Molecular Cloning, Characterization, and Genetic Mapping of an Endogenous Murine Mammary Tumor Virus Proviral Unit I of C3H/He Mice

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Received 26 November 1984/Accepted 1 February 1985

We have cloned and characterized a novel endogenous murine mammary tumor virus proviral unit of the C3H/He strain of mice. The cloned proviral unit is 16 kilobase pairs (kbp) in size and is composed of a 5.6-kbp 5' *EcoRI* segment of an endogenous provirus with 10.4-kbp flanking cellular sequences. A comparison of the restriction map of the cloned proviral DNA with that of an endogenous provirus of the GR strain of mice has revealed minor differences in restriction sites on the two proviruses. The restriction enzyme *SstI*, which does not cleave the 5' *EcoRI* fragment of GR DNA, cleaves the C3H/He proviral sequences once; *MspI* has an additional site in the C3H/He proviral sequences. By using a subcloned fragment containing unique cellular sequences as a hybridization probe, we (i) mapped the C3H/He proviral unit to chromosome 14 by using mouse-hamster somatic cell hybrids, and (ii) demonstrated that this proviral unit is also present in the genome of DBA/2 mice. From these results we conclude that the C3H/He strain of mice acquired this proviral unit from DBA stock by genetic transmission. Our data also indicate that the murine mammary tumor virus sequences present in the *gag*-specific proviral unit of C3H/He mice extend at least 2.45 kbp downstream of the *EcoRI* site in the genomic DNA. Since the structural organization and chromosomal location of this proviral unit are distinct from those of previously reported proviral units represented by similar-sized (16.7-kbp) *EcoRI* fragments, we tentatively propose to designate this proviral unit *Mtv-7a*.

The murine mammary tumor virus (MuMTV) is the etiological agent of mammary adenocarcinomas in several inbred strains of mice (2, 25, 26). The virus is transmitted either endogenously as provirus(es) covalently linked to the mouse genome or exogenously as a milk-borne virus which synthesizes proviral DNA that can integrate at many sites in the mouse genome (7, 32, 34). The identification of individual endogenous proviruses has been largely based on molecular analysis of genomic DNA with restriction endonuclease *EcoRI* and blot hybridization with MuMTV-specific probes. *EcoRI* has been chosen mainly because the cleavage site for the enzyme is uniformly present in the nondefective proviral DNA of all mouse strains (9, 13, 34). The enzyme cleaves at only one site within the nondefective provirus and therefore generates two fragments containing MuMTV sequences which vary in size according to the locations of *EcoRI* restriction sites in the flanking cellular sequences. Restriction endonuclease analyses of genomic DNA indicated that MuMTV proviruses were located at many sites in the mouse genome. Several of the proviruses were found to lack *EcoRI* cleavage sites, suggesting that they were either mutated in this site or are subgenomic (37). The chromosomal location of a few of the proviruses has been determined by genetic segregation analysis and somatic cell hybrid techniques (4, 36). It was found that the MuMTV proviruses were integrated in many chromosomes.

One of the endogenous proviruses, which was originally designated unit I, is characterized by a 16.7-kilobase pair (kbp) *EcoRI*-generated fragment of the genomic DNA. This fragment is present in various strains of C3H, CBA, DBA, BALB/c, GR, and NSF mice (5). The structure of this fragment is still not clearly defined. Earlier studies by Cohen

et al. (6), involving direct physical mapping of the endogenous viral DNA sequences in the genomic DNA of BALB/c mice, indicated the subgenomic nature of unit I. More recently, it has been speculated that the proviral DNA in unit I is mainly composed of MuMTV long terminal repeat (LTR) sequences (39, 40). The specific chromosomal location of the unit I provirus was examined by Traina et al. (36) in two strains of mice, DBA/2 and C3H/He, by the genetic segregation analysis technique. The C3H family of mouse strains is derived from crossing the Bagg albino stock with the DBA stock, the ancestors of the BALB/c and DBA/2 strains, respectively (36). They found that the proviruses generating the 16.7-kbp fragment in DBA/2 and C3H/He are nonallelic and unlinked since unit I, which is linked to chromosome 1 markers in the DBA/2 strain, segregated independently of chromosome 1 markers in the C3H/He strain. On the basis of these observations, they designated unit I in the DBA/2 strain as unit Ia. However, they could not assign unit I of C3H/He to any specific chromosome. Unit I of the BALB/c strain has recently been mapped to chromosome 16. Therefore, although a 16.7-kbp MuMTV-specific fragment is present in *EcoRI* digests of the genomic DNA of several strains of mice, its independent segregation in BALB/c and DBA/2 strains suggests that similar-sized fragments are produced by more than one proviral locus. In fact, MacInnes et al. (20) have recently reported that the 16.7-kbp *EcoRI* fragment is produced by two MuMTV loci in the DBA mouse strain. One of the loci produces 16.7- and 11.7-kbp fragments and maps to chromosome 1, whereas the other generates only a 16.7-kbp fragment and has not yet been mapped. Furthermore, the structure of these two similar-sized fragments is currently unknown.

The widespread occurrence of unit I in several strains of mice, its unknown structure, and its heterogenous segrega-

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tion pattern prompted us to clone this unit from the genomic DNA of C3H/He mice. Our preliminary objective was to use the cloned DNA to characterize the proviral sequences by restriction analysis and to establish the chromosomal location of this unit by hybridization of cloned unique cellular sequences to DNA from a series of somatic cell hybrids which had segregated different mouse chromosomes. In this report we present evidence that the 16.7-kbp designated MuMTV-specific fragment in the C3H/He strain of mice contains a 5' *EcoRI* segment of an endogenous provirus and is structurally quite distinct from the 16.7-kbp *EcoRI* fragment of BALB/c mice. Furthermore, we have assigned the 16.7-kbp *EcoRI* fragment (proviral unit I) of C3H/He mice to chromosome 14. Our results also suggest that this proviral unit is present in the genomic DNA of DBA/2 mice and is located at the same chromosomal site.

