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Genome Structure of Mink Cell Focus-Forming Murine Leukemia Virus in Epithelial Mink Lung Cells Transformed In Vitro by Iododeoxyuridine-Induced C3H/MuLV Cells

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We characterized mink cell focus-forming murine leukemia viruses that were isolated from C3H/MCA-5 cells after induction with 5-iododeoxyuridine in culture. Mink lung epithelial cells malignantly transformed in vitro by induced virus were the source of four molecular clones of mink cell focus-forming virus, CI-1, CI-2, CI-3, and CI-4. Three clones, CI-1, CI-2, and CI-3, had full-length mink cell focus-forming viral genomes, one of which (CI-3) was infectious. In addition, we obtained a defective viral genome (CI-4) which had a deletion in the envelope gene. A comparison between the envelope genes of CI-4 and those of spleen focus-forming virus by heteroduplex mapping showed close homology in the substitution region and defined the deletion as being identical to the p15E deletion of spleen focus-forming virus. The recombinant mink cell focus-forming genomes are not endogenous in C3H/MCA-5 cells and therefore must have been formed in culture after induction by 5-iododeoxyuridine. CI-3, the infectious clone of mink cell focus-forming murine leukemia virus, was dualtropic, and mink cells infected with CI-3 were altered in their response to epidermal growth factor. In the presence of epidermal growth factor at 10 ng/ml, uninfected mink cells retained their epithelial morphology in monolayer culture and did not form colonies in soft agar. In contrast, CI-3 virus-infected mink cells grew with fibroblastic morphology in monolayer culture and showed an increased growth rate in soft agar in the presence of epidermal growth factor.

Mink cell focus-forming (MCF) murine leukemia viruses (MuLVs) form cytopathic foci in Mv1Lu mink cell monolayers (17, 23, 39) and are thought to be recombinants between ecotropic viruses and other endogenous viruses which are related to, but distinct from, isolated xenotropic MuLV (5, 11, 12, 42, 45). The recombination is in the envelope gene and alters the host range of the virus such that it can infect both mouse and nonmouse cells; i.e., the viruses are dualtropic (17, 23, 38). The novel viral host range is due to a recombinant envelope glycoprotein, gp70, with binding specificity for a chromosome 2-coded receptor which is distinct from the cell surface receptor used by ecotropic, xenotropic, and amphotropic MuLV (35). MCF viruses are found in vivo during the preleukemic period in the thymus of high-leukemia strains of mice such as AKR and also during development of leukemia induced in leukemia-negative mice after injection of exogenous ecotropic MuLV (23, 39, 42, 52–54). Since inoculation of young AKR mice with certain of these recombinants accelerates leukemia development, these viruses have been thought to be necessary intermediates in the transformation process (8, 23, 33, 34, 44). Recently, workers in several laboratories have shown that spontaneous lymphomas of AKR mice regularly contain at least one genome of a recombinant MCF-class genome (5, 25, 57), thus providing strong support for this hypothesis.

We have observed that MCF MuLVs are present after 5-iododeoxyuridine (IdUrd) induction of C3H/10T1/2 and C3H/MCA-5 cells in culture (39–41). We have also shown that cells can be transformed in vitro with virus selected from the total population of IdUrd-induced viruses (C3H MuLV). Growing these IdUrd-induced viruses in a variety of target cells resulted in the isolation of viruses which induce carcinomas and sarcomas in vivo (37, 40). In this report we present the structure of viral genomes present in a clone of Mv1Lu epithelial mink lung cells that was transformed in culture by IdUrd-induced C3H MuLV. We also show that these virus-transformed cells are sensitized to the ac-

tion of epidermal growth factor (EGF), which strongly enhances their transformed phenotype.

MATERIALS AND METHODS

Cells and viruses. Cell lines used included NIH 3T3 mouse fibroblasts (29), the feral mouse cell line Sc-1 (22), and Mv1Lu epithelial mink lung cells (24). IdUrd-induced C3H MuLV was obtained from C3H/10T1/2 and C3H/MCA-5 cells as previously described (38). All cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Transformation of Mv1Lu cells with virus from IdUrd-induced C3H cells was performed as previously described (41). Transformed cell colonies were selected after infected cells were seeded in 0.3% soft agar (Noble) (39). When the susceptibility of cells to colony induction by EGF was tested, the soft agar was supplemented with 10 ng of EGF (Collaborative Research, Inc.) per ml.

Transfection of viral DNA. NIH 3T3 Cl 7 cells are a subline of NIH 3T3 cells selected for flat morphology. These cells were transfected with viral DNA by using calcium phosphate according to a modification of the procedure of Graham and van der Eb (18). Cells were seeded at 5×10^5 per T25 flask (Falcon Plastics) and used for transfection on the following day. Test DNA (between 0.5 and 7 μg per reaction) was added to carrier calf thymus or salmon sperm DNA (Worthington Diagnostics) (10 to 20 μg per reaction and sheared 5 to 10 times through a 22-gauge needle) dissolved to yield final concentrations of 140 mM NaCl, 5 mM KCl, 7 mM Na_2HPO_4, 6 mM dextran, and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.0). The DNA was precipitated by addition of 1.3 M $CaCl_2$ to a final concentration of 125 mM, followed by incubation at room temperature for 30 min. Medium was removed from the cells, and 0.55 ml of the precipitated DNA was added to each flask. The flasks were rocked for 20 min at room temperature, and 4.5 ml of culture medium was then added. The cells were incubated for 4 to 5 h at 37°C, and the medium was then replaced by fresh medium containing 20% dimethyl sulfoxide. After exposure of the cells to dimethyl sulfoxide for 3 to 5 min, the medium was removed and the cells were rinsed three times and refed with 5 ml of culture medium. At confluency, the cells were transferred to T75 culture flasks and monitored for production of DNA polymerase-containing particles as previously described (38).

Isolation of unintegrated viral DNA. NIH 3T3 (29) mouse fibroblast cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Cells were infected with virus from IdUrd-induced C3H/MCA-5 Cl 8 cells (38) for 24 h and washed twice with phosphate-buffered saline, and the unintegrated viral DNA was isolated by hydroxyapatite chromatography (48).

Isolation of cellular DNA. Cells suspended in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) were lysed by incubating them for 2 h at 37°C in 1× SSC containing 0.5% sodium dodecyl sulfate and 100 μg of proteinase K (E. Merck AG) per ml. The DNA was then purified as previously described (1).

Gel electrophoresis and blotting. DNA was electrophoresed in 0.7, 0.8, or 1.0% agarose gels (Seakem; ME grade) with a Tris-acetate buffer (pH 7.8) (47). Restriction fragments of λ and φX174 DNA were used as size markers. ^{32}P-labeled markers were made by using avian myeloblastosis virus DNA polymerase to fill in the ends of *Hin*dIII fragments of λ and *Taq*I fragments of φX174 (55). After electrophoresis, DNA was transferred from gels onto 0.45-μm nitrocellulose membranes (Schleicher & Schuell Co.) as described by Southern (50) or onto Genescreen (New England Nuclear Corp.) by a modification of the technique of Southern as recommended by New England Nuclear Corp.

Preparation of probes and hybridization of blots. cDNA was prepared by using viral 70S RNA as the template for avian myeloblastosis virus DNA polymerase as previously described (1). Nick-translated probes were prepared with cloned DNA as previously described (4). Baked nitrocellulose and Genescreen membranes containing the blotted DNA were hybridized according to published procedures (1).

Cloning of viral DNA. Circular viral DNA was purified by electrophoresis of unintegrated viral DNA in a 0.8% agarose gel. The circular DNA, after *Eco*RI digestion, was ligated with 5.0 μg of λgtWES · λB *Eco*RI arms (20). The ligated DNA was packaged into phage particles as described by Enquist and Sternberg (13). Phage were propagated in *Escherichia coli* DP50 *supF*, and plaques were screened for viral DNA by the plaque lift procedure of Benton and Davis (2) with viral cDNA as a probe. Phage particles were purified in CsCl gradients as described by Fred Blattner in his outline accompanying the Charon phage. The method of phage DNA extraction has been published (32).

