Isolation of a Pathogenic Clone of Mouse Mammary Tumor Virus

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Exogenous mouse mammary tumor virus (MMTV) was cloned from a GR mammary tumor. Clone λ GRT39 contained a full-length integrated MMTV(GR) provirus and both 5' and 3' host flanking DNA. The λ GRT39 provirus had no apparent structural changes associated with cloning and retained the exogenous MMTV *gag* gene poison sequence. When introduced into rat mammary adenocarcinoma LA7 cells, the λ GRT39 provirus was fully expressed. λ GRT39-transfected LA7 cells made MMTV RNA, had gp52 SU protein on the cell surface, and produced B-type retrovirus particles characteristic of MMTV. Mammary tumors developed in hormone-stimulated BALB/c females injected with MMTV from λ GRT39-transfected LA7 cells [MMTV (λ GRT39)]. The tumors had new, clonally integrated copies of the MMTV(λ GRT39) provirus and were expressing MMTV antigen. These data indicate that the λ GRT39 provirus is biologically active and pathogenic.

Mouse mammary tumor virus (MMTV) is a replication-competent, slow-transforming retrovirus that causes mammary adenocarcinomas in female mice (41). In addition, MMTV causes a variety of premalignant mammary lesions and is useful for studies of multistep neoplastic development (27). MMTV expression is hormone regulated (32), and there is a strong endocrine influence on the induction of mammary tumors by MMTV (18). Pathogenesis is due in part to mutation of host genes by MMTV provirus insertion (9, 29).

Genetic analysis of MMTV and its role in mammary tumor development has been hampered for many years by the lack of infectious and pathogenic DNA clones of exogenous MMTV proviruses. The failure to generate such clones was attributed to an MMTV sequence localized to the 5' end of the *gag* gene, called the poison sequence (5, 10, 17, 24, 39). This sequence could not be cloned intact in plasmid vectors and was not recovered from lambda libraries screened by standard techniques.

In this report, we present data indicating that (i) full-length exogenous MMTV provirus DNA from the GR mouse strain [MMTV(GR)] can be cloned and propagated in the bacteriophage lambda vector λ L47 by using modifications of standard screening and amplification protocols, (ii) cloned provirus DNA can direct the production of recombinant DNA-derived MMTV(GR) in cultured rat mammary adenocarcinoma cells, and (iii) recombinant DNA-derived MMTV(GR) is tumorigenic. These experiments form the basis for studies, using MMTV(GR) mutants produced in vitro, to address basic problems pertaining to the regulation of MMTV expression, MMTV infectivity, and the molecular mechanisms of MMTV pathogenesis.

MATERIALS AND METHODS

Mice. The GRSA mouse strain (referred to here as GR) was obtained from the Netherlands Cancer Institute, Amsterdam (28), and the BALB/c/Crgl mouse strain (referred to here as BALB/c) was obtained from the Cancer Research Laboratory, University of California, Berkeley. Mammary tumors were identified by weekly palpation of female mice. When tumors were 0.5 to 1.5 cm in the longest dimension,

they were collected and frozen at -70°C . BALB/c mice used in the tumorigenesis experiment were hormone stimulated with pituitary isografts by implantation of two pituitaries from syngeneic donors into the left no. 4 mammary fat pad.

DNA cloning. A genomic library was constructed in bacteriophage lambda of *Mbo*I partials. Cellular DNA from a GR mammary tumor was partially digested with *Mbo*I to give restriction fragments with a modal size distribution of 15 to 20 kilobases (kb). This size fraction was purified on 5 to 20% potassium acetate gradients (25). The fractionated DNA was ligated to *Bam*HI-cleaved bacteriophage λ L47 (22) arms, and the ligation products were packaged in vitro, using strains from Sternberg et al. (37) and standard packaging protocols (19). A total of 1.5×10^6 PFU was plated on *Escherichia coli* KH802 (42) and screened by plaque hybridization (2), using MMTV(C3H) probe pCP4.2 (see Fig. 1). Positive clones were picked and plaque purified.

Minipreps of phage DNAs for mapping and subcloning were prepared from nuclease-treated plate lysates. Plate lysates were prepared as described elsewhere (25), using 1×10^5 to 2×10^5 PFU/100-mm plate. DNase I and RNase I were added to 1 $\mu\text{g}/\text{ml}$, and the samples were incubated for 30 min at room temperature. Phage particles were pelleted at $90,000 \times g$ for 2 h at 4°C . Pelleted phage was suspended in 10 mM Tris (pH 7.5)–10 mM NaCl–10 mM trisodium EDTA–1% sodium dodecyl sulfate (SDS)–100 μg of proteinase K per ml and extracted with buffer-equilibrated phenol. Phage DNA was ethanol precipitated and suspended in 10 mM Tris (pH 7.5)–10 mM NaCl–0.1 mM trisodium EDTA.

Restriction fragments from recombinant lambda clones for use as probes were subcloned in pUC12 (40). Insert and vector DNAs for subcloning were purified by electrophoresis in 5% polyacrylamide gels and electroelution of the appropriate fragments. Ligations were performed at 15°C overnight in 50 mM Tris (pH 7.5)–20 mM NaCl–10 mM MgCl_2 –1 mM dithiothreitol–1 mM ATP–1 U of T4 DNA ligase per μl at a DNA concentration of 10 $\mu\text{g}/\text{ml}$ and a 1:3 molar ratio of vector to insert. Ligated DNAs were used to transform *E. coli* JM83 (40) by standard techniques (25). Miniprep DNA samples (25) from Lac[−] clones were screened by restriction mapping.