MATERIALS AND METHODS

Preparation of tissue DNA. High-molecular-weight DNA was extracted from the livers of BALB/c and C3H/He mice and from the spleens of DBA/2 mice as described previously (11). Briefly, nuclei were isolated from tissue homogenates by centrifugation and treated overnight with sodium dodecyl sulfate (1%) and pronase (1 mg/ml) at 37°C. After extraction with phenol-chloroform (1:1), the samples were dialyzed against three changes of 5 mM Tris-hydrochloride (pH 7.4) with 0.1 mM EDTA and stored at 4°C.

Restriction enzyme digestion of DNA. All the restriction endonucleases except *SstI* were purchased from the New England BioLabs, Beverly, Mass. *SstI* was purchased from Bethesda Research Labs, Rockville, Md. The digestion conditions used with these enzymes were those suggested by the suppliers. To ensure complete digestion of the DNA, 5- to 10-fold excess enzyme was used.

Gel electrophoresis and hybridization analysis. DNA samples digested with restriction endonucleases were electrophoresed in 0.7 to 1% horizontal agarose (SeaKem) gels with the Tris-EDTA-borate buffer system (pH 8.2; TBE) (3). Lambda DNA cleaved with *HindIII* and ϕ X174 DNA cleaved with *HaeIII* were used as molecular weight markers (28, 33) and were visualized under UV light after ethidium bromide staining. The DNA to be hybridized with 32 P probes was transferred to nitrocellulose paper by the procedure of Southern (35), with minor modifications. The filters were treated at 68°C for at least 4 h in hybridization buffer containing 6 $\times$ SSPE (1 $\times$ SSPE is 0.15 M NaCl, 0.01 M NaH₂PO₄, plus 0.001 M EDTA [pH 7.0]), 0.06% bovine serum albumin, 0.06% polyvinylpyrrolidone, 0.06% Ficoll, 0.1% sodium dodecyl sulfate, and 100 μ g of sheared salmon sperm DNA per ml. The filters were then hybridized in the same buffer containing 0.5 $\times$ 10⁶ to 1 $\times$ 10⁶ cpm of 32 P-labeled probe per ml and hybridized for 24 h at 68°C. After hybridization the filters were extensively washed first with 5 $\times$ and then with 1 $\times$ SSPE containing 0.1% sodium dodecyl sulfate. The washed and dried filters were exposed to Kodak X-ray film at -70°C with a Dupont Cronex intensifying screen.

Preparation of 32 P-labeled probes. *Escherichia coli* clones containing a copy of permuted viral DNA, LTR-specific DNA, and *env*-specific DNA were generous gifts of John Majors and Harold Varmus (21). Recombinant plasmid DNAs were purified from these clones by the procedure described in this section. Purified *gag*-specific cloned DNA was kindly supplied by E. Buetti (12). Recombinant plasmid DNA or virus-specific DNA fragments were 32 P labeled by nick translation (31) with the systems from New England Nuclear

Corp., Boston, Mass. After labeling, the reaction was terminated by the addition of stop buffer (10 mM Tris [pH 7.4], 10 mM EDTA, 0.5% sodium dodecyl sulfate), and unincorporated label was removed by spin dialysis through a small column of Sepharose CL-6B.

Isolation of plasmid DNA and cloned DNA fragments. The plasmid DNAs were isolated from 1-liter cultures as described previously (29), except that the 17,000 rpm supernatant was heated at 65°C for 15 min. The resulting precipitate was removed by centrifugation, and the supernatant was made to 0.25 M with NaCl and 10% with polyethylene glycol. After 2 h at 4°C, the precipitated DNA was pelleted, resuspended in TE (10 mM Tris-hydrochloride, [pH 8.0], 1 mM EDTA), extracted twice with phenol (saturated with 0.1 M Tris-hydrochloride [pH 7.5]) and chloroform, and ethanol precipitated. The DNA pellet was resuspended in 8 ml of TE. After the addition of 4 mg of ethidium bromide and 8.29 g of CsCl to give a final density of 1.55, the solutions were spun for 48 h at 35,000 rpm and 20°C. The DNA bands were visualized under UV light and withdrawn into a hypodermic syringe, and the DNA was extracted with water-saturated isobutanol. After dialysis against TE, the DNA was ethanol precipitated.

Virus-specific DNA fragments from recombinant plasmids were prepared as follows: DNA (200 to 300 μ g) was digested to completion by restriction enzymes and fractionated by preparative agarose gel electrophoresis. Regions of the gel containing DNA fragments of interest were cut out and electroeluted in 0.5 $\times$ TBE containing 0.1% Sarkosyl, essentially as described by Maniatis et al. (23). DNA was concentrated by extraction with 2-butanol and ethanol precipitated after 2 to 3 extractions with phenol and chloroform. The DNA was further purified by passage through Elutip-d column (Schleicher and Schuell, Inc., Keene, N.H.) by the recommended procedure.

