Identification and partial characterization of an endogenous form of mouse mammary tumor virus that is transcribed into the virion-associated RNA genome

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Received 31 July 1980

ABSTRACT

Restriction mapping demonstrated the presence of several distinct proviral forms of mouse mammary tumor virus in the genome of GR mice. One of these proviruses (GR-MTV-2) was highly amplified in GR 3A cells, a cell line derived from a GR mammary tumor. By the criterion of restriction mapping, the amplified GR-MTV-2 provirus found in GR 3A cells was identical to the provirus found in M1.19 cells, a rat cell line infected with virions obtained from GR 3A culture fluid. This result clearly implies that the GR-MTV-2 provirus in GR 3A cells was transcribed into the virion-associated viral RNA genome. Cleavage of either GR 3A or M1.19 cell DNAs with the restriction enzyme *Bgl* II gave rise to a 2.6×10^6 dalton GR-MTV-2 proviral fragment (ca. 45% of the viral genome). This fragment was isolated and mapped with thirteen restriction enzymes.

INTRODUCTION

All laboratory strains of mice harbor endogenous, genetically transmitted proviral copies of mouse mammary tumor virus (MuMTV) (1). Liquid hybridization has placed the number of endogenous MuMTV proviruses at 5-7 copies per haploid cell in GR mice (2). During lactation, one or more of the endogenous GR proviruses is transcribed into RNA which is packaged into virions and passed onto newborn mice via the milk (3). The GR milk-borne virus is known to be a causative agent in the development of mouse mammary tumors in low tumor incidence mouse strains (3).

Most, if not all, mouse mammary tumors induced by viral infection contain more MuMTV proviral copies than found in normal tissue (2,4). Cells derived from some of these tumors have been successfully cultured. One such line, designated GR 3A, was derived from a mammary tumor originating in a GR mouse (5). GR 3A cells have approximately 40-60 more copies of MuMTV than normal tissue such as liver (6) and release large quantities of virions (MuMTV-GR) into the culture fluid. These virions are infectious for heterologous cells

such as rat (7,8) and feline (8) cells. One MuMTV-GR infected rat cell line, designated M1.19, contains 10-20 proviral copies of MuMTV (6).

We have recently demonstrated that GR mammary tumors contain additional MuMTV proviral sequences and that these sequences originate from only one class of the 5-7 endogenous proviruses of GR mice. We have designated this provirus GR-MTV-2 provirus because it is absent from the DNA of GR-Mtv-2⁻ mice (9). Further, the GR-MTV-2 provirus found in GR mammary tumors was identical to the amplified provirus found in GR 3A cells (9). Here we show, on the basis of restriction mapping, that the GR-MTV-2 provirus found in GR 3A cells is identical to the MuMTV provirus found in MuMTV-GR infected rat cells. Thus, the GR-MTV-2 provirus in GR 3A cells serves as a template for the virion-associated RNA genome.

MATERIAL AND METHODS

cell Lines and Nucleic Acid Extraction: GR 3A is a cell line derived from a GR mouse mammary tumor (5). M1.19 and Fe/GR are MuMTV-infected rat (7) and feline (10) cell lines respectively. The source of the MuMTV used to infect the heterologous cells was GR 3A tissue culture fluid (M1.19) and GR mouse milk (Fe/GR). The lines were maintained in Dulbecco's Modified Eagle's Medium with 5% calf serum, 5 µg/ml dexamethasone, 10 µg/ml insulin and 25 µg/ml gentamicin. The cells were scraped from tissue culture plates, suspended in 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 10 mM NaCl, then proteinase K and SDS were added to 100 µg/ml and 0.5% respectively (11). After 30 min at 37°C, the mixtures were extracted twice with buffer-saturated phenol and the nucleic acids were dialyzed exhaustively at 4°C against 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

MuMTV virions (from the Mm5mt/cl cell line (12)) were disrupted by adding SDS to 1% and the RNA was extracted twice with phenol. The RNA was heated to 90°C for 3 minutes, then centrifuged in a 5-20% sucrose gradient (13). Material sedimenting in the 23-35S region of the gradient was collected, ethanol precipitated and dissolved in 10 mM Tris-HCl, pH 7.4, 50 mM KCl, 1 mM EDTA.

