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Number and Location of Mouse Mammary Tumor Virus Proviral DNA in Mouse DNA of Normal Tissue and of Mammary Tumors

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The Southern DNA filter transfer technique was used to characterize the genomic location of the mouse mammary tumor proviral DNA in different inbred strains of mice. Two of the strains (C3H and CBA) arose from a cross of a Bagg albino (BALB/c) mouse and a DBA mouse. The mouse mammary tumor virus-containing restriction enzyme DNA fragments of these strains had similar patterns, suggesting that the proviruses of these mice are in similar genomic locations. Conversely, the pattern arising from the DNA of the GR mouse, a strain genetically unrelated to the others, appeared different, suggesting that its mouse mammary tumor proviruses are located in different genomic sites. The structure of another gene, that coding for β-globin, was also compared. The mice strains which we studied can be categorized into two classes, expressing either one or two β-globin proteins. The macroenvironment of the β-globin gene appeared similar among the mice strains belonging to one genetic class. Female mice of the C3H strain exogenously transmit mouse mammary tumor virus via the milk, and their offspring have a high incidence of mammary tumor occurrence. DNA isolated from individual mammary tumors taken from C3H mice or from BALB/c mice foster nursed on C3H mothers was analyzed by the DNA filter transfer technique. Additional mouse mammary tumor virus-containing fragments were found in the DNA isolated from each mammary tumor. These proviral sequences were integrated into different genomic sites in each tumor.

Mouse mammary tumor virus (MMTV) is an RNA-containing virus which causes mammary tumors in mice. In certain inbred strains of mice the virus is exogenously transmitted to the offspring via the milk. Animals which receive this milk-borne virus have a high incidence of mammary tumor occurrence. In addition, mice transmit MMTV-specific information to their offspring via the germ cell. This endogenously transmitted MMTV is present as proviral DNA integrated into the genomic DNA of all cells of the mouse (2, 23, 27).

All tested inbred strains of mice contain endogenously transmitted MMTV proviral sequences (22, 25, 35, 44). The origin and the possible function of proviral DNA remain speculative. In all but the GR mouse strain (3) the endogenously transmitted MMTV proviral sequences appear to have no role in early mammary tumor occurrence. One hypothesis is that these proviral sequences arise from the viral infection of the germ cell of the animal (43). A comparative study of the genomic locations of the endogenous MMTV proviruses in different inbred mouse strains may be informative in determining their origins.

Mammary tumors arising in mice that were exogenously infected by milk-borne virus have been shown by hybridization analyses to contain not only the endogenous proviruses but also additional proviral copies (22, 25). These are assumed to have arisen from the exogenous viral infection of mammary gland cells, followed by reverse transcription and viral integration into genomic DNA. These mammary tumor-specific proviral sequences are thought to be related to mammary tumor formation. The control of their expression may be based, in part, on the primary nucleotide sequence within and around the proviral integration site. Therefore, we have begun a characterization of the integration sites of the exogenously acquired MMTV proviruses of mammary tumor DNA.

We used the Southern DNA filter transfer technique (37) to study both the genomic locations of endogenous MMTV proviruses and the genomic integration sites of the exogenously acquired proviruses in mammary tumor DNA. We compared the endogenous proviruses present in the liver DNA of seven different inbred mouse strains. Six of the strains have been bred in the United States, and of these, two (CBA and C3H)

can be directly traced from a BALB/c and DBA mating (19, 38). The seventh strain, GR, has been bred in Europe (26, 38). The results suggest that the genomic locations of the MMTV proviruses in mouse strains of related genetic origins are similar. Conversely, the MMTV proviral DNA in mouse strains of unrelated origins appears to be located in different genomic sites. We determined the integration sites of the newly acquired MMTV proviruses in mammary tumor DNA isolated from individual C3H or BALB/c mammary tumors. C3H females produce milk-borne MMTV, and their offspring have a high incidence of mammary tumor occurrence. BALB/c mice transmit no milk-borne MMTV, but when foster nursed on C3H mothers (BALB/cfC3H), these animals also have a high incidence of mammary tumor occurrence. Additional MMTV-containing sequences were identified in the mammary tumor DNA of both strains. The additional copies were integrated at different genomic sites in each tumor.