Preparation of phage DNA for cloning. Bacteriophage vector lambda L47 was propagated in *E. coli* K 802 with NZCYM growth medium, and DNA was purified by using the CsCl gradient procedure described by Maniatis et al. (23). Vector arms with *EcoRI* cloning sites were prepared by the potassium acetate gradient procedure, as well as by the procedure described by Maniatis et al. (23).

In vitro packaging and analysis of clones. Sonicated extract and freeze-thaw lysate for in vitro packaging of phage recombinant DNA were prepared from *E. coli* BHB 2690 and BHB 2688, respectively, by the procedure of V. Pirotta and were obtained from Simon Ford (Sloan-Kettering Institute for Cancer Research). The in vitro packaging reaction was carried out by mixing 0.2 to 0.5 μ g of recombinant phage DNA with 5 μ l of sonicated extract and 10 μ l of freeze-thaw lysate. After 1 h of incubation at 25°C, 0.1 ml of phage dilution buffer (100 mM NaCl, 8 mM MgSO₄, 50 mM Tris-hydrochloride [pH 7.5], 0.01% gelatin) and 10 μ l of chloroform were added and the lysate was stored in the cold. Phage recombinants were detected by the in situ plaque hybridization technique (1). Briefly, the plaques from a 150-mm petri dish were transferred to a nitrocellulose circle. The filters were immersed in denaturing solution (1.5 M NaCl, 0.1 M NaOH) for 25 s, followed by neutralization in neutralizing solution (2 $\times$ SSPE containing 0.2 M Tris-hydrochloride [pH 7.5]). The filter was baked for 2 h at 80°C under vacuum and hybridized to 32 P-labeled MuMTV *rep* DNA. The MuMTV-positive recombinant phage plaques were identified by autoradiography on X-ray film. The phages from the positive plaques were eluted and plaque purified by plating and hybridization. DNA was isolated from the MuMTV-pos-

FIG. 1. *EcoRI* restriction pattern of MuMTV-specific sequences in the genomic DNAs of C3H/He (lanes 1 to 3 and 7) and BALB/c (lanes 4 to 6 and 8) mouse liver. The DNAs (15 μ g) were digested with *EcoRI*, electrophoresed in a 0.8% agarose gel, transferred to nitrocellulose filters, hybridized to 32 P-labeled MuMTV *rep*, *gag*, *env*, and LTR probes, and autoradiographed as described in the text. The sizes of the MuMTV-specific fragments given on the left side of the panel were calculated by comparing their migrations with those of the fragments of known size obtained by *HindIII* digestion of lambda DNA and MuMTV-specific *EcoRI* fragments of C3H/He (25, 28) and BALB/c mouse DNA (33). The Roman numerals on the right side of the panel designate endogenous proviral units. The MuMTV-specific fragment which migrates as a 19-kbp fragment in our gel system has been designated as 16.7 kbp in this and subsequent figures to conform with the published results (25, 28, 33).

itive clones by the procedure used for lambda L47 vector. A sample of phage DNA was digested with *EcoRI* and analyzed by gel electrophoresis and blot hybridization to determine the size of the MuMTV-specific insert.

Subcloning and isolation of recombinant DNA. The *EcoRI*-generated MuMTV-specific fragment of the phage DNA was ligated into the *EcoRI* site of dephosphorylated plasmid vector pBR322 (New England BioLabs) and transfected into *E. coli* HB101 by the procedure described previously (29). The transformants were scored on agar plates containing 150 μ g of ampicillin per ml and subsequently screened for proviral DNA-containing fragments by colony hybridization (14) with an MuMTV *rep* probe. Recombinant plasmid DNA was prepared as described above.

Somatic cell hybrids. The somatic cell hybrids used in the chromosomal mapping of the cloned MuMTV proviral unit were derived from the fusion of E36 Chinese hamster cells and cells of three different strains of mice: BALB/c, NFS.Akv-2, and A/HeJ (17-19). The mouse chromosome content of these hybrids was determined by Giemsa-trypsin banding followed by staining with Hoechst 33258 (16) and typing for isozyme markers present on 15 mouse chromosomes (17).

RESULTS

MuMTV proviral unit I of C3H/He mice contains *gag*-specific sequences. MuMTV proviral unit I has been shown to be present in the genome of C3H/He mice. However, no information is available regarding the nature of the proviral sequences in this unit. We therefore set out to study the structure of this unit. As an initial step we examined the genomic DNA of C3H/He mice for the presence of MuMTV-specific proviral sequences. DNA was extracted from mouse liver, digested with restriction enzyme *EcoRI*, and analyzed by gel electrophoresis and blot hybridization (35) with MuMTV *gag* and *env* probes in addition to a probe representative of the entire viral genome (MuMTV *rep*). The

results shown in Fig. 1 (lane 1) revealed the presence of six MuMTV-specific *EcoRI* fragments: 19.0, 8.5, 6.7, 6.5, 5.8, and 4.5 kbp in size, as estimated on the basis of the migration of *HindIII*-generated fragments of λ DNA. The 19-kbp fragment has been previously designated the 16.7-kbp (25, 28) or 18-kbp (11) fragment. Therefore, in this communication we will designate the 19-kbp fragment as 16.7 kbp to conform with the published results. The 8.5- and 6.7-kbp fragments are known to represent a provirus designated unit II, whereas the 6.5- and 4.5-kbp fragments represent unit V (36). The remaining fragments, 16.7 and 5.8 kbp, represent unit I and VII, respectively (36). Examination of the MuMTV-specific DNA fragments with 32 P-labeled *gag*- and *env*-specific probes revealed that unit I has *gag*-specific sequences (lane 2), whereas unit VII has *env*-specific sequences (lane 3).