Cellular DNA and RNA extraction. Cellular DNAs were

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FIG. 1. Restriction map of λ GRT39 and location of the probes used in this study. The λ GRT39 *Pst*I (P) and *Sst*I (S) restriction maps are shown. λ GRT39 contains an entire MMTV(GR) proviral genome as well as 5' and 3' host flanking DNA on a 14.7-kb *Mbo*I partial. The *Sst*I C fragment is unique to MMTV(GR) and was used as a marker for mouse cells infected with MMTV(GR) or MMTV(λ GRT39). The *Pst*I F and *Sst*I D fragments contain the exogenous MMTV poison sequence.

isolated by homogenizing minced tissue or cells scraped from tissue culture plates with a Dounce homogenizer in 10 mM Tris (pH 7.5)–10 mM NaCl–10 mM trisodium EDTA. The suspensions were lysed by adding SDS to a concentration of 0.5% and then extracted once with buffer-equilibrated phenol, once with buffer equilibrated phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform-isoamyl alcohol (24:1). Extracts were dialyzed for 48 h at 4°C against several changes of 10 mM Tris (pH 7.5)–10 mM NaCl–0.1 mM trisodium EDTA to remove residual organic solvents. DNA concentrations were determined by measuring A_{260} .

Total cellular RNAs were prepared by standard techniques (25). Guanidine isothiocyanate lysates of tissue culture cells were pelleted through a 5.7 M CsCl cushion to remove DNA and protein. The RNA pellets were extracted with chloroform–1-butanol (4:1) and then suspended in water.

Southern blot analysis. Restriction enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Cellular DNAs (5 to 10 μ g) were digested with a threefold excess of enzyme for 3 h under buffer and temperature conditions recommended by the manufacturer. Reactions were stopped by adding trisodium EDTA to a concentration of 50 mM. Digested DNAs were electrophoresed in horizontal 1% agarose gels containing 50 mM Tris base, 20 mM sodium acetate, 2 mM tetrasodium EDTA, 22 mM NaCl, and 30 mM acetic acid. After electrophoresis, gels were stained with ethidium bromide and checked by transillumination at 260 nm to verify complete digestion. The restriction fragments were transferred to nitrocellulose by the method of Southern (35) in 3.0 M NaCl–0.3 M trisodium citrate (38). Hybridizations were performed in 2 ml of 45% formamide, 0.45 M NaCl, 0.045 M trisodium citrate, 3 $\times$ Denhardt solution, 100 μ g of yeast RNA per ml, and 50 μ g of base-sheared salmon sperm DNA per ml at 37°C for 3 days with a probe concentration of 5×10^6 cpm/ml. Blots were washed once in 0.3 M NaCl–0.03 M trisodium citrate–0.1% SDS at room temperature for 10 min, twice in 0.03 M NaCl–0.003 M trisodium citrate–0.1% SDS at room temperature for 10 min, and once in 0.3 M NaCl–0.03 M trisodium citrate–0.1% SDS at 65°C for 30 min. Filters were wrapped in Saran Wrap and autoradiographed for 2 to 7 days, using XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) with a Cronex

Lightning-Plus intensifying screen (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) at -70°C .

RNA dot blot analysis. RNAs samples for dot blot analysis were bound to nitrocellulose by manifold filtration in saturated NaI (3). Residual DNA and rRNA does not bind efficiently to nitrocellulose in high-molarity NaI solutions. Hybridizations were performed as described for Southern blot analysis with the following exceptions: the formamide concentration of the hybridization solution was increased to 50%, and 1% SDS was added.

Probes. The probes used in this study are diagrammed in Fig. 1. pBR322 clones of three of the four internal *Pst*I restriction fragments from unintegrated exogenous MMTV(C3H) provirus were provided by Majors and Varmus (24). The *gag-pol*, *env*, and long terminal repeat (LTR) clones were subcloned into pUC12 and named pCP4.2, pCP1.8, and pCP1.4, respectively. An equal-mass mixture of the inserts from these three plasmids is referred to as rep probe in this report. pDM5 is a 1.3-kb *Pst*I-to-*Pvu*II fragment from λ GRT39 containing the MMTV(GR) 5' LTR cloned in *Pst*I- and *Sma*I-cleaved pUC12.

Preparation of radiolabeled DNAs. ^{32}P -labeled MMTV (C3H) cDNA was prepared by reverse transcription of random-primed viral RNA. A mixture of 500 ng of viral RNA and 200 μ g of base-sheared calf thymus DNA primers was heated to 100°C for 1 min and allowed to anneal for 20 min at 37°C in 25 mM KCl. The reverse transcription reaction was carried out with 100 μ Ci of [α - ^{32}P]dATP (3,000 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) in a 50- μ l reaction mixture containing 50 mM Tris (pH 8.1), 10 mM MgCl_2 , 5 mM KCl, 2 mM dithiothreitol, 25 μ g of actinomycin D per ml, 0.2 mM each dCTP, dGTP, and dTTP, 0.03 mM dATP, 10 μ g of MMTV(C3H) viral RNA per ml, and 1 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) per ml for 30 min at 37°C . The viral RNA template was base hydrolyzed after the reaction for 3 h in 0.3 M NaOH–50 mM trisodium EDTA–0.5% SDS. Unincorporated [α - ^{32}P]dATP was removed by gel filtration of the hydrolyzed reaction product on a Sephadex G-50 column.