Phage DNA containing the C3H viral DNA inserts was digested with *Eco*RI, and the inserts were purified by electrophoresis in low-melting-temperature agarose (Seaplaque) gels (56). Insert DNA (1.0 μg) was ligated to pBR322 DNA (0.2 μg) under the reaction conditions described above. The pBR322 DNA had previously been digested with *Eco*RI and bacterial alkaline phosphatase (Bethesda Research Laboratories). *E. coli* HB101 was transformed (9), and ampicillin-resistant colonies were screened for C3H viral DNA (19) by using viral cDNA as a probe. The CI-3 clone was also subcloned in a form colinear with viral RNA by ligating the *Eco*RI ends, cutting the long terminal repeat (LTR) sequences with *Pst*I, and inserting the digested DNA into the *Pst*I site of pBR322.

Preparation of heteroduplexes. Cloned viral DNAs used for these experiments were separated from their cloning vectors by restriction endonuclease digestion and preparative gel electrophoresis, followed by phenol extraction and ethanol precipitation. Residual impurities were removed by DEAE-cellulose chromatography. Viral DNA was suspended in 10 mM Tris (pH 7.4)–1 mM EDTA to a final concentration of 100 μg/ml. Approximately 0.1 μg of each DNA to be heteroduplexed was mixed and then denatured by incubation in 0.1 N NaOH for 10 min at 37°C. The solution was neutralized by the addition of 1/5 volume of 1 M Tris-hydrochloride (pH 7.0). Deionized formamide was added to a final concentration of 50%. Renaturation was allowed to occur for 20 to 30 min (58).

Electron microscopy. The heteroduplexes were mounted for electron microscopy by the basic protein film technique of Davis et al. (10). Hyperphases usually consisted of 55% formamide, 100 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic

acid], 10 mM EDTA, cytochrome *c* at a concentration of 30 μg/ml, and the duplexed DNA. Hyperphases were spread onto a hypophase of 15% formamide and 1/10 the electrolyte (= 10 mM TES, 1 mM EDTA), picked up on Parlodion-covered 300-mesh copper grids, dehydrated in 80% ethanol, and rotary shadowed with platinum-palladium. Photographed heteroduplex molecules were measured with a digital length calculator from actual micrographs. Since many of the genomes were cloned in a permuted fashion, many heteroduplexes formed circles. We used these circular DNA molecules as internal standards for length calculations, equating them to the sizes of the DNA molecules as determined from gel electrophoresis experiments.

RESULTS

Derivation of transformed Mv1Lu cells. NIH cells chronically infected with IdUrd-induced C3H MuLV from C3H/MCA-5 cells were mixed with Sc-1 cells at ratios of 40:60, 20:80, 10:90, 5:95, and 2.5:97.5. The presence of Sc-1 cells in this mixture was required for the detection of mink-plating virus since this virus is not detectable upon direct infection of mink cells with type C virus induced from C3H/10T1/2 cells (36, 41). The SC-1 cells are presumably necessary to allow unmasking (16, 36, 41) of dualtropic viruses. The mixed cell cultures were cocultivated with an equal number of Mv1Lu Cl 12 cells in T75 culture flasks in the presence of Polybrene at 4 μg/ml. Culture fluids were assayed for reverse transcriptase at every subculture, and the Mv1Lu cells were observed for the emergence of transformed foci. As foci became apparent, the transformed cells were dislodged mechanically and transferred to a new culture vessel. At each of the following four subcultures, virus produced by these cells was used to infect non-neoplastic Mv1Lu Cl 12 cells which were then assayed for colony formation in soft agar. In this fashion a large number of transformed clones from soft-agar colonies were obtained, including agar clone 31 (ACl 31) which resulted from a coculture that had a mixing ratio of 20% virus producer to 80% virus-free Sc-1 cells. The viral genomes present in the transformed ACl 31 cells were characterized. In addition to ACl 31 cells, which are positive for virus production, several nonproducer transformed clones were isolated after ACl 31 was replated in agar.

Southern blot analysis of ACl 31 cell DNA with AKR MuLV cDNA. Figure 1 shows a blot of cellular DNA from ACl 31 cells before and after digestion with *Eco*RI. Intense bands of unintegrated linear and nicked circular proviral genomes were observed in the undigested DNA. The two size classes of nicked circular DNA presumably correspond to full-length viral genomes containing one and two terminal repeats. These circles were cut once by *Eco*RI to yield fragments of 8.6 and 8.0 kilobases (kb), whereas the linear genome was cut into two fragments of 1.8 and 6.8 kb. The additional minor bands presumably resulted from the cleavage of integrated genomes (see below).

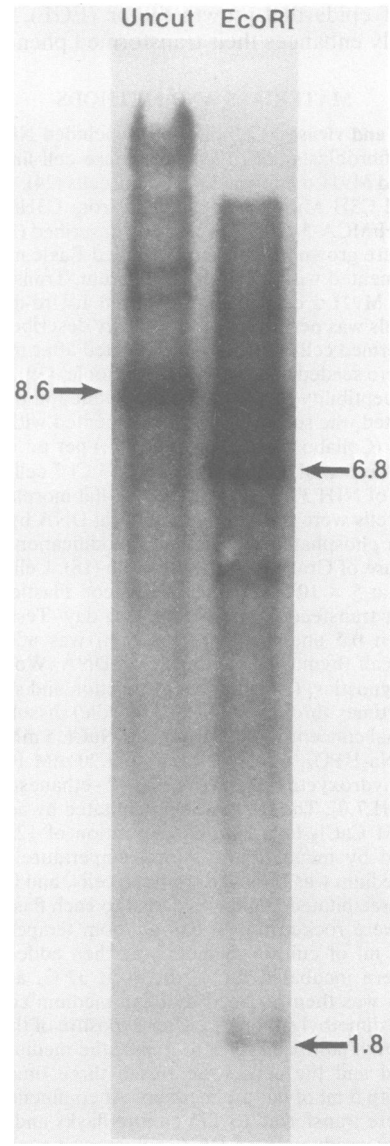

FIG. 1. Southern blot analysis of ACl 31 cell DNA (5 μg) with AKR MuLV cDNA, before and after digestion with restriction endonuclease *Eco*RI. The DNA was electrophoresed on a 1.0% agarose gel, transferred to nitrocellulose paper, and hybridized with AKR [^{32}P]cDNA.

Cloning of the persistent unintegrated genome. Unintegrated proviral DNA was separated from high-molecular-weight ACl 31 cellular DNA by hydroxyapatite chromatography. The circular DNA fraction was purified by agarose gel elec-