MATERIALS AND METHODS

Mouse strains and genetic origins. BALB/cfC3H mice bearing mammary tumors and GR mice were obtained from the Netherlands Cancer Institute, Amsterdam, The Netherlands. C3H/HeJ, BALB/c, CBA/H-T6, C57BL/6J, DBA/2J, and A/J mice were obtained from colonies maintained by the animal care center of the Swiss Institute for Experimental Cancer Research. All of the inbred strains, with the exception of GR, have been bred in the United States. Approximately 60 years ago a Bagg albino female was mated with a DBA male. A number of different strains, including C3H and CBA, can be traced directly from this mating. In addition, the BALB/c strain originated directly from the Bagg albino stock, and the A strain descended from a cross of a Bagg albino with another mouse stock. The C57BL strain cannot be traced back directly from the above-mentioned crosses but it descends from stocks which were used for breeding other American strains (19, 38). Thus, five of these strains are known to be genetically related, and the possibility that the C57BL strain is also related to other American-bred strains is not unlikely. The GR strain originated and has been bred in Europe (26, 38).

Purification of MMTV viral RNA. GR cells from a mouse mammary tumor cell line obtained from K. Yamamoto were grown in roller bottles in Dulbecco-modified Eagle medium containing 10% fetal calf serum and 10^{-6} M dexamethasone. The medium from confluent cultures was collected at 24-h intervals and clarified by centrifugation, and the virus was pelleted by a second centrifugation for 45 min at 25,000 rpm in a Beckman SW27 rotor. The pelleted virus was suspended in TNE (0.1 M NaCl, 5 mM EDTA, 10 mM Tris-hydrochloride [pH 7.5]) and repelleted in a Beckman SW65 rotor by centrifugation for 30 min at 35,000 rpm. The viral pellet was resuspended in TNE, and proteinase K and sodium dodecyl sulfate (SDS) were added to, respectively, 100 μg/ml and 0.5%. After incubation for 1 h at 37°C, the viral RNA was extracted with phenol-chloroform and chloroform and precipitated by the addition of 2 volumes of ethanol. The RNA was dissolved in 10 mM Tris-hydrochloride (pH 7.5)–5 mM EDTA and layered onto a sucrose gradient (10 to 30% sucrose in 0.1 M Tris acetate [pH 8.0], 0.2% SDS) which was centrifuged for 2 h at 50,000 rpm at 2°C in a Beckman SW65 rotor. The 70S region of the gradient was collected, the RNA was precipitated by the addition of 2 volumes of ethanol, and the precipitate was collected by centrifugation. The RNA was redissolved in 10 mM Tris-hydrochloride (pH 7.5)–5 mM EDTA and heat denatured for 2 min at 70°C. The solution was layered onto a second sucrose gradient made as described above and centrifuged for 4.5 h at 50,000 rpm in a Beckman SW65 rotor. The RNA sedimenting in the 20 to 40S region was collected, precipitated by the addition of 2 volumes of ethanol, and isolated by centrifugation. The RNA was dissolved in sterile water and stored at −20°C.

Isolation of DNA from mouse tissues. Mammary tumors and organs were removed from the animals, frozen in liquid nitrogen, and stored at −70°C. One gram of tissue was thawed in 10 ml of homogenization buffer (0.3 M sucrose, 10 mM NaCl, 1.5 mM magnesium acetate, 10 mM Tris-hydrochloride [pH 8.0], 1 mM dithiothreitol, 0.1% Nonidet P-40) and homogenized. The nuclei were sedimented by centrifugation for 5 min at 3,000 × *g*, the pellet was washed 3 times with 10 ml of homogenization buffer and suspended in 10 ml of TNE, and proteinase K and SDS were added to, respectively, 100 μg/ml and 0.5%. After incubation for 12 h at 37°C, the DNA was extracted with phenol-chloroform and chloroform and precipitated by the addition of 2 volumes of ethanol. The DNA was collected by centrifugation and suspended in 1 mM EDTA to a concentration of approximately 500 μg/ml.