^{32}P -labeled plasmid DNA was prepared by nick translation of gel-purified insert DNA. Insert DNAs from various plasmids were purified by gel electrophoresis of restriction

enzyme digests in 5% polyacrylamide gels, followed by electroelution of the desired fragments. Purified insert DNAs (200 ng) were nick translated with 100 μ Ci of [α - 32 P]dCTP (3,000 Ci/mmol; ICN Biomedicals Inc., Irvine, Calif.) in a 20- μ l reaction mixture containing 50 mM Tris (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, 50 μ g of bovine serum albumin per ml, 0.1 mM each dATP, dGTP, and dTTP, 10 pg of DNase I (Sigma Chemical Co., St. Louis, Mo.) per ml, and 0.5 U of DNA polymerase I (Bethesda Research Laboratories) per μ l at 15°C for 0.5 to 2 h (31). Unincorporated [α - 32 P]dCTP was removed by gel filtration of the reaction product on a Sephadex G-50 column. Probes routinely had a specific activity of 2×10^8 to 4×10^8 cpm/ μ g.

Transfections. The LA7 cell line selected for the gene transfer experiments was derived from a dimethylbenz(a)anthracene-induced rat mammary adenocarcinoma (1, 11). Cells were maintained on Dulbecco modified Eagle medium supplemented with 5% calf serum and 5 μ g of dexamethasone per ml. LA7 cells were seeded at a density of 5×10^5 /30-mm plate 48 h before transfection. λ GRT39 DNA was cotransfected with pSV-1/neo from P. A. Luciw (a pBR328 derivative of pSV2neo) carrying the Tn5 *neo* gene, which confers G-418 resistance to mammalian cells (36). Approximately 60 μ g of λ GRT39 or wild-type λ DNA was mixed with 20 μ g of pSV-1/neo DNA, ethanol precipitated, and suspended in 2 ml of Hanks buffered saline solution with a carefully adjusted pH of 7.10. Then 0.2 ml of 1.25 M CaCl₂ was added dropwise while the solution was gently mixed by bubbling air through the sample. The precipitate was allowed to form for 20 min at room temperature and then diluted with 2 ml of Hanks balanced salt solution. A 0.5-ml aliquot of precipitate solution was added per plate and adsorbed for 20 min at room temperature before the addition of 5 ml of growth medium. After incubation at 37°C for 4 h, the cells were washed and glycerol shocked for 3 min in Hanks balanced salt solution containing 15% glycerol (23). Selection for gene transfer was carried out by the addition of 400 μ g of G-418 per ml to the growth medium beginning at 24 h after transfection. More than 100 foci per plate were observed. Individual foci were picked and expanded into cell lines for analysis of λ GRT39-directed expression of MMTV.

Immunoperoxidase staining. Mammary tissues were fixed in 70% ethanol-acetic acid-formaldehyde (20:1:1) for at least 24 h (21). Fixed tissues were embedded in paraffin and sectioned. The sections were immunoperoxidase stained with polyclonal rabbit anti-MMTV antisera and a Vectastain ABC kit (Vector Laboratories, Burlingame, Calif.) according to the instructions of the manufacturer and counterstained with hematoxylin. A 1:1,000 dilution of anti-MMTV antiserum was used. This antiserum was prepared by using MMTV(C3H), purified from a BALB/cC3H tumor primary culture, as an immunogen (7).

Indirect immunofluorescent staining. Live cells were analyzed for cell surface expression of the MMTV SU protein (20) gp52 by indirect immunofluorescent staining, using the anti-gp52 rat monoclonal antibody 72H3 from A. Sonnenberg. Approximately 10^4 cells were passaged onto ethanol-washed glass cover slips in 24-well tissue culture plates and maintained in 0.25 ml of growth medium containing 5 μ g of dexamethasone per ml. Then 60 μ l of primary antibody 72H3 was added directly into the growth medium, and the cells were incubated at 37°C for 30 min. The cells were washed three times to remove the unbound primary antibody, and 20 μ l of fluorescein isothiocyanate-labeled goat anti-rat immunoglobulin G antiserum in 1 ml of growth medium was added. After incubation at 37°C for 30 min, the cells were

washed three times, fixed in 1 ml of 2% paraformaldehyde, covered with cover slips, and photographed with a fluorescence microscope.

Electron microscopy. Tissue culture cells for transmission electron microscopy were fixed in 2.5% glutaraldehyde-0.1 M sodium cacodylate (pH 7.2) for 2 h at room temperature and then overnight at 4°C. The fixed cells were washed twice with 0.1 M sodium cacodylate (pH 7.2), postfixed with 1% OsO₄-0.1 M sodium cacodylate (pH 7.2) for 1 h at 4°C, washed for 30 s in distilled water, and stained for 2 h with 2% aqueous uranyl acetate. In a modification of the technique of Brinkley et al. (4), the cells were embedded in situ, using an epon-araldite resin. Thin sections of selected areas were cut with a diamond knife, mounted on 200-mesh grids, stained with uranyl acetate and lead citrate, and examined in a Philips EM-400 electron microscope.