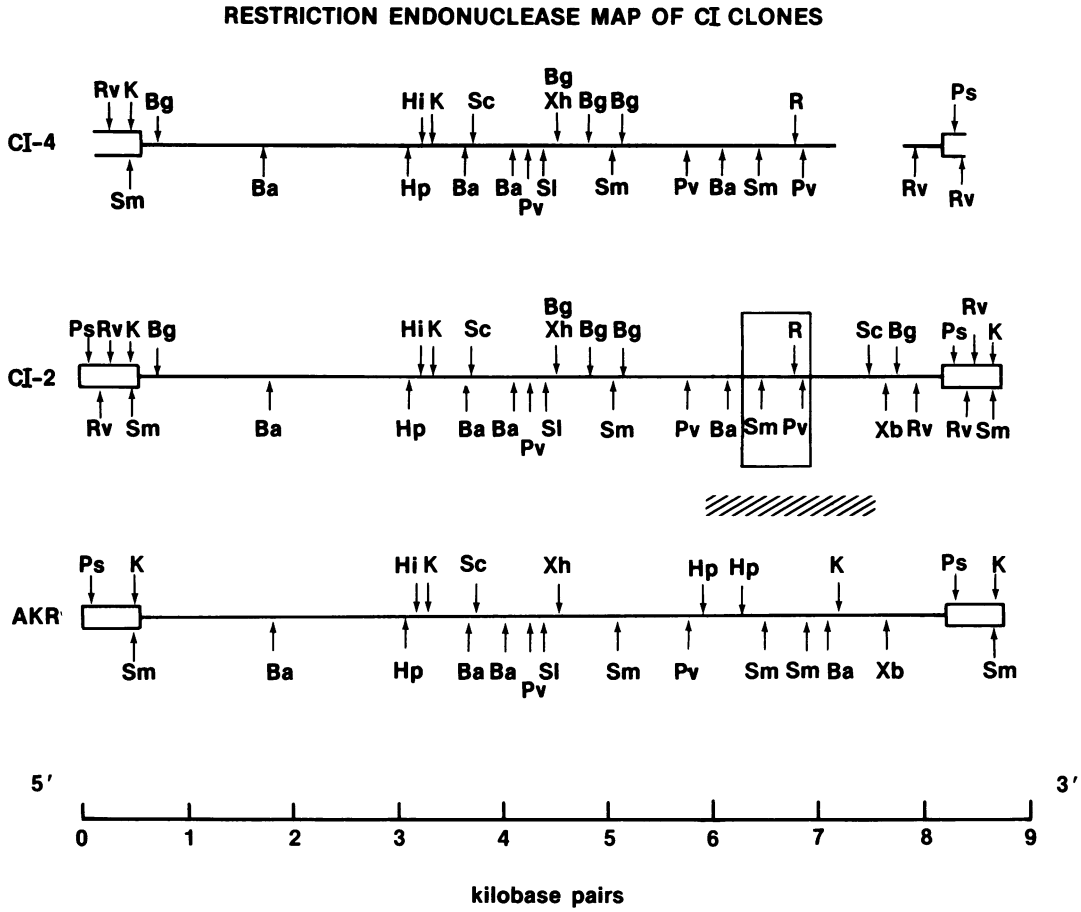

FIG. 2. Restriction endonuclease map of CI clones. The boxed area on the map of CI-2 delineates the location of a substitution loop that is apparent in heteroduplexes between DNAs from CI-2 and from AKR virus. The shaded band indicates the area where the CI-2 map differs from the map of AKR ecotropic MuLV. Restriction endonucleases *Eco*RV and *Bgl*II have not been located on the AKR map. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; Hi, *Hin*dIII; Hp, *Hpa*II; K, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*II; R, *Eco*RI; Rv, *Eco*RV; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I; Xb, *Xba*I; Xh, *Xho*I.

trophoresis, digested with *Eco*RI, and cloned in the *Eco*RI site of λgtWES · λB. After plaques were screened with viral cDNA, four clones (for C3H, IdUrd induced), CI-1, CI-2, CI-3, and CI-4, were obtained. Restriction maps (Fig. 2) were made of the viral genomes as previously described (4). CI-1 was identical to CI-2 and CI-3 except that CI-1 contained only a single LTR. CI-3 has the same restriction map as CI-2. A comparison of the maps of CI-2 and CI-4 with that of AKR ecotropic MuLV (31) showed near identity except for a region between 5.9 and 7.9 kb. A comparison of the CI-3 and CI-4 restriction maps indicates that CI-4 has a deletion of 0.70 kb located between the *Pvu*II site at 6.9 kb and the *Eco*RV site at 7.9 kb. Since the *Sac*I, *Xba*I, and *Bgl*II sites of CI-2 are missing, the 3' end of the deletion is presumably between 7.8 and 7.9 kb of CI-2. In addition, CI-4 contains only a single LTR.

Heteroduplex analyses of cloned viral genomes. To further compare the similarities and differences which exist between the cloned CI viral genomes and that of the cloned AKR ecotropic MuLV (kindly provided by D. Lowy), heteroduplex analyses were performed. A heteroduplex analysis of CI-3 and AKR cloned genomes is shown in Fig. 3. Both viral DNAs were subcloned at the *Pst*I site in the LTR, and they are therefore colinear with respect to their viral message. A single unequal substitution was localized (near the 3' end of the viral genome) in the *env* gene region. This substitution appeared in two forms. The majority of heteroduplexes (form I, Fig. 3A) consisted of the single large substitution. A minor population (form II, Fig.

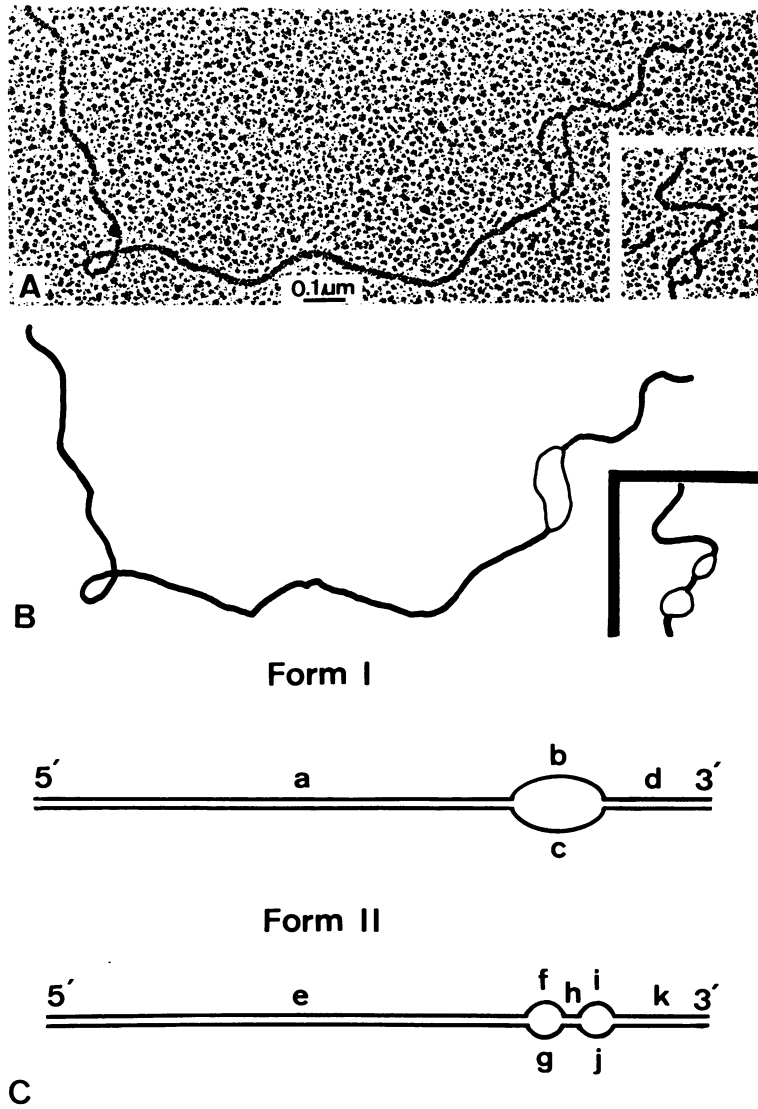

FIG. 3. Heteroduplex analysis of the relationship between CI-3 and AKR cloned genomes. These viral DNAs were cloned in the LTR in a 5′-to-3′ orientation and are colinear with respect to their viral message. (A) Actual heteroduplex. (B) Interpretive drawing. (C) Schematic representation. There were two forms of substitutions with these heteroduplexes. The majority of heteroduplexes (form I) appear in panels A and B. The inserts (A and B) show the minor population (form II). The dimensions given in panel C are based on measurements of more than 20 molecules. Contour lengths (in kb) were as follows: a, 6.20 ± 0.20; b, 0.69 ± 0.06; c, 0.83 ± 0.12; d, 1.32 ± 0.09; e, 6.20 ± 0.20; f, 0.32 ± 0.03; g, 0.49 ± 0.04; h, 0.13 ± 0.02; i, 0.25 ± 0.02; j, 0.26 ± 0.03; k, 1.28 ± 0.07. Form I of CI-3 includes a, b, and d; form I of AKR includes a, c, and d; form II of CI-3 includes e, f, h, i, and k; and form II of AKR includes e, g, h, j, and k.

3A) was also encountered. With these heteroduplexes, a small region of homology (0.13 kb) was detected approximately in the middle of the large substitution. The longer arm (0.83 kb) of the form I substitution was assigned to AKR, whereas the shorter arm (0.69 kb) was assigned to CI-3 based on the known sizes of each of the cloned DNAs from electron microscope and gel electrophoresis measurements. When stronger denaturation conditions, i.e., 60 to 70% formamide, were used in the spreading solution, only form I was present. We used this structural difference to map the substitution in the following heteroduplexes.