Since the genomic DNA of BALB/c strain of mice has been shown to contain a 16.7-kbp *EcoRI* fragment designated unit I (6), it was of interest to examine the nature of the MuMTV-specific sequences in this proviral unit by using MuMTV *rep*-, *gag*- and *env*-specific probes. The results presented in Fig. 1 (lane 4) indicated that the MuMTV *rep* probe hybridized very poorly to the 16.7-kbp fragment as compared with other MuMTV-specific *EcoRI*-generated fragments (10.0, 8.5, 7.8, and 6.7 kbp). Moreover, no hybridization of unit I to MuMTV *gag*- or *env*-specific probes was observed (lanes 5 and 6), suggesting that the 16.7-kbp MuMTV fragment from the BALB/c strain of mice did not contain *gag*- or *env*-specific sequences. These results, with the observation that both MuMTV *rep* and *gag*-specific probes hybridized efficiently with the 16.7-kbp fragment of C3H/He genomic DNA, indicated that similar-sized fragments from these two strains of mice are structurally distinct. To determine whether the 16.7-kbp *EcoRI*-generated fragments of C3H/He and BALB/c genomic DNA contained LTR sequences, we hybridized the DNA fragments with the MuMTV-LTR probe. It was found that the 16.7-kbp frag-

FIG. 2. Identification of the molecularly cloned 16-kbp MuMTV-specific fragment of C3H/He genomic DNA. Genomic DNA (15 μ g) (lane 1) and recombinant phage DNA (0.5 μ g) (lane 2) were digested with *Eco*RI and analyzed by gel electrophoresis on a 0.7% agarose gel and blot hybridization with a *gag*-specific probe.

ments in both strains of mice hybridized to the LTR probe, indicating the presence of LTR sequences in both (lanes 7 and 8). However, a much sharper band with BALB/c DNA (lane 8) was observed in the autoradiograms, compared with a broad and intense band seen with C3H/He DNA (lane 7), although the same amount of DNA was used in both cases. It is therefore possible that more than one MuMTV-specific bands of slightly different sizes, which were not resolved under our experimental conditions, might be present in the *Eco*RI digests of C3H/He mouse DNA.

Molecular cloning and characterization of the MuMTV-specific 16.7-kb *Eco*RI fragment. To characterize unit I of C3H/He mice and to determine its chromosomal location, we prepared molecular clones of the *Eco*RI-generated 16.7-kbp MuMTV-specific DNA fragment in lambda L47 phage vector. Liver DNA was digested to completion with *Eco*RI and electrophoresed on preparative agarose gels. DNA fragments 12 to 20 kbp in size were isolated by electroelution and inserted into the dephosphorylated arms of lambda L47 vector. Approximately 1 μ g of vector arms was ligated with 0.3 μ g of the DNA fragments, and the recombinant DNA was packaged into phage particles in vitro. The yield of recombinant phage by plaque assay was 6×10^5 plaques per 1.3 μ g of DNA. Recombinant phage plaques containing MuMTV-specific sequences were identified by screening 4×10^5 plaques by the plaque lift procedure followed by hybridization with 32 P-labeled MuMTV *rep* DNA. Seven MuMTV-specific plaques were obtained, and their DNA was analyzed by *Eco*RI digestion and blot hybridization. All the recombinant plaques were found to contain a single insert that hybridized to *gag*-specific probe (data not presented). The size of the insert was 16 kbp in all except one, for which the size was found to be 13 kbp. DNA from one of the recombinant phages containing the 16-kbp *Eco*RI fragment was purified and analyzed further by extended electrophoresis on a 0.7% agarose gel along with

C3H/He genomic DNA. The sizes of the MuMTV-specific fragments were determined by comparison with *Hind*III lambda fragments of known size. The results of these analyses are presented in Fig. 2. The cloned DNA fragment migrated faster than the genomic DNA fragment (lane 1) and was found to be 16 kbp in size (lane 2). The observed difference in the migration of the two DNA fragments prompted us to examine the migration of the cloned DNA in the presence of a large excess of genomic DNA. Therefore, 0.2 μ g of recombinant phage DNA was mixed with 15 μ g of BALB/c liver DNA, digested with *Eco*RI, and analyzed on an 0.8% agarose gel. The cloned DNA digested with *Eco*RI was used as a control. The results of such an analysis indicate that the migration of the cloned proviral DNA in the presence of genomic DNA was much slower than that of the cloned DNA alone (Fig. 3). These observations suggest that the considerable size difference between the cloned fragment (16.0 kbp) and the genomic fragment (16.7 kbp) is not due to any deletion in the cloned fragment.

To characterize the proviral sequences and to identify the unique cellular sequences in the cloned DNA, restriction enzyme analyses of the 16-kbp fragment were performed, and its restriction map was compared with the maps of other proviruses. The 16-kbp *Eco*RI fragment was subcloned in pBR322, and the recombinant plasmid DNA was isolated. Twelve restriction endonucleases with known cleavage sites in MuMTV proviral DNA in conjunction with *Eco*RI were used to analyze the recombinant phage DNA by gel electrophoresis and blot hybridization with MuMTV LTR, *gag*- and *rep*-specific probes. As an example, the results obtained with the *rep* probe are shown in Fig. 4. The restriction map of the cloned DNA is shown in Fig. 5. Three enzymes, *Sall* (Fig. 4, lane 5), *Bam*HI (lane 7), and *Hind*III (lane 10), did not cleave within the viral sequences. Four enzymes, *Bgl*II (lane 4), *Pvu*II (lane 8), *Sst*I (lane 9), and *Xho*I (lane 11), cleaved viral DNA once, and one enzyme, *Bgl*I (lane 3),