MMTV infections of BALB/c neonates. MMTV for injection into BALB/c neonates was prepared from the λ GRT39-transfected LA7 cell line L16-9B10 or the GR mouse mammary tumor cell line GR3A. Cell cultures were supplemented with 5 μ g of dexamethasone per ml to increase virus expression. Cells were scraped from the culture surface with a rubber policeman, and both cells and medium were homogenized gently in a Dounce homogenizer. The suspension was clarified by low-speed centrifugation in a clinical centrifuge for 5 min. Additional debris was removed by centrifugation at $15,000 \times g$ for 30 min in a refrigerated centrifuge. Virus particles were pelleted from the supernatant at 4°C by centrifugation at $150,000 \times g$ for 30 min in an ultracentrifuge. Virus pellets were suspended in approximately 200 μ l of Hanks balanced salt solution per confluent T75 culture flask. One-day-old BALB/c pups were injected intraperitoneally with 100 μ l of virus suspension.

RESULTS

Cloning of a full-length integrated exogenous MMTV(GR) provirus in bacteriophage lambda. A full-length integrated exogenous provirus was cloned from mouse mammary tumor sample GRTu7, which was collected from our GR mouse strain breeding colony. This tumor had a single new, clonally integrated MMTV(GR) exogenous provirus. A genomic library was constructed in bacteriophage lambda vector λ L47 by using high-molecular-weight tumor DNA and screened with radiolabeled probe pCP4.2 DNA from the *gag-pol* region of MMTV(C3H) (Fig. 1). A total of 39 positive plaques were observed, and 24 were successfully plaque purified. Phage miniprep DNAs were prepared from these recombinants and compared by Southern blot analysis of *Sst*I digests, using probe pCP1.4. A clone containing the full-length exogenous GRTu7 provirus, λ GRT39, was identified by the presence of (i) the 6.1-kb MMTV(GR)-specific *Sst*I restriction fragment (13) and (ii) low-molecular-weight host-virus junction restriction fragments that comigrated with novel exogenous proviral restriction fragments in the original tumor DNA. λ GRT39 contained the entire MMTV(GR) provirus and both 5' and 3' host flanking DNA (Fig. 1). The provirus was integrated upstream of the *int-2* gene (D. W. Morris, P. A. Barry, H. D. Bradshaw, Jr., and R. D. Cardiff, manuscript in preparation).

When λ GRT39 was amplified to prepare phage DNA for molecular analysis, a deletion mutant appeared (apparently by homologous recombination between the two LTRs) that removed the exogenous provirus poison sequence. Normal λ GRT39 plaques were small and had a relatively low titer. The deletion mutants formed large, high-titer plaques. Dur-

ing successive amplifications the original λ GRT39 clone was rapidly lost, particularly if the clone was amplified by using liquid culture techniques (25).

The deletion problem was circumvented by starting with early picks of λ GRT39, amplifying the clone by plate lysate methods, and limiting the number of sequential amplifications. Secondary picks of λ GRT39 were identified by plaque hybridizations of low-density platings of the primary pick. Individual secondary picks were amplified as 100-mm plate lysates, and DNA was purified as described above. DNA from each amplified secondary pick was checked for deletion mutant DNA by digestion with *Hind*III, which cleaves MMTV(GR) once near the middle of the genome. Amplifications that had large plaques or restriction pattern evidence of the deletion mutant were discarded. The remaining DNA preparations were pooled. The λ GRT39 DNA yield from each individual amplified secondary pick was approximately 1 to 2 μ g. With this procedure, sufficient DNA for molecular analysis and gene transfer experiments could be obtained.

Exogenous MMTV poison sequence in λ GRT39. The restriction map of the λ GRT39 provirus was the same as previously reported maps of exogenous MMTV(GR) (13, 14, 34). Figure 1 shows the λ GRT39 restriction map for the enzymes relevant to this report, *Pst*I and *Sst*I. To verify that λ GRT39 contains the exogenous MMTV poison sequence, the MMTV(GR) restriction patterns of λ GRT39 and a GR mammary tumor cell line, GR3A, were compared (Fig. 2). The GR3A cell line has a high copy number of the exogenous MMTV(GR) provirus (13). An MMTV viral RNA cDNA probe was used because it recognizes the entire MMTV genome, including the poison sequence. All internal MMTV(GR) restriction fragments in the GR3A cell line were also present in λ GRT39 (Fig. 2). In particular, the 1.1-kb *Pst*I fragment that originally localized the exogenous MMTV poison sequence (24) was present in λ GRT39 (Fig. 2, arrow). No differences in MMTV restriction fragment mobilities between λ GRT39 and GR3A DNAs were detected that would suggest that major structural changes had occurred during cloning or amplification. Although these data did not conclusively demonstrate that λ GRT39 had an intact, un-rearranged provirus, they were sufficient to justify testing the biological activity of the clone.