A heteroduplex analysis of CI-4 and AKR cloned genomes is shown in Fig. 4. The CI-4

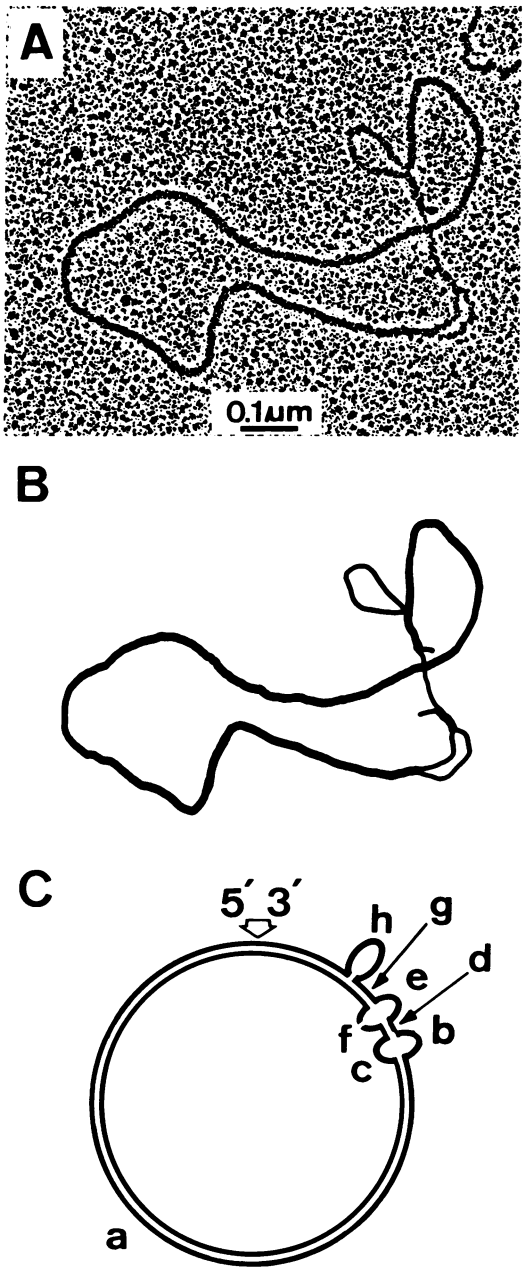

FIG. 4. Heteroduplex analysis of the relationship between CI-4 and AKR cloned genomes. The CI-4 viral DNA was cloned in a permuted fashion in relation to its viral message. The AKR viral DNA was the same colinear clone used for Fig. 3. (A) Actual heteroduplex. (B) Interpretive drawing. (C) Schematic representation. The dimensions given in panel C are based on measurements of more than 20 molecules. Contour lengths (in kb) were as follows: a, 6.41 ± 0.20; b, 0.43 ± 0.06; c, 0.34 ± 0.04; d, 0.11 ± 0.04; e, 0.27 ± 0.05; f, 0.19 ± 0.06; g, 0.23 ± 0.06; h, 0.77 ± 0.09. CI-4 includes a, c, d, f, and g; AKR includes a, b, d, e, g, and h. Note that loop f is broken in the middle at the *Eco*RI site of CI-4.

viral DNA was cloned in a permuted fashion in relation to its viral RNA. The AKR viral DNA was the same colinear clone used in CI-3 × AKR heteroduplexes shown in Fig. 3. The presence of a circular DNA molecule after heteroduplexing is a consequence of using two circularly permuted DNA molecules which were cloned at different enzyme sites. CI-4 × AKR heteroduplexes were circular, with a substitution (form II) in the *env* gene region similar to that noted for CI-3 × AKR heteroduplexes. The *Eco*RI ends of CI-4 occur within loop f. As expected from the restriction map, a deletion of 0.77 kb was observed in CI-4 which was located 3′ of the *env* substitution. Since the *Pst*I-cut AKR and CI-4 molecules both contain a single LTR, there is no additional feature.

To verify that the large substitutions present in CI-3 and CI-4 were the same, heteroduplexes between *Eco*RI clones of these viral DNAs were performed (Fig. 5). The *Eco*RI clones are permuted relative to their viral RNA, but linear heteroduplexes were observed because both clones were inserted into pBR at their common *Eco*RI site. With these heteroduplexes, two deletions were observed in CI-4, whereas the rest of the genome was totally homologous to CI-3. One of the deletions is of the same size and location as the one observed between CI-4 and AKR. The second deletion of 0.53 kb in CI-4 represents a deleted LTR which was evident in the restriction map of CI-4. Presumably the cloning of CI-4 DNA was from an unintegrated circular viral DNA intermediate with a single LTR. These heteroduplexes establish that the large substitutions present in CI-3 and CI-4 noted in heteroduplexes to AKR are identical.

Because the MCF activity of CI-3 suggests a similar origin of envelope sequences to other MCF viruses, we wanted to determine the relationship of CI-3 and CI-4 to other MCF recombinants. In a recent heteroduplex study between Friend-MCF and -MuLV (M. A. Gonda, J. Kamenchik, A. Oliff, S. Anderson, J. Menke, and E. M. Scolnick, manuscript in preparation), a similar large substitution in the *env* gene region was observed to have two forms at different stringencies, similar to those observed here between CI-3 and AKR viral DNAs. Furthermore, spleen focus-forming virus (SFFV), which is a defective recombinant virus of the Friend virus complex, was shown to share *env* sequence homology with Friend-MCF but to have a deletion of p15(E) in the *env* gene. This finding is in accord with the protein studies of Schultz et al. (46) that demonstrated the absence of p15(E) antigen in SFFV nonproducer cells. Because of the similarities which exist between the relationship of AKR, CI-3, and CI-4 and that of the members of the pathogenic Friend virus com-

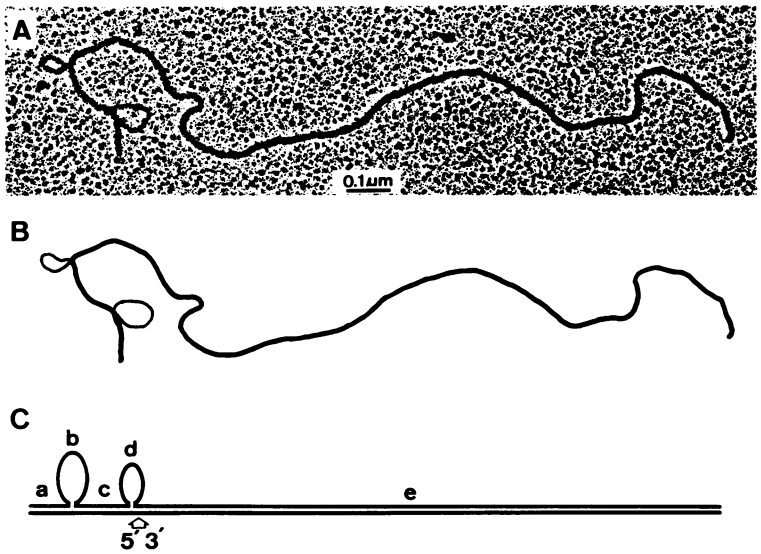

FIG. 5. Heteroduplex analysis of the relationship between CI-3 and CI-4. These viral DNAs were cloned in a permuted fashion with respect to their viral message. These *Eco*RI clones cleave in the *env* gene region. (A) Actual heteroduplex. (B) Interpretive drawing. (C) Schematic drawing. The dimensions given in panel C are based on measurements of more than 20 molecules. Contour lengths (in kb) were as follows: a, 0.36 ± 0.04; b, 0.69 ± 0.10; c, 0.48 ± 0.05; d, 0.53 ± 0.05; e, 6.47 ± 0.30. CI-3 includes a, b, c, d, and e; CI-4 includes a, c, and e.

plex, heteroduplexes were performed with a 2.4-kb piece of SFFV containing the *env* gene. A heteroduplex of CI-3 and SFFV (Fig. 6) showed a 0.65-kb deletion in the *env* gene of SFFV in the same location as seen in comparisons between CI-3 and CI-4. The rest of the *env* gene was totally homologous, indicating that the large *env* gene substitution in CI-3 was closely related to the MCF sequences in SFFV. Heteroduplexes of CI-4 and SFFV (Fig. 7) showed no substitutions or deletions, which implies that they have nearly identical p15(E) deletions (46; Gonda et al., in preparation).

