gel in high-salt E buffer (18) for 18 h at 1.5 V/cm. Mouse liver DNAs in lanes D and E were electrophoresed through a 0.7% agarose gel in low-salt E buffer for 19 h at 2 V/cm. (A) DNAs hybridized with a ^{32}P-labeled representative probe for AKR MuLV; (B) DNAs hybridized with MCF-13 MuLV [^{32}P]cDNA; (C) DNAs hybridized with ^{32}P-labeled 300-bp MCF-13 MuLV DNA. Lanes A, *Sma*I-digested AKR MuLV DNA; lanes B, *Eco*RI- and *Sma*I-digested clone MCF 13-7; lanes C, *Eco*RI- and *Sma*I-digested clone MCF 13-15; lane D, *Eco*RI-digested 129 mouse liver DNA; and lane E, *Eco*RI-digested NZB mouse liver DNA. Numbers to the left of the lanes refer to viral DNA fragment sizes in base pairs for lanes A through C only. *Eco*RI-digested λ DNA was electrophoresed as a marker on the same gel as the mouse liver DNAs. These fragment sizes are indicated to the right of the figure and apply to lanes D and E.

ized to all of the DNA fragments shared by the two MuLV DNAs but not to the 400-bp *Sma*I fragment from AKR MuLV (Fig. 4B, lane A). It also hybridized to the 300-bp fragment from both clones MCF 13-7 and MCF 13-15 (Fig. 4B, lanes B and C) and to the 600-bp fragment from clone MCF 13-15 (Fig. 4B, lane C), which corresponded to a putative LTR fragment.

Because of the apparent specificity of the 300-bp *Sma*I-*Eco*RI DNA fragment, it was chosen for further analysis. DNA corresponding to this fragment was eluted from an agarose gel after electrophoresis and nick-translated. The ^{32}P-labeled 300-bp fragment hybridized only to clones MCF 13-7 and MCF 13-15 (Fig. 4C, lanes B and C) but not to AKR MuLV (Fig. 4C, lane A) DNAs. To further test whether this DNA fragment recognized other xenotropic virus-related sequences, it was hybridized to 129 or NZB mouse liver DNAs, which possess xenotropic but no ecotropic MuLV-related sequences (Fig. 4C, lanes D and E). Complex patterns of *Eco*RI-digested DNA with homology to the 300-bp fragment were generated from both 129 and NZB mouse liver DNAs.

The 300-bp fragment, which represents part of the glycoprotein region of the MCF-13 MuLV genome, apparently corresponds to the xenotropic MuLV sequences that are present in MCF MuLVs (7, 14). A corresponding *Sma*I DNA fragment from the glycoprotein region of AKR MuLV DNA was previously identified to be specific for ecotropic viral sequences (5, 18). Use of this ecotropic MuLV-specific probe has resulted in unambiguous analysis of the fate of AKR ecotropic provirus DNA in leukemic thymuses in the AKR mouse (18). The availability of the complementary xenotropic MuLV-specific probe should also prove useful in further analysis of this type.

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