FIG. 3. Effect of relative DNA concentration on the mobility of cloned DNA in agarose gels. Recombinant phage DNA (0.2 μ g) alone (lane 1) and mixed with 15 μ g of BALB/c liver DNA (lane 2) was digested with *Eco*RI, electrophoresed in an 0.8% agarose gel, and blot hybridized with *gag*-specific probe. The sizes of the MuMTV-specific fragments were calculated by comparing their migrations with those of the fragments of known size obtained by *Hind*III digestion of lambda DNA. Note that the bands observed in the figure are due to the cloned DNA alone. The *gag*-specific endogenous proviral DNA fragments in the *Eco*RI digest of the genomic DNA were not detected because of very short exposure (1 h) of the X-ray film.

FIG. 4. Restriction endonuclease analysis of the cloned 16-kbp proviral unit of C3H/He genomic DNA. The recombinant pBR322 DNA was first digested with *EcoRI* (lane 1). Samples containing 0.5 μ g of the DNA were taken for further digestion with other restriction endonucleases: *AvaI* (lane 2), *BglI* (lane 3), *BglII* (lane 4), *SalI* (lane 5), *MspI* (lane 6), *BamHI* (lane 7), *PvuII* (lane 8), *SstI* (lane 9), *HindIII* (lane 10), *XhoI* (lane 11), *HaeIII* (lane 12), and *PstI* (lane 13). The digested DNA samples were analyzed on 1% agarose gels with an MuMTV-*rep* probe. The sizes of the restriction fragments (in kilobase pairs) were measured in relation to the restriction fragments of known size from λ and ϕ X174 DNA.

cleaved the DNA twice. The cleavage sites for *PstI* (lane 13), an enzyme which is used to distinguish endogenous from exogenous provirus (8), and the cleavage sites for the other above-mentioned enzymes were comparable with that of an endogenous provirus such as the endogenous provirus of GR mice (15). However, this proviral DNA fragment was distinguishable from the GR provirus in that it had a cleavage site for *SstI* (lane 9) and an additional site for *MspI* (lane 6). Two of the enzymes, *AvaI* (lane 2) and *HaeIII* (lane 12), cleaved within the viral DNA, but we could not map the sites for these enzymes. The cellular sequences flanking the viral sequences were found to have sites for the restriction

FIG. 6. Identification of unique cellular sequences in the cloned 16-kbp proviral unit. Samples containing 0.5 μ g of *EcoRI*-digested recombinant plasmid DNA were digested with *PstI*, and the resulting fragments were resolved in 1% agarose gels, stained with ethidium bromide, and photographed (lane 1). The DNA was then transferred to nitrocellulose paper and hybridized with MuMTV-*rep* probe (lane 2) and BALB/c genomic DNA probe (lane 3). The 1.7-kbp DNA fragment was considered to contain unique cellular sequences, since it did not hybridize to either of the probes. Two other fragments (3.6 and 0.75 kbp) shown in lane 1 represent the *EcoRI*-*PstI*-generated fragments of pBR322 DNA. The sizes of the fragments were determined as described in the legend to Fig. 3.

enzymes *BglII*, *BglIII*, *MspI*, *XhoI*, *PvuII*, and *SstI* (Fig. 5). On the basis of restriction endonuclease analysis, we conclude that the 16.0-kbp cloned fragment is composed of a 5' *EcoRI* segment (5.6 kbp) of an endogenous provirus and 10.4-kbp flanking cellular sequences.

To identify the mouse chromosome in which the proviral unit I of C3H/He mice is located, we isolated unique cellular sequences from the cloned DNA. For this purpose the

FIG. 5. Restriction endonuclease map of the 16-kbp DNA that was cloned from the genomic DNA of C3H/He mice. Specific cleavage sites for *BglI* (B1), *BglII* (B2), *MspI* (M), *PstI* (P), *PvuII* (Pv), *SstI* (S), and *XhoI* (X) are shown. No cleavage sites for *BamHI*, *HindIII* and *SalI* were found. The wavy line represents cellular DNA, the solid line represents proviral DNA, and the hatched box represents LTR sequences. The map is drawn to scale (in kbp).

FIG. 7. Hybridization of unique cellular sequence-specific probe to *Hind*III-restricted DNA obtained from a series of mouse-hamster hybrid cells. DNAs (15 μ g) were digested with *Hind*III, electrophoresed on an 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized to the nick-translated 1.7-kbp DNA fragment containing unique cellular sequences from the cloned proviral unit I of C3H/He mice. The sizes of the fragments were determined by comparing their migration with respect to the migration of fragments of known size obtained by *Hind*III digestion of lambda DNA.

recombinant plasmid DNA was digested with restriction enzymes *Bgl*I, *Bgl*II, *Msp*I, *Pst*I, *Sst*I, *Xho*I, *Hae*III, *Pvu*II, and *Ava*I in combination with *Eco*RI and blot hybridized with BALB/c genomic DNA and MuMTV *rep* probes. It was found that *Eco*RI-*Pst*I generated a 1.7-kbp DNA fragment that did not hybridize to MuMTV *rep* or BALB/c genomic

FIG. 8. Hybridization analyses of *Eco*RI-restricted liver DNA of BALB/c (lanes 1 and 4), C3H/He (lanes 2 and 5), and DBA/2 (lanes 3 and 6) mice with MuMTV *gag*-specific (lanes 1 to 3) and unique cellular sequence-specific (lanes 4 to 6) probes. *Eco*RI-digested DNAs (15 μ g) were electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose filters and hybridized to the probes indicated above. The sizes of the fragments were determined as described in the legend to Fig. 1.