Origin of the recombinant viral genome. Our selection scheme for the isolation of mink cell-transforming virus from IdUrd-induced C3H/MCA-5 cells had several steps, including transfer of virus to Sc-1 and Mv1Lu cells. Thus, the MCF viral genome either was preexisting in the population of induced viruses or was generated during infection of Sc-1 or Mv1Lu Cl 12 mink cells. To determine whether the viral genomes that we have molecularly cloned were present among the IdUrd-induced viruses, we prepared unintegrated provirus from NIH 3T3 cells that had been acutely infected with IdUrd-induced C3H MuLV. A Southern blot analysis showed that the vast majority of full-length linear genomes were resistant to digestion by *Eco*RI, as expected for ecotropic MuLV (31). In an attempt to test for the presence of genomes with an *Eco*RI-sensitive site similar to that of the virus that we cloned from transformed Mv1Lu cells, circular proviral DNA was digested with *Eco*RI and cloned in the RI site of λgtWES · λB. We obtained three molecular clones which had an insert that was indistinguishable by restriction mapping from that of the full-length ACl 31-derived clones. Thus, we conclude that the virus induced from C3H fibroblast cells in culture either contains CI genomes or easily gives rise to these recombinants upon acute infection of Sc-1 or NIH 3T3 cells.

The MCF MuLV present in C3H/MCA-5 cells that were chronically infected after IdUrd induction did not exist in the DNA of cells before induction (Fig. 8). A Southern blot of *Eco*RI-*Xba*I-digested cellular DNA (Fig. 8A) from CI-3 virus-infected NIH 3T3 cells (lane 3) compared with DNA from uninfected NIH 3T3 (lane 2) and C3H/MCA-5 (lane 1) cells shows the recombinant virus-specific fragment of 0.9 kb only in the infected cells. A comparison with lane 4, which contains the equivalent of a single copy of CI-2 cloned DNA per haploid genome of NIH 3T3 DNA, indicates that the infected cells contained approximately two viral copies per haploid genome (or four copies per cell) and clearly demonstrates that a single copy of recombinant virus would have been detected in the uninfected cells. This result is corroborated by the *Bam*HI-*Xba*I digests (Fig. 8B), which detected the recombinant band of 1.55 kb in the infected cells but not in the uninfected cells. Since the

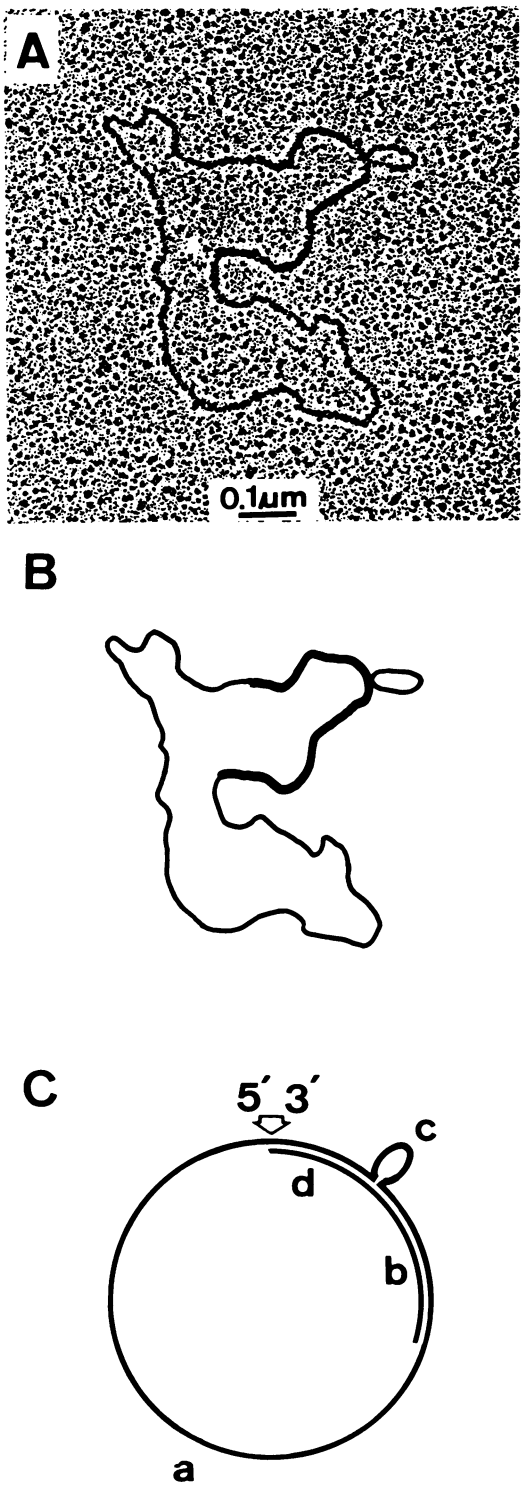

FIG. 6. Heteroduplex analysis of the relationship between CI-3 and a 2.4-kb clone of SFFV representing all of the *env* gene and the LTR region. (A) Actual heteroduplex. (B) Interpretive drawing. (C) Schematic representation. The dimensions given in panel C are based on measurements of more than 20 molecules. Contour lengths (in kb) were as follows: a, 5.06 ± 0.21; b, 1.53 ± 0.11; c, 0.65 ± 0.06; d, 0.83 ± 0.07. CI-3 includes a, b, c, and d; SFFV includes b and d.

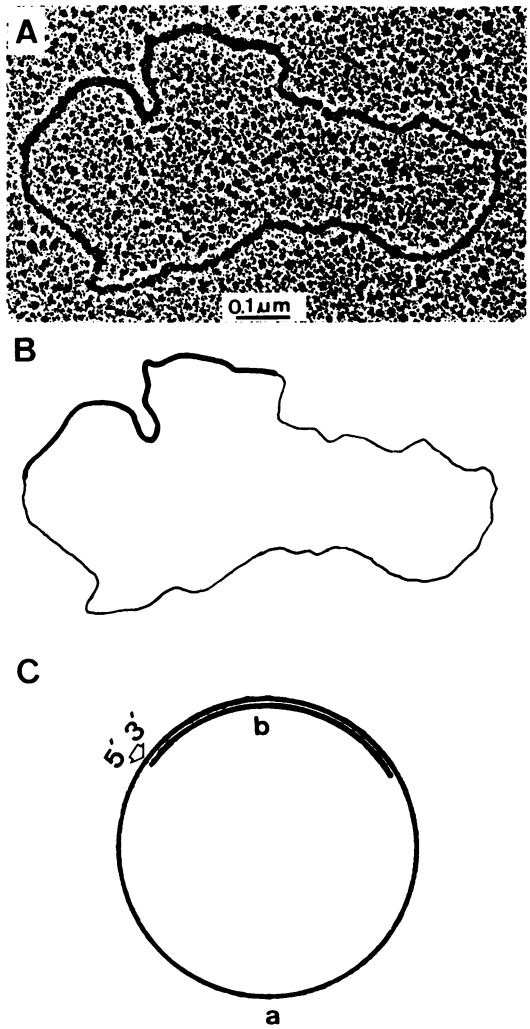

FIG. 7. Heteroduplex analysis of the relationship of CI-4 and a 2.4-kb clone of SFFV representing all of the *env* gene and the LTR region. (A) Actual heteroduplex. (B) Interpretive drawing. (C) Schematic representation. The dimensions given in panel C are based on measurements of more than 10 molecules. Contour lengths (in kb) were as follows: a, 4.81 ± 0.38; b, 2.43 ± 0.25. CI-4 includes a and b; SFFV includes b.