preparation of cDNA: MuMTV-specific (³²P)-cDNA was prepared in a 50 µl reaction mixture containing: 50 mM Tris-HCl, pH 8.1, 75 mM KCl, 10 mM MgCl₂, 2 mM dithiothreitol, 0.2 mM each of dGTP, dCTP and dTTP, 50 µCi (³²P)-dATP (New England Nuclear, 2,000 Ci/mmol), 25 µg/ml actinomycin D, 0.5 µg MuMTV 23-35S RNA, 600 µg calf DNA primers (14) and 50 units of avian myeloblastosis

virus reverse transcriptase (15). The mixture was incubated for 30 minutes at 37°C and then EDTA, SDS and NaOH were added to final concentrations of 0.05 M, 0.5% and 0.3 M respectively. After incubation at 65°C for 3-4 hours to hydrolyze the RNA, the (³²P)-cDNA was passed through a Sephadex G-50 column, ethanol precipitated and dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

Enzyme Digests and Blotting: Restriction enzymes were purchased from New England Biolabs, Beverly, MA. Digests were done in 100 mM Tris-HCl, pH 7.4, 10 mM NaCl, 5 mM MgCl₂. Lysozyme was added to 100 µg/ml to digests done at 37°C. No lysozyme was added to digests performed at 65°C (eg. Taq I) since this appeared to result in the aggregation of the DNA. In our hands certain enzymes (eg. Eco RI, Hind III, Kpn I) failed to cleave DNA isolated from agarose gels via hydroxylapatite as described by Tabak and Flavell (16). The inclusion of lysozyme, but not bovine serum albumin, in the cleavage mixture overcame the inhibitory effect. The DNAs to be examined were mixed with approximately 1 µg of bacteriophage lambda DNA so that the completeness of the digestion could be verified by the restriction pattern of the added lambda DNA. Total cellular DNA digests were run in 1% agarose gels, isolated fragments were run in 1.7% agarose gels. The gels contained 50 mM Tris-HCl, pH 8.1, 20 mM NaOAc, 22 mM NaCl, 2 mM EDTA which was also the composition of the reservoir buffer. The transfer of DNA fragments from the gels to nitro-cellulose filters and the subsequent hybridization was done essentially as described by Southern and others (17,18). The isolation of DNA fragments from agarose gels was done by a slight modification of the procedure of Tabak and Flavell (16).

RESULTS AND DISCUSSION

We extracted the nucleic acids from several GR mouse livers and digested the DNAs with the restriction enzymes Sac I, Bgl II and Taq I. The digests were analyzed by the blot-transfer method of Southern (17). Liver cells are not infected by MuMTV (2). Thus, the restriction patterns should reveal the endogenous, genetically transmitted proviruses. An autoradiogram of one analysis is presented in Figure 1A. It is apparent from this figure that each enzyme generated a unique pattern of proviral fragments and that there was little, if any, amplification of specific sequences.

In contrast to the patterns seen in GR liver DNA, the MuMTV restriction patterns of GR 3A and M1.19 DNAs indicated that one, or one class of, MuMTV

Figure 1. Comparison of MuMTV proviral sequences in GR liver DNA, GR 3A cell DNA and M1.19 cell DNA. (A) GR liver DNA (9 µg) was digested with either Sac I (slot 1), Bgl II (slot 2) or Taq I (slot 3) and analyzed the Southern procedure and autoradiography. (B) Approximately 5 µg of either GR 3A (slots 1, 3 and 5) or M1.19 (slots 2, 4 and 6) cell DNAs were digested with either Sac I (slots 1 and 2), Bgl II (slots 3 and 4) or Taq I (slots 5 and 6) and analyzed as in (A). The dots in (A) identify the fragments in GR liver DNA that are amplified in GR 3A and M1.19 DNAs (B). The dot in (B) between slots 2 and 3 identifies the 2.6×10^6 dalton Bgl II fragment.

provirus was extensively amplified (Figure 1B; the liver fragments corresponding to the amplified fragments are indicated by dots in Figure 1A). We have previously shown that this virus is amplified in GR mammary tumors and have designated it the GR-MTV-2 provirus (9). The molecular weights of the major GR-MTV-2 proviral sequences found in GR 3A and M1.19 cellular DNAs were: Sac I, 1.6 and 4.0×10^6 daltons; Bgl II, 2.6×10^6 daltons; Taq I, 1.2 , 1.6 and 1.9×10^6 daltons. Proviral sequences that are flanked by mouse (rat) sequences would be expected to migrate to many positions in the gel since MuMTV can integrate at many positions in the host genome (18) and the restriction sites in the mouse (rat) genome should differ in distance from integration site to integration site. Thus, we attribute the appearance of discrete bands to cleavage at sites within the amplified GR-MTV-2 provirus.