Expression of the λ GRT39 provirus after transfection into rat mammary adenocarcinoma cells. To test the biological activity of λ GRT39, the clone was introduced into LA7 cells. The LA7 cell line was derived from a chemically induced rat mammary adenocarcinoma (1, 11). This cell line was selected for two reasons. First, MMTV expression is tissue specific; therefore, a mammary cell line was used to maximize the chances of efficient production of λ GRT39-encoded retrovirus particles. Second, all mouse mammary cell lines have endogenous MMTV proviruses which could hypothetically be induced by the experimental manipulations involved in these experiments; therefore, a cell line from a closely related species was used.

λ GRT39 was cotransfected into LA7 cells with a pSV2neo derivative, and G-418-resistant transformants were selected. Twenty-one cell lines were established from individual G-418-resistant foci. Southern blot analysis with MMTV(C3H) rep probe showed that 76% (16 of 21) of the cell lines had stably integrated the unselected λ GRT39 DNA. Four of the λ GRT39-transfected cell lines (L15-2, L15-7, L16-5, and L16-9) and three control cell lines transfected with wild-type λ DNA (L8-1, L8-2, and L8-4) were arbitrarily selected for further analysis. Figure 3A shows a Southern blot of DNAs from these cell lines digested with *Sst*I and hybridized with

FIG. 2. Southern blot comparison of the λ GRT39 provirus and exogenous MMTV(GR) proviruses in a GR mammary tumor cell line. The *Pst*I and *Sst*I restriction patterns of λ GRT39 (lanes 2) and the MMTV(GR)-containing GR3A mammary tumor cell line (lanes 1) were compared by Southern blot analysis, using 32 P-labeled MMTV(C3H) cDNA as a probe. Letters in the margins refer to specific restriction fragments diagramed in Fig. 1. GR3A cells contain >50 copies of exogenous MMTV(GR). Consequently, the prominent GR3A bands are from internal restriction fragments of the high-copy-number exogenous provirus. λ GRT39 DNA was spiked into DNA from an MMTV-free mouse line (8) so that the λ GRT39 and GR3A lanes contained identical amounts of total DNA. All MMTV(GR) *Pst*I and *Sst*I internal restriction fragments, including the 1.1-kb *Pst*I poison sequence fragment (24), were present in λ GRT39, and these fragments all precisely comigrated with their counterparts in the GR3A cell line. The 1.1-kb *Pst*I poison sequence is labeled with an arrow. Fragment A' is the λ GRT39 *Pst*I A fragment (Fig. 1) annealed via the *cos* site to the first small *Pst*I fragment from the left arm of λ L47. The restriction patterns of *Hind*III-cleaved λ DNA and *Hae*III-cleaved ϕ X174 DNA markers are also shown.

32 P-labeled MMTV(C3H) rep probe. All λ GRT39-transfected samples had the *Sst*I C, D, F, and G fragments from the λ GRT39 provirus. In addition, several faint fragments were detected that could have been from rearrangements during transfection or reintegration of reverse-transcribed virus transcripts. On long exposures, several MMTV-related rat restriction fragments were detected that could be partially eliminated by increasing the stringency of the hybridization and wash steps of the Southern blot procedure. None of the rat fragments comigrated with the internal *Sst*I C fragment diagnostic for MMTV(GR) and MMTV(λ GRT39). In addition, there was no evidence of expression of the rat sequences in the wild-type λ -transfected controls by RNA dot blot analysis, immunofluorescent staining, or electron microscopy (see below).

Expression of the λ GRT39 provirus was first observed in the λ GRT39-transfected cell lines by RNA dot blot analysis (Fig. 3B). RNA was bound to nitrocellulose membranes by manifold filtration in the presence of saturated NaI (3) and

FIG. 3. Expression of the λ GRT39 provirus in rat mammary adenocarcinoma cells. (A) Southern blot analysis of λ GRT39-transfected LA7 cells. LA7 cells (lane 8) transfected with λ GRT39 DNA (lanes 5 through 7) or transfected with wild-type λ DNA (lanes 1 through 3) were analyzed by Southern blot analysis. Cell line DNAs were digested with *Sst*I and probed with MMTV(C3H) rep probe. Letters in the margins refer to specific restriction fragments diagrammed in Fig. 1. (B) RNA dot blot analysis of λ GRT39-transfected LA7 cells. Total cellular RNAs from the cell lines analyzed in panel A were applied to nitrocellulose by manifold filtration in saturated NaI and hybridized with MMTV(C3H) rep probe. MMTV RNA was detected only in cell lines that were transfected with λ GRT39 DNA.

probed with 32 P-labeled MMTV(C3H) rep probe. MMTV transcripts were detected in all four λ GRT39-transfected cell lines, although the level of expression in L15-2 and L16-5 was low. Equivalent RNA loading was verified by staining the RNA in situ on the nitrocellulose filter with methylene blue (25). As analysis of these cell lines was proceeding, we determined that the original stock of LA7 cells was contaminated with mycoplasma virus. Since mycoplasma virus is known to affect the level of expression of retroviruses in cell culture (26), no attempts were made to quantitate the levels of MMTV RNA expression in the cell lines or to correlate the levels of expression with the amount of transfected λ GRT39 DNA in each line. On the basis of the DNA and RNA data, L16-9 was selected for further analysis.

