NOTES

Molecular Cloning of Friend Mink Cell Focus-Inducing Virus: Identification of Mink Cell Focus-Inducing Virus-Like Messages in Normal and Transformed Cells

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Friend mink cell focus-inducing virus (Fr-MCF) is a leukemogenic murine retrovirus that was isolated from a leukemic NIH Swiss mouse. We molecularly cloned the genome of this virus into pBR322. Restriction enzyme mapping of this cloned Fr-MCF DNA revealed a 1.0-kilobase-pair region in the envelope gene which differs from the restriction map of the Friend ecotropic virus (Friend murine leukemia virus). A 400-base-pair Fr-MCF envelope gene fragment was subcloned from this region and designated pLEK. Probes made with pLEK hybridize to Fr-MCF DNA but do not hybridize to either Friend or amphotropic murine leukemia virus DNA. Polyadenylic acid-selected RNA was prepared from the hematopoietic tissues of normal NIH Swiss mice, Fr-MCF-infected erythroleukemia cells (TP-1), and uninfected chemically transformed T lymphocytes (RB-1). The pLEK probe identified 34S and 22S messages in the TP-1 cells and in the normal hematopoietic tissues. RB-1 cells contain 32S and 26S messages that hybridize to pLEK. However, the pLEK-like RNAs found in the normal hematopoietic tissues and in the RB-1 cells were 400-fold less abundant than the RNAs found in the TP-1 cells.

Friend mink cell focus-inducing virus (Fr-MCF) is a replication-competent, dual-tropic, type C retrovirus (14). Fr-MCF is invariably found in the hematopoietic tissues of leukemic mice inoculated with the ecotropic Friend erythroleukemia virus-Friend murine leukemia virus (F-MuLV) (11, 12). Fr-MCF is not found in mice that are resistant to F-MuLV-induced leukemia. However, F-MuLV replicates to high titers in leukemia-resistant mice (11). Fr-MCF is capable of causing leukemia in either newborn or adult mice, whereas F-MuLV only causes disease upon inoculation into newborn mice. A biologically cloned isolate of Fr-MCF is capable of inducing erythroleukemia in the absence of F-MuLV (11). These observations (the association of Fr-MCF with F-MuLV-induced leukemia and the ability of Fr-MCF to independently cause leukemia) have led us to propose that Fr-MCF is the proximal cause of F-MuLV-induced disease (11, 12). This model implies that F-MuLV is not the immediate cause of leukemia. Instead, F-MuLV would induce disease by recombining with endogenous murine cellular sequences to generate Fr-MCF. One implication of this theory

is that the genetic differences between F-MuLV and Fr-MCF are important determinants for the pathogenic potential of these viruses. We wished to identify the genetic differences between F-MuLV and Fr-MCF by using molecular clones of F-MuLV and Fr-MCF DNAs. The genome of F-MuLV was previously cloned (9). We molecularly cloned the genome of Fr-MCF.

Fr-MCF was isolated from an F-MuLV-infected NIH Swiss mouse and purified by biological cloning in mink lung cells (Fr-MCF cl-1) (14). Fr-MCF cl-1 was used to acutely infect *Mus dunii* cells (a gift from Sisir Chattopadhyay). *M. dunii* cells produce a two- to fivefold-higher level of viral DNA than do NIH 3T3 cells upon acute infection with Fr-MCF (unpublished data). The cells were lysed 24 h later, and the viral DNA was isolated by the method of Hirt (6). The form I, II, and III viral DNA species were isolated by preparative electrophoresis on an agarose slab gel (10). Virus-specific DNA was identified by use of a ³²P-labeled Fr-MCF cDNA probe made from Fr-MCF 70S viral RNA by reverse transcription (13). Restriction enzyme mapping of form III linear viral DNA revealed a single *Sph*I