DNA probe (Fig. 6, lanes 2 and 3), indicating that the cellular sequences contained in this fragment were unique. The 3.6- and 0.75-kbp fragments (lane 1) which did not hybridize to either of the probes (lanes 2 and 3) were identified as the plasmid DNA fragments on the basis of the migration of *Eco*RI-*Pst*I-generated fragments of pBR322 DNA. The 1.7-kbp unique sequence fragment was isolated in microgram quantities by using the techniques of preparative gel electrophoresis and was used in subsequent experiments.

The *gag*-specific MuMTV proviral unit I maps in the mouse chromosome 14. To map the MuMTV proviral unit I of C3H/He mice, we used a series of Chinese hamster and mouse somatic cell hybrids which had segregated different mouse chromosomes (17, 18, 19). DNAs from 20 such hybrids were analyzed by two restriction enzymes, *Eco*RI and *Hind*III, followed by electrophoresis and blot hybridization with a probe specific to the unique 1.7-kbp cellular sequences.

Figure 7 shows an example of the results obtained with six hybrid cell DNAs digested with *Hind*III. In our analyses the unique sequence probe detected 12-kbp *Hind*III-generated fragments in five of the 20 hybrid cell DNAs examined. This probe also detected a 3.9-kbp fragment, which was common to all hybrid cell DNAs, indicating that the 12-kbp fragment is specific to the mouse chromosome, whereas the 3.9-kbp fragment is specific to hamster chromosome.

Analysis of the hybrid DNAs with *Eco*RI demonstrated the presence of the unique sequences in five hybrid cell DNAs which were also scored positive in studies with *Hind*III. Analysis of the mouse chromosome content of all the hybrids showed that chromosome 14 was present in the

FIG. 9. *Apa*I restriction pattern of the cloned 16-kbp MuMTV *gag*-specific proviral unit. Recombinant plasmid DNA (0.5 μ g) was digested with *Eco*RI (lanes 1, 3, and 5) and *Eco*RI plus *Apa*I (lanes 2, 4, and 6), electrophoresed on a 0.8% agarose gel, stained with ethidium bromide, and photographed (lanes 1 and 2). The DNA was transferred to a nitrocellulose filter and hybridized with a *gag*-specific probe (lanes 3 and 4) and a unique cell-specific probe (lanes 5 and 6). The sizes of the DNA fragments were determined as described in the legend to Fig. 5.

hybrid cells that were positive for the presence of the unique sequences (Table 1). Since no chromosome showed any correlation with the unique sequences, we conclude that the 16.7-kbp proviral unit of C3H/He is present on chromosome 14.

The *gag*-specific proviral unit I of C3H/He is also present in the genome of DBA/2 mice and maps at chromosome 14. Since the C3H/He strain of mice is genetically related to the DBA/2 strain of mice (8, 36), one might expect that the 16.7-kbp *gag*-specific unit of C3H/He should also be present in the DNA of DBA/2 mice. We therefore analyzed the genomic DNA of DBA/2 mice by gel electrophoresis and blot hybridization with the MuMTV *gag*-specific probe as well as a probe specific to the 1.7-kbp unique cellular sequences isolated from the cloned proviral unit I of C3H/He mice. Since we have shown that, unlike C3H/He mouse DNA, the *gag*-specific 16.7-kbp *Eco*RI fragment is not present in the genomic DNA of BALB/c mice (Fig. 1), we used both C3H/He and BALB/c mouse DNA as controls. The results of these experiments are presented in Fig. 8. In addition to other *gag*-specific proviral fragments endogenous to each strain of mice, the *gag*-specific probe detected DNA fragments of the same size (16.7 kbp) in the *Eco*RI digests of C3H/He (lane 2) and DBA/2 (lane 3) mouse DNA but not in the BALB/c mouse DNA (lane 1). The unique sequence probe also detected the same 16.7-kbp fragments in the genomic DNA digests of C3H/He (lane 5) and DBA/2 mice (lane 6) but not in the genomic DNA of BALB/c mice, in which a much smaller fragment was detectable (lane 4). These results indicate that the *gag*-specific 16.7-kbp unit is common to C3H/He and DBA/2 strains of mice. Further-

TABLE 1. Correlation between mouse chromosomes and the 16.7-kbp *Eco*RI MuMTV-specific DNA fragment from C3H/He mice in 20 mouse-hamster hybrids^a

Mouse chromosomes	No. of hybrid clones (16.7-kbp MuMTV <i>Eco</i> RI fragment/chromosome retention)				Discordance (%)
	+/+	-/-	+/-	-/+	
1	0	7 ^b	2 ^b	7 ^b	56
2	3	7	1	7	44
3	1	7	2	4	43
4	2	11	1	3	47
5	0	11	5	3	42
6	3	7	1	7	44
7	5	4	0	11	55
8	3	11	1	2	18
9	4	10	1	4	26
10	1	14	4	1	25
11	0	13	4	0	24
12	2	4	0	6	50
13	1	7	0	4	33
14	4	15	0	0	0
15	2	1	0	9	75
16	1	8	2	5	44
17	4	6	0	8	44
18	2	6	0	4	33
19	3	8	1	6	39
X	3	6	0	6	40

^a Twelve of the hybrids were typed for mouse chromosomes by direct karyotyping; eight hybrids were typed for specific chromosomes by typing for isozyme markers.