To determine whether the MMTV RNA expressed in L16-9 cells was capable of directing expression of MMTV protein, live cells were analyzed for cell surface expression of MMTV SU protein gp52. The rat anti-gp52 monoclonal antibody 72H3 was used in an indirect immunofluorescent staining procedure on L16-9, GR3A, and L8-2 cells. Patches of cells in the L16-9 culture were positive for gp52 antigen (Fig. 4). The level of expression was comparable to that of cells from the positive control GR3A cell line. No viral protein was detected in control L8-2 cultures. Nonexpressing cells in the L16-9 culture did not contain λ GRT39 DNA (see below).

Production of MMTV(λ GRT39) retrovirus particles by λ GRT39-transfected rat mammary adenocarcinoma cells. From the data presented above, it appeared that λ GRT39 was biologically active. To determine whether retrovirus particles were being produced, L16-9 and control cultures were examined by transmission electron microscopy. L16-9 cells were found to produce B-type retrovirus particles characteristic of MMTV (Fig. 5). Budding, immature, and mature B particles as well as intracytoplasmic A particles were observed. The mature B particles were characterized by electron-dense, eccentric nucleoids and the presence of spikes on the virus membrane. The particles observed in L16-9 cultures could not be morphologically distinguished from B particles observed in mammary tumor cultures by using the same techniques. No retrovirus particles were detected in the negative control culture L8-2. On the basis of these data, we concluded that λ GRT39 was fully expressed in LA7 cells and that these cells were making MMTV (λ GRT39) encoded by the λ GRT39 provirus.

Induction of mammary tumors in BALB/c mice by MMTV(λ GRT39) from λ GRT39-transfected LA7 cells. The B particles observed by electron microscopy in λ GRT39-transfected LA7 cells were tested in vivo for tumorigenicity by injection into hormone-stimulated females from the exogenous virus-free BALB/c mouse strain. This strain was selected because of its sensitivity to carcinogenesis by exogenous MMTV and its very low (<1%) spontaneous mammary tumor incidence (18).

A 100% immunofluorescence-positive, mycoplasma virus-free subline of L16-9, L16-9B10, was established for use as a source of MMTV(λ GRT39). Ten sublines were cloned from L16-9 by endpoint dilution. DNAs from five lines were analyzed by Southern blot analysis with 32 P-labeled probe pDM5. Three lines did not have detectable λ GRT39 DNA. Two lines had MMTV restriction patterns identical to that of the parental L16-9 cell line, which suggested that the original L16-9 focus was picked before stable integration of the unselected λ GRT39 DNA and that horizontal spread of MMTV(λ GRT39) does not occur in LA7 cultures. The L16-9B10 subline was treated for mycoplasma virus contamination with BM-Cycline (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) according to the instructions of the manufacturer and then recharacterized to verify that the cells were still producing MMTV antigen and B particles.

Virus particles from L16-9B10 cultures were injected intraperitoneally into BALB/c neonates. Control neonates were injected with similar preparations from the L8-2 negative control cell line or the GR3A positive control cell line. Neonates were used to avoid an immune response to the rat proteins present in the virus preparations. Female mice were stimulated by pituitary isografts at 1 month of age, and male mice were removed from the experiment.

Table 1 shows the tumor incidence in these groups at 10 months. Only females that survived to maturity are included.

FIG. 4. Expression of viral envelope SU protein gp52 on the surface of λ GRT39-transfected LA7 cells. Cell line L16-9 was analyzed for cell surface expression of gp52 by indirect immunofluorescent staining of live cells, using the anti-gp52 monoclonal antibody 72H3. A patch of fluorescent gp52-positive cells is shown in the top panel. The negative cells did not contain λ GRT39 DNA (see text) and serve as an internal negative control for background fluorescence. A phase photomicrograph of the same region is shown in the bottom panel.

Tumors began to appear at 9 months postinjection, and one-third of the experimental group had tumors by 10 months of age. At this time, we cannot project what the final tumor incidence will be with this induction protocol, although we anticipate more tumors. The animals will be held for 2 years.

Expression of viral antigens in MMTV(λ GRT39)-induced BALB/c mammary tumors. Immunohistochemical analysis of the first mammary adenocarcinoma to appear in the MMTV(λ GRT39)-injected animals is shown in Fig. 6. A paraffin section of the tumor was treated with polyclonal rabbit anti-MMTV antiserum (7) and immunoperoxidase stained. The tumor is a mammary adenocarcinoma, the predominant histopathological type of mammary tumor associated with MMTV infection. Concentrations of MMTV

antigen were readily detectable in the tumor, particularly on the apical surfaces of alveolar structures. The other three tumors in mice injected with L16-9B10 MMTV(λ GRT39) and the two tumors in mice injected with GR3A MMTV (GR) were also mammary adenocarcinomas and expressed MMTV antigen.