FIG. 1. Restriction enzyme maps of F-MuLV, Fr-MCF cl-1, and 54-B viral DNAs. Shown is a schematic representation of the restriction endonuclease sites present in F-MuLV and Fr-MCF cl-1 by enzyme mapping of form III linear viral DNAs obtained from Hirt lysates of acutely infected fibroblast cultures. Restriction mapping of the 54-B viral DNA was performed on molecularly cloned 54-B DNA. Note that the genome of 54-B is permuted in orientation relative to those of F-MuLV and Fr-MCF cl-1 such that the LTR region is located near the center of the 54-B DNA clone. Abbreviations: Ac, *AccI*; Av, *AvaI*; B, *BamHI*; C, *Clal*; E, *EcoRI*; H, *HindIII*; K, *KpnI*; P, *PstI*; Pv, *PvuI*; S, *SphI*; Sc, *SacI*; Sm, *SmaI*; and X, *XbaI*. The heavy lines represent the region of nonhomology between F-MuLV and Fr-MCF.

site (Fig. 1). Form I closed circular viral DNA was linearized by using *SphI* and cloned into the unique *SphI* site of pBR322. Of 5,000 recombinant clones, 3 were identified as containing Fr-MCF sequences by hybridization with the Fr-MCF cDNA probe as previously described (9). One of these clones (54-B) was found to contain an 8.2-kilobase-pair (kbp) retroviral DNA insert. Restriction enzyme mapping of this insert confirmed its identity as a permuted copy of the Fr-MCF viral DNA. However, 54-B contained only a single copy of the Fr-MCF long terminal repeat (LTR) region (Fig. 1).

A comparison of the restriction enzyme maps of the molecularly cloned Fr-MCF DNA versus the F-MuLV DNA reveals that both of these viruses share the same enzyme sites throughout their *gag*, *pol*, and LTR regions. In contrast to these similarities, multiple restriction site differences occurred over a 1.0-kbp region of the viral envelope gene (Fig. 1). These restriction enzyme studies are in agreement with a heteroduplex study of the same cloned DNAs (M. A. Gonda, J. Kamenchik, A. Oliff, S. Anderson, J. Menke, and E. Scolnick, manuscript in preparation). Only a portion of the envelope genes of Fr-MCF and F-MuLV failed to anneal to one another

during the heteroduplex analysis. This region of differing restriction enzyme sites corresponds to the general area of nonhomology between MCFs and their ecotropic MuLV counterparts (4). In the published nucleotide sequence of F-MuLV (7), this area of nonhomology occurs in the N-terminal two-thirds of the gp70 coding region. A more precise localization of these differences will have to await the sequencing of the corresponding region of the Fr-MCF genome.

At least some of the differences between the envelope genes of F-MuLV and Fr-MCF must be responsible for the different host range patterns of these viruses. The similarity between F-MuLV and Fr-MCF throughout the remainder of their genomes suggests that this nonhomologous envelope gene region also encodes the information responsible for the pathogenic differences between these viruses. Fr-MCF is able to infect cells which cannot be infected by F-MuLV. This expanded host range could enable Fr-MCF to penetrate a critical target cell involved in leukemogenesis, or the Fr-MCF envelope glycoprotein may have different functions which contribute to the development of leukemia. Alternatively, our restriction enzyme analysis could have missed differences in the genetic

sequence of the other MuLV genes. Such differences could be responsible for the pathogenicity of Fr-MCF.

MCFs are generated by the infection of murine cells with an ecotropic retrovirus and by the subsequent recombination between this exogenous infecting virus and endogenous host cell sequences which are homologous to either xenotropic or MCF viral sequences (2, 5). We wished to examine the endogenous host cell sequences which participate in the generation of Fr-MCF virus after infection by F-MuLV. Some of these sequences must be present in the Fr-MCF genome. However, these host cell sequences should not be found in F-MuLV DNA. To prepare probes with this specificity, we subcloned a 400-base-pair (bp) fragment of Fr-MCF