^b Seven hybrids lacked 16.7-kbp *Eco*RI fragment-specific sequences and chromosome 1 (-/-), two retained the sequences but lacked chromosome 1 (+/-), and seven lacked the sequences but retained chromosome 1 (-/+).

FIG. 10. Hybridization analyses of *Apal*-restricted liver DNA of BALB/c (lanes 1 and 4), C3H/He (lanes 2 and 5), and DBA/2 (lanes 3 and 6) mice with MuMTV *gag*-specific (lanes 1 to 3) and *env*-specific (lanes 4 to 6) probes. *Apal*-digested samples of DNA (15 µg) were electrophoresed on a 0.7% agarose gel, transferred to a nitrocellulose filter, and hybridized to the probes indicated above. The sizes of the fragments were determined as described in the legend to Fig. 1.

more, it appears from the results obtained with the unique-sequence probe that the proviral unit in the DBA/2 strain of mice is localized on the same chromosome (chromosome 14) as it is in C3H/He mice (Fig. 7).

Unit I of C3H/He mice contains MuMTV sequences downstream of the *Eco*RI site. Since the cloned proviral unit was found to contain a complete 5' *Eco*RI segment of an endogenous provirus, the possibility existed that this proviral unit was part of a full-length endogenous provirus. To test this possibility, we took advantage of the known DNA sequence of the MuMTV envelope gene region (30) and used a computer search to look for restriction enzymes which had no other sites in the 3' *Eco*RI fragment of a proviral DNA except a single site, proximal to the right LTR, within the *env* gene. These enzymes were then tested for their ability to cleave the cloned proviral DNA only once in the flanking cellular sequences. The rationale of this approach was that if the provirus that generates the 16.7-kbp *Eco*RI fragment is complete or extends beyond the restriction site of any of these enzymes, a fragment of known size will be generated. This fragment then can be characterized by hybridization to *gag*-, *env*- and unique cellular sequence-specific probes. We found that *Apal* was most suitable for our purpose since the cleavage site for this enzyme was located 2.45 kbp downstream of the *Eco*RI site toward the end of the *env* gene. In addition, this enzyme was found to cleave the cloned fragment only once within the cellular sequences, generating two DNA fragments which were 6.5 and 8.8 kbp in size (Fig. 9). The 6.4-kbp fragment (lane 6) hybridized to the unique cellular sequence probe, whereas the 8.8-kbp fragment hybridized to the MuMTV-specific *gag* probe (lane 4). The 16-kbp fragment hybridized to both probes (lanes 3 and 5). In our experimental strategy described above, therefore, the

restriction enzyme *ApaI* should generate a virus-specific fragment of 11.3 kbp from the genomic DNAs of C3H/He and DBA/2 mice, and this DNA fragment should hybridize to *gag*-, MuMTV-, and *env*-specific probes but not to the unique sequence probe since the unique cellular sequences are located upstream of the *ApaI* site. The results of such an experiment demonstrated the presence of a unique 11.3-kbp fragment in the *ApaI* digest of C3H/He (Fig. 10, lanes 2 and 5) and DBA/2 (lanes 3 and 6) genomic DNA which hybridizes to both *gag*- and *env*-specific probes. The genomic DNA of BALB/c mice which lacks the *gag*-specific 16.7-kbp proviral unit did not generate the 11.3-kbp fragment that hybridized to both probes. These observations suggested that the *gag*-specific 16.7-kbp *EcoRI* fragment of C3H/He and DBA/2 mouse DNA constituted part of the provirus that extended at least 2.45 kbp downstream from the *EcoRI* site.

DISCUSSION

In this study we have cloned and characterized one of the endogenous MuMTV proviral units of the C3H/He strain of mice that has been described as a 16.7-kbp *EcoRI*-generated fragment and is designated unit I or *Mtv-6* according to the alphanumerical system (36). The proviral unit in the *EcoRI* digest of genomic DNA migrates as an 18- to 19-kbp fragment in agarose gels (11; this work). The cloned unit, however, is 16 kbp in size and is composed of a 5' *EcoRI* segment (5.6 kbp) of an endogenous provirus with flanking 10.4-kbp cellular sequences. Our results indicate that the difference in the migrations of the two DNA fragments is due to the difference in their concentrations in the two DNA samples. It should be noted that many investigators encountered difficulties in cloning the 5' half of the MuMTV proviral DNA because of the presence of a putative "poison" sequence (21, 22) in this region. However, such a poison sequence does not appear to be present in our cloned proviral DNA, since we did not encounter any difficulty in cloning or subcloning, nor did we observe any detectable deletion in the DNA. This observation, along with the fact that endogenous MuMTV proviruses from GR (15) and A/J (10) strains of mice were cloned without apparent difficulty, would suggest that many MuMTV proviruses do not contain a poison sequence.

A comparison of the restriction map of the cloned proviral DNA with the restriction map of another cloned endogenous provirus of the GR mouse strain (15) revealed minor differences. One of the enzymes, *SstI*, which did not cleave the 5' *EcoRI* fragment of GR DNA (15), cleaved the C3H/He mouse DNA sequences once, generating a 2.7-kbp *SstI*-*EcoRI* fragment from the proviral DNA. The other enzyme, *MspI*, was found to cleave the C3H/He proviral sequences at an additional site, generating three internal fragments of 1.9, 1.6, and 0.6 kbp, as compared with two internal fragments (3.0 and 1.9 kbp) in the 5' *EcoRI* segment of GR mouse DNA. It therefore appears that, although endogenous proviruses are very similar in their structure, some variations in their restriction maps exist which can be used to distinguish one provirus from another.