The major MuMTV restriction fragments of GR 3A cell DNA were virtually identical with those of the MuMTV-GR infected rat cell DNA. The only exception was a small Bgl II fragment present in GR 3A cell DNA, but not in M1.19 cell DNA (Figure 1B, compare slots 3 and 4). However, this fragment arose from cleavage within one or more of the MuMTV proviruses that were not amplified since GR-Mtv-2⁻ mice, which lack the GR-MTV-2 provirus, still contained the fragment (9).

The results of Figure 1B clearly imply that the amplified GR-MTV-2 provirus found in GR 3A cellular DNA was also present in MuMTV-GR infected rat cells. To test this further we isolated the 2.6×10^6 dalton Bgl II proviral

fragment (Figure 1B) from both GR 3A and M1.19 cellular DNAs by the method of Tabak and Flavell (16). We did this because (a) preliminary data indicated that many of the enzymes that cleave the GR-MTV-2 provirus a limited number of times did so within this region, (b) the "background" caused by cleavage of the endogenous, nonamplified proviruses present in GR 3A cells, and by the unique provirus-host sequences (Figure 1B, slots 3 and 4; the bands above the amplified fragments represent MuMTV proviral-host sequences) could be eliminated and (c) other investigators (19) had shown that this fragment is near the 3'-end of the genome (*ie.* in an area that probably codes for the *env* proteins and might code for a transforming protein).

We digested both isolated 2.6×10^6 dalton Bgl II fragments with eleven restriction enzymes. An example of one analysis is shown in Figure 2. The major digestion products of each fragment were identical in molecular weight. In addition, the partial digestion products were identical (Figure 2, slots 1, 2, 5, 6, 7, 8). Double digests of each fragment with Eco RI plus Hind III and Pst I plus Bgl I also gave identical patterns. We conclude, on the basis of restriction mapping, that the two MuMTV proviral fragments, one from mouse cells and one from rat cells, are identical. Thus, we conclude that the GR-MTV-2 provirus in GR 3A cells is transcribed into viral genomic RNA that is incorporated into infectious virions which are released into the culture fluid. This identifies the GR-MTV-2 provirus as the high oncogenic virus of GR mice since viruses obtained from GR 3A culture fluid cause mammary tumors when injected into low tumor incidence strains of mice (20).

We found one exception to the results presented above. The restriction enzyme Hha I cleaved the 2.6×10^6 dalton Bgl II fragment isolated from rat

Figure 2. Restriction maps of the MuMTV 2.6×10^6 dalton Bgl II proviral fragments of GR 3A and M1.19 cell DNAs. Each fragment was digested with either Bam HI (slots 1 and 2), Hind III (slots 3 and 4), Alu I (slots 5 and 6) or Taq I (slots 7 and 8) and analyzed by the Southern procedure and autoradiography. The digestion products of the fragment from GR 3A DNA were run in slots 1, 3, 5 and 7; those of the fragment from M1.19 DNA in slots 2, 4, 6 and 8.

cells at a site not cleaved in the fragment isolated from mouse cells. However, we have data which indicates that the Hha I enzyme cleaves the two proviral DNAs differentially at several sites. We attribute the differential cleavage patterns to the fact that the Hha I enzyme contains a CpG dinucleotide in its recognition sequence. A large percentage of the CpG dinucleotides in mouse and rat DNAs contain methylcytosine (21) which inhibits the Hha I enzyme (22). Thus, differential degrees of DNA methylation in mouse and rat cells probably accounts for the different patterns we observe between the two proviral DNAs after cleavage with Hha I (Fanning, Puma, Cardiff, manuscript in preparation).

The 2.6×10^6 dalton Bgl II fragment isolated from GR 3A cell DNA was digested with 24 restriction enzymes. Eight enzymes (BstE II, Hpa I, Kpn I, Pvu II, Sac I, Sal I, Xba I, Xho I) did not cleave within the fragment. Three enzymes (Hae III, Hinf I, Mbo I) cleaved the fragment so frequently that mapping the majority of the cleavage sites proved impossible. The remaining enzymes cleaved at a limited number of sites. In most cases we mapped these cleavage sites in relation to the single Eco RI site. An example of such an analysis is shown in Figure 3.