MCA-5 cells represent the progeny from a single clone of transformed C3H/10T1/2 cells, it appears unlikely that only a small fraction of these cells would contain CI virus. Thus, if CI virus is present, it should be present at a level of at least one copy per cell.

Viral DNA in nonproducer transformed ACI

31-2 cells. Upon replating in soft agar, the productively transformed ACl 31 mink lung cells gave rise to transformed cell clones which were negative for virus production as judged by assay for particle-bound reverse transcriptase released into the culture fluid. To characterize the mouse viral genomes that were present in these cells, their DNA was subjected to a Southern blot analysis. Since we had observed the presence of persistent unintegrated viral genomes in these cells, the unintegrated DNA was first removed by preparative gel electrophoresis. Blots were hybridized at 1.5× SSC at 60°C with a nick-translated subclone of the CI-3 LTR in pBR322

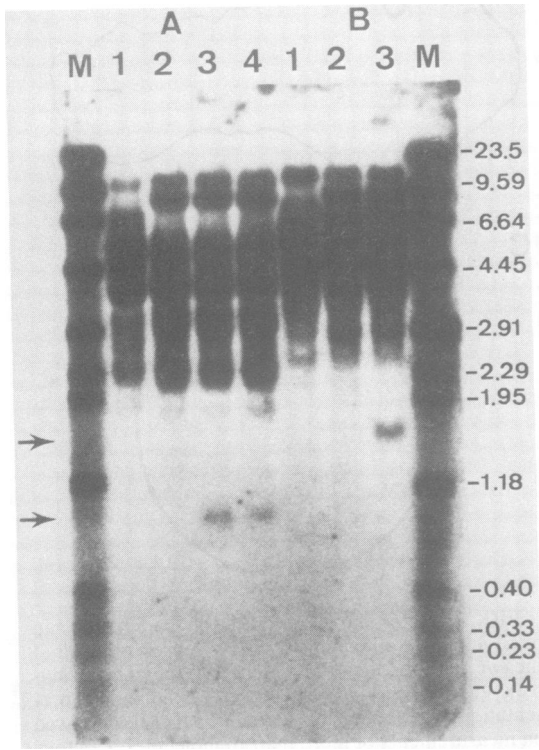

FIG. 8. Southern blot analysis of C3H/MCA-5 and NIH 3T3 cellular DNAs. Cellular DNAs were digested with *Eco*RI-*Xba*I (A) or *Bam*HI-*Xba*I (B). Lanes: 1, C3H/MCA-5 DNA; 2, NIH 3T3 DNA; 3, DNA from NIH 3T3 cells infected with CI-3 virus recovered from transfection with CI-3 DNA; 4, mock single copy containing 3 pg of CI-2 DNA per μg of NIH 3T3 DNA. Marker sizes (lane M) (in kb pairs): λ DNA digested with *Hin*dIII, 23.5, 9.59, 6.64, 4.45, 2.29, and 1.95; ϕX174 DNA digested with *Taq*I; 2.91, 1.18, 0.40, 0.33, 0.23, and 0.14. DNAs were sequentially digested with *Xba*I followed by *Eco*RI or *Bam*HI, electrophoresed in a 1% agarose gel, transferred to Genescreen (New England Nuclear Corp.), and hybridized with a nick-translated *Eco*RI-to-*Bgl*II fragment of CI-3 DNA (3′ probe; map position, 6.8 to 7.8 kb). Only panel A, lanes 3 and 4, and panel B, lane 3, show fragments of appropriate size for the recombinant virus.

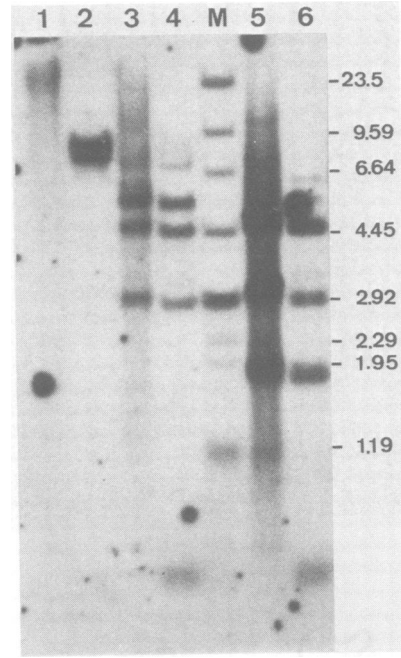

FIG. 9. Digestion of unintegrated and high-molecular-weight ACl 31-2 cell DNA. Lanes: 1, 3, and 5, high-molecular-weight DNA; 2, 4, and 6, unintegrated DNA; 1 and 2, undigested DNA; 3 and 4, *Kpn*I digest; 5 and 6, *Kpn*I-*Eco*RI sequential digests; M, markers as described in the legend to Fig. 8. DNAs were electrophoresed, blotted, and hybridized as described in the legend to Fig. 8, except that the probe contained the CI-3 LTR.

(Fig. 9). Lanes 1 and 2 show undigested DNAs from the high (>23 kb)- and low (<23 kb)-molecular-weight DNAs, respectively. The low-molecular-weight DNA clearly shows unintegrated DNA fragments of 8.6 and 7.7 kb; furthermore, the *Kpn*I digest of these two DNAs (lanes 3 and 4) shows bands at 5.4, 4.5, and 2.85 kb, as would be expected from the maps of CI-3 and CI-4. However, *Kpn*I-*Eco*RI double digests of these DNA fractions (lanes 5 and 6) did not show the 4.5-kb *Kpn*I band being cut by *Eco*RI as expected for CI-4. Similar *Pvu*II-*Kpn*I digests (not shown) also did not produce the band expected for CI-4. Instead, there was a band of 1.9 kb, suggesting a deletion of 0.9 kb which includes the *Pvu*II site at 6.8 kb on the map.

To further establish the nature of the integrated viral genomes in ACl 31-2 cells, cellular DNA was digested with a series of restriction enzymes that yield fragments characteristic of the CI-3 and CI-4 viral genomes (Fig. 10). Lanes 1 and 2 show the 0.9-kb *Pvu*II-*Xba*I and *Eco*RI-*Xba*I fragments expected from the map of CI-3. Lane 3 shows the presence of a 2.1-kb *Bam*HI-*Pst*I fragment corresponding to CI-3 and a second

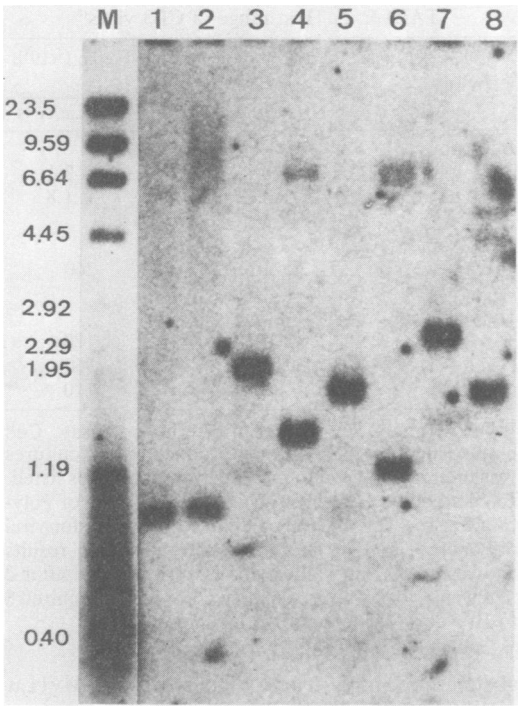

FIG. 10. Double-digest analysis of high-molecular-weight DNA from ACl 31-2 cells. Lanes: 1, *Pvu*II-*Xba*I; 2, *Eco*RI-*Xba*I; 3, *Bam*HI-*Pst*I; 4, *Eco*RI-*Pst*I; 5, *Bam*HI-*Eco*RV; 6, *Eco*RI-*Eco*RV; 7, *Bam*HI-*Kpn*I; 8, *Eco*RI-*Kpn*I; M, markers as described in the legend to Fig. 8. DNA from ACl 31-2 (high-molecular-weight fraction) was restricted sequentially, electrophoresed, transferred to Genescreen, and hybridized to a 3′ probe as described in the legend to Fig. 8.