Our present studies, as well as those reported by other investigators, show that there is great diversity in the structural organization and chromosomal distribution of *EcoRI*-generated 16.7-kbp MuMTV-specific fragments derived from C3H/He, BALB/c, and DBA/2 mice. The MuMTV sequences in the proviral unit of C3H/He mouse DNA consist of the sequences that are present in the 5' *EcoRI* segment of an endogenous provirus (this work). By contrast, the MuMTV sequences in unit I of BALB/c mouse strain

appear to be composed of mainly LTR sequences. Furthermore, the chromosome mapping data with Chinese hamster-mouse somatic cell hybrid DNAs and cloned unique cellular sequence probe revealed that the 16.7-kbp proviral unit of C3H/He mice is located on chromosome 14; the 16.7-kbp proviral unit of BALB/c mice is located on chromosome 16 (4). We have also found that the *EcoRI* digestion of the genomic DNA of DBA/2 mice generates a 16.7-kbp fragment which hybridizes to MuMTV *gag*-specific probe. This 16.7-kbp proviral unit also maps at chromosome 14 like the proviral unit of C3H/He mice. Since the C3H strain of mice is derived from Bagg albino $\times$ DBA mice (6), we conclude that the C3H/He strain of mice acquired the 16.7-kbp *gag*-specific unit from DBA stock by genetic transmission and not by independent infection of the germinal tissues.

Recently, MacInnes et al. (20) have reported that two 16.7-kbp *EcoRI* fragments are generated from the genomic DNA of DBA/2 mice by more than one proviral locus. One of these fragments segregated with an 11.7-kbp fragment and was assigned to chromosome 1 (36). The other 16.7-kbp proviral unit, originally described as subgenomic (36), was not mapped to any chromosome. The proviral unit that we have found in DBA/2 mice neither maps to chromosome 1 nor appears to be subgenomic. Furthermore, this unit cannot segregate with the 11.7-kbp fragment since this fragment is not present in the C3H/He mouse DNA. We therefore conclude that the *gag*-specific 16.7-kbp *EcoRI* fragment of C3H/He mouse DNA characterized in this study is different from either of the 16.7-kbp fragments described previously and should be designated as a new proviral unit. Historically, endogenous MuMTV proviruses of the inbred strains of mice were designated as units by arbitrarily assigned Roman numerals (8). Recently the units have been enumerated with an alphanumerical system from *Mtv-1* to *Mtv-17* (36, 37) with no attached biological significance except for *Mtv-1* to *Mtv-3* (24, 27, 38). Since all endogenous proviruses segregate as stable genetic elements and several of them have been assigned to specific chromosomes, it would seem more appropriate to classify the endogenous MuMTV proviruses by their chromosomal location. Therefore, even though we have tentatively designated this unit as *Mtv-7a*, perhaps this *gag*-specific 16.7-kbp proviral unit of C3H/He and DBA/2 mice should be designated as *Mtv-14* on the basis of its localization in chromosome 14.

In this study we have made no efforts to investigate into the possibility of another 16.7-kbp proviral unit in the *EcoRI* digests of C3H/He mouse DNA. In view of the production of fragments that are the same size but structurally different by more than one proviral locus, we must consider the possibility that the designated unit I (*Mtv-6*) of C3H/He mice (36) is yet another 16.7-kbp fragment which is analogous to BALB/c unit I or to the 16.7-kbp subgenomic proviral unit of DBA/2 mice. Our preliminary results suggest that the MuMTV sequences in the 16.7-kbp *gag*-specific proviral unit are part of an intact provirus. Therefore, this provirus should generate a 3' *env*-specific fragment after *EcoRI* digestion. Interestingly, a 5.8-kbp fragment designated unit VII is known to be present in the *EcoRI* digests of C3H/He DNA (36). We have shown that this fragment hybridizes specifically to MuMTV *env*-specific sequences, indicating that the proviral sequences in this fragment represented the 3' half of the *EcoRI*-generated proviral DNA fragment. Although genetic segregation studies have failed to establish its linkage with the 16.7-kbp fragment containing the 5' half of proviral DNA, our observation, along with the fact that unit VII showed a fairly high degree of cosegregation (9 out

of 11 of the recombinant strains derived from C57BL/6J $\times$ C3H/HeJ) with the 16.7-kbp *EcoRI* fragment (36), suggests that unit VII and the 16.7-kbp *gag*-specific fragment form a full-length proviral unit. As shown above, the 16.7-kbp *gag*-specific proviral unit found in the *EcoRI* digests of C3H/He mouse DNA is also present in the genomic DNA of DBA/2 mice. This proviral unit is distinct from the two other 16.7-kbp fragments observed by MacInnes et al. (20) in this strain of mice. Since the *EcoRI* digests of DBA/2 mouse DNA contain a 15-kbp fragment which segregates with the 5.8-kbp fragment (20), the possibility exists that this fragment is the same as the 16.7-kbp proviral unit identified in this study.

ACKNOWLEDGMENTS

We thank John Majors and Harold Varmus for their generous gift of clones containing a copy of permuted MuMTV DNA, and *env*- and LTR-specific DNA. We also thank Elena Buetti and Heidi Diggelman for providing us the cloned MuMTV *gag*-specific DNA fragment and James Eperwine and James Roberts of Columbia University for the computer analysis of the DNA sequence information of MuMTV-*env* for various restriction sites.

This investigation was supported by Public Health Service grant no. CA-17129 from the National Institutes of Health.

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