New copies of clonally integrated exogenous MMTV (λ GRT39) proviruses in MMTV(λ GRT39)-induced BALB/c mammary tumors. If the mammary adenocarcinomas appearing in the BALB/c mice injected with L16-9B10 virus preparations were induced by MMTV(λ GRT39), then two predictions could be made. First, the tumors should have the *Sst*I C restriction fragment diagnostic for exogenous provirus from the GR mouse strain (Fig. 1). Second, the tumors should have newly integrated copies of exogenous provirus

FIG. 5. Detection of B-type retrovirus particles in λ GRT39-transfected LA7 cells by electron microscopy. Budding, immature, and mature B particles were detected by transmission electron microscopy of L16-9 cultures grown in the presence of dexamethasone. A mature B particle (arrow) and several budding B particles are shown.

TABLE 1. Tumor incidence 10 months postinjection^a

Source of inoculum	Tumor incidence (%) ^b
L8-2.....	0 (0/2)
L16-9B10	36 (4/11)
GR3A.....	67 (2/3)
Uninjected	0 (0/8)

^a All mice were hormone stimulated with pituitary isografts.
^b Values in parentheses are number of tumor-bearing mice to total number of mice.

in clonal (although not necessarily monoclonal) stoichiometries.

Southern blot analysis of the first putative MMTV (λ GRT39)-induced BALB/c mammary adenocarcinoma is shown in Fig. 7. Restriction patterns of the tumor DNA (lane 2) and negative control spleen DNA (lane 1) were compared by using ³²P-labeled probe pDM5 (Fig. 1). The MMTV (λ GRT39)-diagnostic *Sst*I C fragment was readily detectable in the tumor sample, which indicated the presence of provirus from λ GRT39-encoded virus. The *Sst*I D fragment comigrated with an endogenous BALB/c proviral fragment (the lowest-molecular-weight fragment in lane 1) and was not used to track MMTV(λ GRT39) in these experiments. The

FIG. 6. MMTV antigen in a BALB/c tumor induced by injection with virus from λ GRT39-transfected LA7 cells. Shown is an immunoperoxidase analysis of two serial sections of the first tumor that arose in the BALB/c mice injected as neonates with virus from L16-9B10 cells. (A) Section treated with a polyclonal anti-MMTV primary antiserum; (B) section treated without the primary antiserum. The staining pattern is typical for MMTV-induced tumors, with concentrations of antigen most evident along the apical borders of alveolar structures.

*Sst*I F and G host-virus junction fragments from the λ GRT39 provirus were not detected, as expected, because of integration of the MMTV(λ GRT39) proviruses into new genomic locations in the BALB/c tumors. In addition, several unique restriction fragments representing host-virus junction fragments from the new, clonally integrated MMTV(λ GRT39) exogenous proviruses were detected. New copies of clonally integrated exogenous proviruses were also observed in the other three MMTV(λ GRT39)-induced tumors and the two MMTV(GR)-induced control tumors. All tumor DNAs had the *Sst*I C fragment and unique exogenous MMTV restriction patterns when cleaved with either *Pst*I or *Sst*I.

DISCUSSION

In this report, we describe the cloning and characterization of λ GRT39, a pathogenic clone of exogenous MMTV from the high-tumor-incidence GR mouse strain. Several lines of evidence indicated that the λ GRT39 provirus was biologically active. First, the λ GRT39 provirus was transcribed when transfected into rat mammary adenocarcinoma LA7 cells. Second, virus gp52 surface protein was made by λ GRT39-transfected LA7 cells, which suggested that viral RNA splicing and polyprotein processing were occurring in the LA7 cells. Third, dexamethasone-regulated, particle-

FIG. 7. Southern blot analysis of the first MMTV(λ GRT39)-induced BALB/c tumor. Tumor and negative control spleen DNAs were digested with *Sst*I and probed with a 32 P-labeled pDM5 insert. The *Sst*I C fragment diagnostic for exogenous MMTV from the GR strain (closed arrow) is clearly detectable in the tumor DNA and absent from the control DNA, demonstrating the presence of MMTV(λ GRT39) in the tumor. In addition, novel restriction fragments (open arrows) are detected. These fragments are host-virus junction fragments from new, clonally integrated copies of MMTV(λ GRT39).

associated reverse transcriptase activity was detected in cell-free culture medium from λ GRT39-transfected LA7 cells (unpublished data). Fourth, λ GRT39-transfected LA7 cells produced intracytoplasmic A particles and mature, immature, and budding B particles characteristic of MMTV. Finally, normal mouse mammary epithelial cells from endogenous and exogenous MMTV-free mice (8) could be efficiently infected in vitro by culture on irradiated λ GRT39-transfected LA7 cell feeder layers (12), as evidenced by transmission of restriction fragments characteristic of the λ GRT39 provirus to the mouse cells in culture (unpublished data). From these data, we concluded that the λ GRT39 provirus was fully expressed in LA7 cells and named the recombinant DNA-derived virus produced by these cells MMTV(λ GRT39) to distinguish it from the naturally occurring, GR mouse-derived MMTV(GR) virus. Additional experiments will be required to determine whether the two viruses are genetically identical.