fragment of 1.2 kb which represents a deletion of 0.9 kb. Double digests of cellular DNA with *Bam*HI and *Kpn*I as well as with *Eco*RI and *Kpn*I also yielded fragments as expected from the map of CI-3 (lanes 7 and 8) and, in addition, fragments corresponding to a 0.9-kb deletion. The deleted genome does not contain an *Eco*RI site, since lane 4 contains the 1.4-kb *Eco*RI-*Pst*I band expected from CI-3 but no 0.5-kb deletion fragment. The 3′ end of the deletion does not extend beyond the *Eco*Rv site that defines the 3′ end of the deletion in CI-4 (Fig. 10, lane 5). This is apparent from the presence in lane 5 of a 1.0-kb band derived from the deleted genome, which is 0.9 kb smaller than the corresponding CI-3 fragment of 1.9 kb. Very weak hybridization to the deleted genome with this probe and strong hybridization with the LTR probe (Fig. 9) implies that the deletion contains most of the 3′ probe. The fact that the deletion fragment is detected with the *Eco*RI-*Bgl*II probe indicates that the 3′ end of the deletion is 5′ of the *Bgl*II site. In conclusion, we have shown that nonproducer ACl 31-2 cells contain CI-3 virus in both unintegrated and integrated forms. CI-4 virus was not present at significant levels; however, we identified another genome in both unintegrated and integrated forms which had a more extensive *env* deletion, encompassing the *Eco*RI site and the adjacent *Pvu*II site of CI-2.

To quantitate the average number of unintegrated viral genomes per infected cell, a comparison (Fig. 11) was made between the intensity of bands of defined amounts of *Eco*RI-digested CI-2 in pBR322 and that of bands of undigested DNA from ACl 31-2 cells. The intensity of unintegrated viral DNA bands in lane 4 is greater than that of the lower band in lane 3 (33 pg of pBR322 DNA) and less than that of the upper band in lane 3 (67 pg of CI-2 viral DNA). We conclude that the combined intensity of the two viral DNA bands in lane 4 corresponds to approximately 50 pg, or 5×10^6 copies of viral DNA, per 10 μg of cellular DNA. Since 10 μg is equivalent to the DNA content of 2×10^6 cells, we conclude that there are about three copies of unintegrated viral DNA per cell.

Biological activity of cloned proviral DNA from ACl 31 cells. Cloned proviral DNA in pBR322

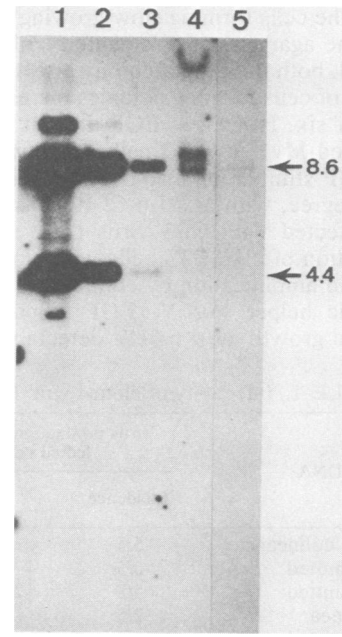

FIG. 11. Average number of unintegrated genomes per infected cell. pBR322 with CI-2 insert DNA was digested with *Eco*RI and loaded in wells at 10 ng (lane 1), 1 ng (lane 2), 100 pg (lane 3), and 10 pg (lane 5). Total cellular DNA from ACl 31-2 cells was loaded at 10 μg per well (lane 4). DNA was electrophoresed, blotted, and hybridized as described in the legend to Fig. 8. The probe was nick-translated DNA from CI-2 virus cloned in pBR322.

was used for transfection onto sensitive NIH 3T3 cells. In the case of CI-3, the colinear form was cut out of pBR322 with *PstI* and then used for transfection directly or after ligation to itself. In the cases of CI-2 and CI-4, the viral DNA insert was separated from pBR322 by *EcoRI* digestion. The viral DNA was ligated to itself, and the resulting concatenated DNA was used for transfection. Only the ligated CI-3 viral DNA was infectious (Table 1). Infectious virus emerged slowly, requiring 8 weeks to reach plateau levels of virus production (Table 1). The host range of this poorly infectious virus identifies it as a dualtropic MuLV (Table 2). At present we do not have an explanation for the lack of infectivity observed with CI-2 viral DNA, which by heteroduplex and restriction enzyme mapping was identical to CI-3. However, it is not the result of using *PstI* digests of CI-3 and *EcoRI* digests of CI-2, since CI-3 is also infectious when ligated *EcoRI* fragments of the permuted genome are used.

Transformed phenotype of ACl 31 producer and nonproducer derivative clones: sensitization to the action of EGF. Table 3 shows a unique property of ACl 31 and derivative clones of this line after exposure to EGF. ACl 31 showed a heterogenous response in soft agar, with 10 to 20% of the cells forming slow-growing colonies. When the agar was supplemented with EGF at 10 ng/ml, both the rate of colony growth and the fraction of cells forming colonies increased by a factor of six. However, EGF did not stimulate uninfected Mv1Lu Cl 12 cells to grow in agar. This EGF stimulation also occurred, albeit to a lesser degree, with Mv1Lu Cl 12 cells that had been infected with CI-3 virus recovered after transfection of NIH 3T3 cells with cloned viral DNA and amplification by coinfection with amphotropic helper MuLV. EGF stimulation of soft-agar growth was barely detectable in the absence of amphotropic helper MuLV. Mv1Lu cells that were induced for anchorage-independent growth by infection with CI-3 and amphotropic helper MuLV, as well as the parental ACl 31 cells, were tumorigenic in nude mice.

TABLE 1. Infectivity of cloned viral DNA[a]

Viral DNA	Virus production by transfected cells	
	Incidence	Latency (weeks)
LEUK-1, colinear	5/5	3
CI-2, permuted	0/5	>16
CI-3, permuted	5/5	8
CI-3, colinear	2/5	8
CI-4, permuted	0/5	>16

[a] Cloned viral DNAs were transfected onto NIH 3T3 cells by the procedure of Graham and van der Eb (18). As a positive control we used cloned viral DNA (LEUK-1) from a rapid leukemia-inducing virus that is closely related to Moloney MuLV. Transfected cultures were grown in the presence of Polybrene at 4 μg/ml and periodically assayed for the production of reverse transcriptase-containing particles.

TABLE 2. Host range of CI-3 virus[a]

Virus	[^{3}H]dTMP incorporated (cpm [$\times 10^3$]) by:	
	NIH 3T3 cells	Mv1Lu cells
Ecotropic		
LEUK-1	740	2.1
BALB-1	380	1.8
Xenotropic		
BALB-2	1.5	260
Dualtropic		
AKR-247	240	180
Z-6	710	560
CI-3	80	110

[a] Determined by reverse transcriptase assay. Cell culture medium from chronic producer stock cultures was filtered and used to infect the host cell lines listed. Infected cultures were grown in the presence of Polybrene at 4 μg/ml, and the medium was assayed for viral polymerase activity at 2-week intervals. The results shown are plateau values which were reached after 2 to 4 weeks for all viruses except CI-3, which required 8 to 10 weeks of incubation after infection.

DISCUSSION

We have previously reported the induction of sarcomas (40; U. R. Rapp, F. H. Reynolds, Jr., and J. R. Stephenson, J. Virol., in press) as well as lung and ovarian carcinoma (41) by type C viruses selected from IdUrd-induced C3H MuLVs for their ability to transform either mouse fibroblast or epithelial mink lung cells. In our present characterization of the viral genomes present in a particular soft-agar clone of in vitro-transformed mink lung cells, we found that both productively transformed cells and their nonproductively transformed offspring contained persistent unintegrated genomes with the structure of MCF MuLV. Cloning of unintegrated virus yielded four clones, three with an apparently complete viral genome (CI-1, CI-2, and CI-3), only one of which (CI-3) was infectious, and a fourth (CI-4) which was identical to the first but for a deletion in the p15E portion of the envelope gene and one copy of the LTR. The MCF MuLV originated from the IdUrd-induced C3H cells, and comparison of their genomes by heteroduplex mapping with that of another pathogenic MCF MuLV (SFFV) showed apparent identity in the envelope gene substitution present in both of these viruses. Moreover, the CI-4 genome contains a deletion of p15E that is similar to that of SFFV.