Complete cleavage maps could not be obtained for several of the enzymes we tested. For example, three fragments were detected after digestion of the 2.6×10^6 dalton Bgl II fragment with Mbo II. We were able to place all three

Figure 3. Restriction maps of the MuMTV 2.6×10^6 dalton Bgl II proviral fragment from GR 3A cell DNA. The isolated fragment was digested with the following enzymes and the digests were analyzed by the Southern procedure and autoradiography: slot 2, no enzyme; slot 3, Eco RI; slot 4, Pst I; slot 5, Pst I plus Eco RI; slot 6, Hind III; slot 7, Hind III plus Eco RI; slot 8, Bgl I; slot 9, Bgl I plus Eco RI; slot 10, Hha I; slot 11, Hha I plus Eco RI. Slots 1

and 12 contain (^{32}P)-lambda DNA cleaved with Hind III and (^{32}P)- ϕX 174 RF DNA cleaved with Hae III. The molecular weights of some of the marker DNA fragments are given (in 10^6 daltons) on the left.

fragments in the middle of the larger fragment (see Figure 4), but fragments corresponding to both the right and left ends were not detected. Our experience with Hae III digested (32 P)- ϕ X 174 RF DNA has been that fragments smaller than 250-300 base pairs (Ca. 1.8×10^5 daltons) are not detectable in our system. Thus, some of the enzymes (eg. Mbo II, Alu I) appear to have cleavage sites on the fragment that are maximally 250-300 base pairs apart.

We determined the Sac I, Bgl II and Taq I cleavage sites within the GR-MTV-2 provirus by double digesting either Bgl II or Sac I digests of GR 3A cell DNA with Taq I. In addition, we digested a partial Taq I digest with Hind III (which cleaves the GR-MTV-2 provirus once) to independently align the Taq I fragments on the provirus (data not shown). The completed restriction map is shown in Figure 4. The 2.6×10^6 dalton Bgl II fragment is approximately 4,000 base pairs long which represents about 45% of the GR-MTV-2 genome (19). We feel that the map positions within the Bgl II fragment shown in Figure 4 are accurate to within $\pm 2\%$ (± 80 base pairs) and the Sac I, Bgl II and Taq I sites in the proviral map to within $\pm 5\%$ (± 450 base pairs).

In addition to MuMTV-GR infected rat cells, we have examined MuMTV-GR infected feline cells (Fe/GR). The source of MuMTV-GR used to infect the cells was GR mouse milk (10). DNA from the MuMTV-GR infected feline cells was

Figure 4. Restriction map of the amplified MuMTV provirus (GR-MTV-2) in GR 3A cell DNA. The restriction sites in the upper map are Sac I ($\square$), Bgl II ($\circ$) and Taq I ($\circ$). The parentheses around the Taq I sites within the Bgl II fragment indicate that their number and positions are unknown. The parentheses around the Taq I sites to the left of the Bgl II fragment indicate that it is not known if such sites exist in this part of the provirus. The cleavage sites within the Bgl II fragment are given in the lower map. The sites for each enzyme are represented by a circle. The horizontal bars between circles, or to the ends of the fragment, indicate the fragment was detected. Open spaces indicate that no fragment was detected. The half-circles in the Alu I map are suspected sites based upon partial cleavage products. The units at the bottom of the Bgl II map are in base pairs. The orientation of the map is 3' (left) to 5' (right) relative to the viral RNA (19).

indicate that no fragment was detected. The half-circles in the Alu I map are suspected sites based upon partial cleavage products. The units at the bottom of the Bgl II map are in base pairs. The orientation of the map is 3' (left) to 5' (right) relative to the viral RNA (19).

cleaved with ten restriction enzymes. The restriction patterns were identical to those obtained after cleavage of GR 3A cell DNA with the same ten enzymes (data not shown). Thus, both heterologous cell systems (rat and feline) harbored the GR-MTV-2 provirus after infection with MuMTV-GR.

Recently we have demonstrated that GR mammary tumors contain more proviral copies of MuMTV than nonmalignant tissue and that only one of the endogenous GR proviruses (GR-MTV-2) was amplified (9). Those results, together with the results presented here demonstrating the infectious nature of GR-MTV-2, suggest that the GR-MTV-2 provirus plays a major role in mammary tumorigenesis in the GR mouse.

ACKNOWLEDGEMENTS

We thank Mr. L.J.T. Young for technical assistance. This work was supported by Grant Number 5 R01 CA 21454 awarded by the National Cancer Institute U.S. Public Health Service DHEW.

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