The conclusion that the λ GRT39 provirus is pathogenic was based primarily on the presence of the MMTV(GR)-specific 6.1-kb *Sst*I restriction fragment in four mammary tumors that appeared in BALB/c mice injected with MMTV(λ GRT39) from λ GRT39-transfected LA7 cells. There are considerable data suggesting that the GR provirus uniquely identified by the 6.1-kb *Sst*I restriction fragment is both expressed and pathogenic. The 6.1-kb *Sst*I fragment has been shown to be present in increased copy number in (i) GR mammary tumors (13), plaques (15), and hyperplastic outgrowths (15), (ii) heterologous cells infected with MMTV from GR milk (14) or a mammary tumor cell line (14), and

(iii) BALB/c tumors induced by foster-nursing BALB/c pups on lactating GR females or injection of sucrose density gradient-banded virus from a GR mammary tumor cell line (unpublished data).

The BALB/c tumors had three other features common to virtually all MMTV-induced neoplasms. First, the tumors had new host-virus junction fragments detectable by Southern blot analysis, which indicated the presence of newly integrated copies of exogenous provirus in clonal stoichiometries. Second, the tumors expressed MMTV antigen. Third, the tumors were adenocarcinomas, the predominant histopathological type of mammary tumor associated with MMTV infection.

Therefore, the weight of evidence indicates that the BALB/c tumors described here were induced by MMTV(λ GRT39) from the λ GRT39 provirus genome. This evidence includes (i) transmission of the MMTV(GR)-specific 6.1-kb *Sst*I restriction fragment and (ii) comparisons with naturally occurring MMTV-induced tumors. Formal proof that the λ GRT39 provirus is pathogenic will require introduction of the λ GRT39 provirus genome directly into normal mouse mammary cells or embryos. Experiments along these lines are in progress.

Despite the technical difficulties of working with λ GRT39, we elected to develop systems to genetically analyze the native clone because of the large amount of virological data in the literature concerning MMTV(GR) (18). λ GRT39 was identified by chance during experiments to clone the host DNA flanking a single clonally integrated exogenous MMTV(GR) provirus observed in a mammary tumor from the GR mouse strain. Our experience with λ GRT39 was consistent with previous reports of attempts to clone exogenous MMTV provirus DNA. λ GRT39 was unstable and tended to delete the MMTV coding sequences by homologous recombination between the proviral LTRs during sequential amplifications. In addition, we were unable to subclone the *Sst*I D fragment containing the poison sequence (Fig. 1). An overlapping clone of this provirus truncated downstream of the poison sequence, λ GRT6, was stable in both lambda and plasmid vectors.

An alternate strategy for cloning biologically active MMTV proviral DNA is to construct chimeric proviruses in plasmids by substituting plasmid-stable endogenous provirus sequences for the unstable region of an exogenous provirus (33; G. M. Shackleford and H. E. Varmus, personal communication). Shackleford and Varmus constructed an infectious and pathogenic chimeric provirus from the weakly oncogenic *Mtv-1* endogenous provirus (30) and exogenous MMTV(C3H). The *Mtv-1*/MMTV(C3H) chimera was expressed in XC cells, was transmitted to uninfected XC cells in coculture, and produced mammary tumors in force-bred BALB/c females. Salmons et al. (33) constructed a chimeric provirus from the nononcogenic *Mtv-8* endogenous provirus and MMTV(GR). The *Mtv-8*/MMTV(GR) chimera was fully expressed in feline kidney cells; however, it was not determined whether this chimeric virus was infectious or tumorigenic.

Another exogenous MMTV(GR) clone has been reported by Buetti and Diggelmann (6). These authors cloned unintegrated circular MMTV(GR) provirus DNA from the Hirt fraction of MMTV(GR)-infected rat hepatoma cells. Intact clones containing a single LTR and rearranged clones containing two LTRs were recovered. Partial expression of a concatemer of a single-LTR clone was observed in L cells. The poison sequence region of the single-LTR clone was successfully subcloned and sequenced (16). We speculate

that the single-LTR clones were a MMTV(GR) variant present in the cell line and that the rearranged clones were clones of the exogenous MMTV(GR) virus that mutated during cloning as a consequence of the poison sequence (5). A comparison of the λ GRT39 poison sequence region (sequenced directly by using a λ GRT39 template and synthetic oligonucleotide primers) and the published sequence of the same region of the single-LTR isolate should resolve the question and may shed additional light on the nature and location of the exogenous MMTV poison sequence.

The experiments described in this report are a prelude to mutational analysis of the MMTV(GR) genome. Procedures must now be devised to efficiently shuttle small subclones of the λ GRT39 provirus back into the lambda clone after mutagenesis *in vitro*. In addition, LA7 cell transfection and culture conditions must be optimized for maximal virus production to eliminate the need for isolation and characterization of individual subclones. Finally, biological systems to assay MMTV function, such as the *in vitro* infection system alluded to above, must be developed. Analysis of MMTV mutants in cell culture and *in vivo* systems will be important in the solution of a number of fundamental problems in the MMTV field.

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ADDENDUM

Virus from the *Mtv-8*/MMTV(GR) chimeric provirus reported by Salmons et al. (33) has recently been shown to be infectious (N. H. Sarkar, personal communication). Also, at 15 months postinfection, the tumor incidence of the positive control group infected with MMTV(GR) from GR3A cells was 100% (3 of 3) and the tumor incidence of the experimental group infected with MMTV(λ GRT39) from L16-9B10 cells was 82% (9 of 11). Several animals developed multiple tumors, and premalignant lesions were observed in mammary gland whole mounts. No tumors have been observed in either of the negative control groups.

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