TABLE 3. Agar growth of Mv1Lu cells infected with CI-3

Cells	Growth without EGF		Growth with EGF[a]	
	% Cells growing	Colony size[b]	% Cells growing	Colony size
Mv1Lu	0	NA[c]	0	NA
ACl 31-infected Mv1Lu	12	8–12	76	>20
CI-3-infected Mv1Lu	0.1	8–12	0.1	8–12
CI-3-infected Mv1Lu[d]	10	8–12	28	>20
4070-infected Mv1Lu	0	NA	0	NA

[a] EGF was at 10 ng/ml.
[b] Measured in multiples of the single-cell diameter at 10 days after feeding.
[c] NA, Not applicable.
[d] Mv-1Lu cells infected with CI-3 were superinfected with 4070 amphotropic MuLV.

Origin of CI MCF virus. MCF MuLV was originally isolated from preleukemic AKR mice, and it was thought that the generation of such recombinants would be limited to certain tissues in vivo, presumably within or in the vicinity of the target cells for transformation (22, 23). The MCF MuLVs that we describe here must have been generated after induction by IdUrd of endogenous MuLVs in C3H cells, since they are present in the induced virus population and are not present in the cells before induction. We have established the presence of CI-3-like genomes in the IdUrd-induced population of C3H MuLV by isolating an identical genome from unintegrated DNA present in mouse cells acutely infected with induced C3H/MuLV. The major component present in this Hirt extract was a genome which lacked an *Eco*RI restriction enzyme site and presumably represented the ecotropic C3H MuLV. The selection of virus with an *Eco*RI cleavage site from this Hirt extract, however, did yield genomes similar to that of CI-3. The possibility of a preexistent CI-3-like virus in the genome of C3H mouse cells was examined by restriction analysis of C3H/MCA-5 cellular DNA. Since MCA-5 cells represent a single cell clone, there should be a minimum of a single copy of recombinant virus per cell. Using conditions that allowed the detection of single-copy DNA, we did not detect the 0.8-kb *Eco*RI-*Xba*I or 1.5-kb *Bam*HI-*Xba*I signature fragments of CI-3 virus.

The induction with IdUrd of a xenotropic MuLV (B-Mux) with an unusual structure, possibly related to MCF MuLV, from Ki-BALB cells has been reported previously by Ihle et al. (26). B-Mux emerged rapidly after treatment of cells with IdUrd and was thought to be derived from ecotropic MuLV by a complete exchange of its envelope gene for that of endogenous xenotropic MuLV (26). The CI viral genomes that we obtained from IdUrd-induced C3H/MCA-5 cells differ from that of B-Mux in host range (dual versus xenotropic) as well as in the extent of envelope gene exchange. It has not yet been established whether B-Mux is a recombinant or in fact an endogenous virus in BALB/c mice.

Nature of the envelope substitution present in CI viral genomes. The substitution present in our molecularly cloned CI viruses is related, as evidenced by restriction analysis and heteroduplexing, to that present in SFFV, which in turn is closely related in the envelope gene to Friend MCF MuLV and MCF virus of AKR origin (7, 30; Gonda et al, in preparation). Thus, it appears that pathogenic MuLVs with several distinct target cell specificities for transformation in vivo contain closely related envelope gene substitutions. Experiments in which we have determined the specificity of MCF gp70s for cell surface receptors have indicated that viruses of differing pathogenicity may share the same receptor specificity. Two in vivo-derived MCF MuLVs, the highly pathogenic isolate Z6 and the weakly pathogenic virus Z9 (11), both use a chromosome 2-coded receptor to enter cells (37), as does CI-3 (Rapp, unpublished data). Chattopadhyay et al. have recently shown for several MCF MuLVs of AKR origin that the gp70 segment of the envelope gene is apparently derived from an endogenous MCF-like viral genome (5).

Biological activity of CI viral genomes. Only one of the two cloned full-length CI viral genomes (CI-3) was infectious upon transfection to NIH 3T3 cells. The basis for the lack of infectivity of CI-2 viral DNA remains to be determined. Its size and restriction enzyme map are identical to those of the infectious CI-3 viral genome. Since both genomes were cloned from persistent unintegrated provirus, it is conceivable that we were selecting flawed proviral DNA with impaired integration function. The transfected CI-3 virus had a low spreading rate as compared with a rapidly replicating MuLV such as Moloney MuLV (data not shown) and was dualtropic. The low replication efficiency of this virus might also be due to the fact that we cloned the proviral DNA from persistent unintegrated proviral DNA.

The persistent unintegrated genomes from which CI-1 through CI-4 were cloned were present at approximately three copies per cell. Accumulation of unintegrated reticuloendotheliosis provirus has been linked to retroviral toxicity in the avian system (51), and it has been shown that

delayed development of superinfection interference is responsible. The basis for such a delay may well lie in cell-type-specific differences in the processing and deposition of viral envelope glycoproteins, thereby allowing for several cycles of infection and the consequent accumulation of unintegrated provirus (51). A high copy number of unintegrated proviral DNA has also been observed in vivo in the thymus of preleukemic AKR mice (3, 25) as well as during the course of Moloney MuLV-induced leukemia (27, 28) where it appeared transiently before the development of overt disease and presumably was due to spread of toxic MCF MuLV through the thymus tissue. Whether or not such an accumulation of unintegrated viral DNA contributes to the cell-type-specific oncogenicity of these viruses remains to be determined. Conceivably, such a condition may increase the number of integration events per infected cell and thus may increase the probability of activation or mutation of genes involved in carcinogenesis.

Faulty posttranslational processing of viral envelope glycoprotein may also lead to an unusual association of this molecule with other cellular structures and thereby exert a toxic or growth-promoting (34, 35) effect on cells. A cell-type-specific effect on the maturation of *env* gene products of dualtropic MCF virus has been observed by Famulari et al., who described the inefficient cleavage of MCF viral envelope precursor protein into gp70 in leukemia cells but not in fibroblasts (14, 15). Differently processed envelope glycoproteins may differ in their biological effects. For example, it has been shown that differences exist in the posttranslational processing of envelope glycoproteins from the anemia versus the polycythemia strain of SFFV and that these differences correlate with differences in the histology of virus-induced tumors (44).

The phenotype of epithelial mink lung cells transformed in vitro by IdUrd-induced C3H MuLV includes an increased sensitivity towards EGF, which alters these cells morphologically and increases their growth rate in soft agar. This enhancement of transformation was apparent at a concentration of 10 ng/ml, which does not affect untransformed mink Mv1Lu cells. An enhancement of transformation frequency by EGF was previously described (21) for ovarian granulosa cells upon infection with Kirsten sarcoma virus. Since the in vitro-transformed producer and nonproducer mink cells contained multiple viral genomes, it is not clear at present whether any of the CI viral genomes that we have described here are solely responsible for the sensitization of infected mink cells towards EGF. Transfer of the infectious CI-3 virus to mink epithelial lung cells did confer sensitivity toward EGF to these cells, but superinfection with amphotropic helper MuLV was required to achieve agar growth comparable to that of the parental mink cell clone AC1 31. We are currently involved in determining by transfection with cellular DNA from ACl 31 and several of its nonproducer subclones as well as with DNA from the virus-induced lung carcinomas whether the transforming DNA in fact contains a CI-3- or CI-4-like viral genome.

In conclusion, we have shown here the isolation and genetic structure of infectious and defective MCF-class virus from IdUrd-induced C3H/MCA-5 cells and have demonstrated an association of these genomes with in vitro-transformed epithelial mink lung cells. The role of viral genomes from these cells in the induction of alveologenic lung adenocarcinomas upon inoculation of newborn NFS/N mice with virus from in vitro-transformed Mv1Lu cells will be the subject of a separate report.

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