FIG. 2. Southern blot analysis of F-MuLV, amphotropic MuLV, and Fr-MCF viral DNAs with the pLEK probe. Southern blot of a 0.5% agarose gel after electrophoresis at 35 V for 18 h. After blotting, the filter was prepared for hybridization as described previously (9) and hybridized to a nick-translated probe made with total pLEK DNA (i.e., viral DNA insert plus pBR322 vector sequences). Hybridization was performed in 50% formamide-4X SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.2% Ficoll-0.2% polyvinylpyrrolidone-0.2% bovine serum albumin-0.1% sodium dodecyl sulfate-0.05 mg of low-molecular-weight salmon sperm DNA per ml at 45°C for 24 h, followed by filter washes in 0.1% sodium dodecyl sulfate- $0.1 \times$ SSC at 55°C until the level of filter radioactivity returned to background. Lane 1, Molecularly cloned F-MuLV DNA (1.0 μ g; 8.2-kbp viral DNA) digested free from its plasmid vector (pBR322) with *Eco*RI; lane 2, molecularly cloned amphotropic MuLV DNA (1.0 μ g; 8.2-kbp viral DNA) digested free from its plasmid vector (pBR325) with *Eco*RI; lane 3, molecularly cloned 54-B viral DNA (1.0 μ g; 8.2-kbp viral DNA) digested free from its plasmid vector (pBR322) with *Sph*I. Note the presence of pBR322 (4.3 kbp) or pBR325 (5.4 kbp) DNA bands in each lane due to hybridization of these vectors with the radiolabeled pBR322 sequences present in the total pLEK DNA probe. Molecular length markers in kbp were obtained by digestion of wild-type lambda phage DNA with *Hind*III and electrophoresis of these DNA fragments in an adjacent lane.

FIG. 3. Southern blot analysis of murine and human cellular DNAs by use of the pLEK probe. High-molecular-weight DNA was obtained from NIH 3T3 cells and human placentas by phenol extraction and ethanol precipitation of sodium dodecyl sulfate (0.5%)-proteinase K (50 mg/ml)-lysed cells or tissue. Twenty micrograms of each DNA was digested with 200 U of *Eco*RI for 2 h and electrophoresed on a 0.5% agarose gel. Subsequent blotting and hybridization were performed as described in the legend to Fig. 2. Lane 1, human placental DNA; lane 2, NIH 3T3 cell DNA. Molecular length markers in kbp were obtained by digestion of wild-type lambda phage DNA with *Hind*III and electrophoresis of these DNA fragments in an adjacent lane.

DNA from the area of nonhomology between Fr-MCF and F-MuLV. The sequences lying between the *Eco*RI and *Kpn*I sites in the Fr-MCF envelope gene were cloned into plasmid pSM804 (a gift from E. Stavnezer). The resulting plasmid was designated pLEK (Fig. 1). Probes made with pLEK DNA hybridized to Fr-MCF DNA but did not hybridize to F-MuLV or amphotropic MuLV DNA (3) (Fig. 2). pLEK also hybridized to multiple bands of *Eco*RI-digested NIH Swiss mouse embryo DNA but did not hybridize to *Eco*RI-digested human placental DNA (Fig. 3). In this regard, our pLEK probe is similar to the *Bam*HI-*Eco*RI probe of spleen focus-forming virus, which hybridizes to the endogenous MCF and xenotropic sequences present in normal murine tissues (1). However, pLEK is not derived from the identical region of the MuLV genome as the *Bam*HI-*Eco*RI probe of spleen focus-forming virus. The 400 bp contained in the pLEK probe lie immediately 3' to the *Bam*HI-*Eco*RI segment of the spleen focus-forming virus-MuLV genome.

pLEK probes were used to examine the RNA from NIH 3T3 fibroblasts infected with Fr-MCF. Polyadenylic acid [poly(A)]-selected RNA from the infected NIH 3T3 cells contained two species of RNA identified by northern blot analysis and hybridization to pLEK (Fig. 4). As

FIG. 4. Northern blot analysis of poly(A)-selected RNAs hybridized with the pLEK probe. Poly(A)-selected RNAs were prepared by lysis of 10^8 nucleated cells from each tissue or cell culture in 4 M guanidinium thiocyanate. RNA was separated from the cell lysates by pelleting through a cesium chloride cushion (5.7 M) at $150,000 \times g$ for 18 h. Poly(A) selection was then carried out with an oligodeoxythymidylate cellulose column. The bound RNA was eluted from the column in sterile distilled water and ethanol precipitated before use. Northern gels were run as 0.8% agarose gels containing 2.2 M formaldehyde. RNA samples were dissolved in 50% formamide–2.2 M formaldehyde– $1 \times$ running buffer (20 mM morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA) and heated to 60°C for 10 min before electrophoresis. Gels were run at 50 V for 18 h and then blotted onto nitrocellulose paper. Prehybridization and hybridization were then performed as described in the legend to Fig. 2. Gels A, B, and C were run separately. Lanes 1 and 5, RNA (100 ng) from NIH 3T3 cells infected with Fr-MCF cl-1; lane 2, RNA (20 μg) from adult mouse liver; lane 3, RNA (20 μg) from adult mouse spleen; lanes 4, 8, and 11, RNA (20 μg) from uninfected NIH 3T3 cells; lane 6, RNA (20 μg) from adult mouse bone marrow; lane 7, RNA (20 μg) from fetal mouse liver; lane 9, RNA (100 ng) from TP-1 cells; lane 10, RNA (20 μg) from RB-1 cells. Note the 34S and 22S viral RNA messages in lanes 1 to 3, 5 to 7, and 9, and the pLEK-like 32S and 26S messages in lane 10. In addition, several minor RNA species are present in lanes 1 to 3, 5 to 7, 9, and 10. These minor bands are inconsistent findings which vary from preparation to preparation and may represent partial degradation products of the 34S or 32S viral RNAs. Molecular size markers represent the migration distances for the 28S and 18S rRNAs present as contaminants in each preparation of poly(A)-selected RNA.

expected, the 34S and 22S RNAs were identified. These RNA species correspond to the complete viral genome (34S) and the envelope gene (22S) messages of Fr-MCF (15). No RNAs homologous to pLEK were found in uninfected NIH 3T3 cells. Poly(A)-selected RNA was prepared from the following normal uninfected NIH Swiss mouse tissues: fetal liver, adult liver, adult spleen, and adult bone marrow. Each of these tissues exhibits low levels of the 34S and 22S RNAs which hybridize to pLEK. However, to attain comparable signals between the RNAs from the infected cells versus the RNAs from uninfected tissues, it was necessary to use 200 times more RNA from each of the uninfected tissues. Densitometer tracings of these signals indicated that the pLEK-like messages in the uninfected cells were present at ca. 1/400 the concentration of the pLEK-like RNAs in the infected cell lines (Fig. 4).

RNA was also prepared from hematopoietic cell lines derived from NIH Swiss mice. TP-1 is an erythroleukemia cell line obtained from a Swiss mouse inoculated with F-MuLV (8). TP-1 cells produce both F-MuLV and Fr-MCF. RB-1

cells are T lymphocytes obtained from a chemically induced T-cell leukemia of a Swiss mouse (unpublished data). As expected, TP-1 cells expressed high levels of 34S and 22S RNAs which hybridized to pLEK. The pLEK probe also identified two species of RNA in the RB-1 cell line. The RB-1 cell messages migrated at 32S and 26S. Again, the relative abundance of the pLEK-related messages in the uninfected RB-1 cells was ca. two logs less than the levels found in the TP-1 cell line.

The pLEK probe described in this report identifies host cell sequences which are homologous to MCF or xenotropic virus-specific sequences. It is unclear whether the pLEK sequences found in Fr-MCF are identical to the MCF or xenotropic sequences present in lymphatic leukemia-inducing MCFs (e.g., AKR MCF 247). However, preliminary studies indicate that pLEK probes do hybridize strongly with AKR MCF 247 DNA (data not shown). Normal NIH Swiss mouse tissues express pLEK-related messages, which indicates that some of the endogenous xenotropic or MCF sequences in these mice are transcriptionally

active. These transcriptionally active pLEK-like sequences may be involved in the recombination event which generates Fr-MCF.

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