Samples of DNA used in hybridization analyses were brought to 0.3 N NaOH and incubated at 100°C for 10 min to denature and decrease the size of the DNA and to digest the nuclear RNA. The solution was neutralized, and the DNA was precipitated by the addition of 2 volumes of ethanol and collected by centrifugation. The pellet was dissolved in 1 mM EDTA, and the DNA concentration was determined by the diphenylamine analysis (6).

Preparation of cDNA. MMTV-specific complementary DNA (cDNA) was synthesized with 20 to 40S viral RNA as the template in a reaction mixture (0.1 ml) containing 0.05 M Tris-hydrochloride (pH 8.3); 0.05 M KCl; 8 mM $MgCl_2$; 5 mM dithiothreitol; 1 mM dATP, 1 mM dGTP, 1 mM dTTP, 0.1 mM [^{3}H]dCTP (25.5 Ci/mmol), or 0.05 mM [^{32}P]dCTP (40 Ci/mmol); actinomycin D (100 μg/ml); RNA (15 to 20 μg/ml); and avian myeloblastosis virus reverse transcriptase (100 U/ml). The synthesis was primed by denatured fragments of DNA prepared from *Micrococcus luteus* DNA treated with DNase I as described previously (40). The DNA fragments at a final concentration of 260 μg/ml were used to prime the synthesis. The mixture was incubated for 2 h at 25°C, and the cDNA was isolated as described previously (15, 16).

For determining the extent to which the MMTV RNA was reverse transcribed into cDNA, increasing

amounts of cDNA were hybridized with a fixed amount of ^{125}I-labeled MMTV RNA prepared as described previously (41). At a cDNA/RNA ratio of 2:1, 50% of the hybridizable RNA was in a cDNA-RNA hybrid, suggesting that the cDNA is a fairly homogeneous copy of the template RNA. At a 10:1 cDNA/RNA ratio, a maximum of 80% of the RNA became resistant to RNase A (data not shown). The purity of the cDNA was assessed by analyzing for the presence of sequences complementary to rRNA, the most likely contaminant of the viral RNA preparation. With the RNA filter transfer technique (1) total polysomal RNA isolated from a GR cell line was bound to paper and hybridized with MMTV cDNA. There was no evidence of hybridization in the region corresponding to mouse rRNA (see Fig. 3 of reference 12).

cDNA hybridization to total cellular DNA. The number of MMTV proviral genomes in the various preparations of cellular DNA was determined by a cDNA excess hybridization technique (4). The DNA (1.6 to 10 μg) was lyophilized together with amounts of ^{3}H-labeled MMTV cDNA (specific activity, 19 cpm/pg) ranging from 30 to 300 pg to achieve the ratios indicated in the figures. The lyophilized nucleic acids were dissolved in 0.24 M sodium phosphate buffer (pH 6.8) containing 1 mM EDTA at a concentration of 0.5 mg/ml, denatured at 100°C for 10 min, and annealed under paraffin oil at 69°C for 48 h. The total content of each tube was removed into 0.5 ml of S1 nuclease assay buffer (0.3 M NaCl, 0.03 M sodium acetate [pH 4.5], 3 mM $ZnSO_4$, denatured *M. luteus* DNA [10 μg/ml]) and the proportion of cDNA which hybridized was determined as described previously (15, 16).

With this technique (4) it is also possible to determine the kinetic complexity of the cDNA, providing an additional measure for the representativeness of the probe. For measuring the kinetics of hybridization, samples containing 1,000 cpm of cDNA were removed at various time intervals into 0.5 ml of S1 nuclease assay buffer, and the proportion of hybridized cDNA was determined. Figure 1 shows the kinetics of hybridization of MMTV cDNA with the total DNA isolated from a C3H mouse liver. From the initial slope of the curve the $D_0t_{1/2}$ value of 6.9×10^{-4} ml^{-1} s was calculated. The substitution of this value into Bishop and Freeman's equation 3 (4) yielded a value for the kinetic complexity of the MMTV cDNA. After a correction was made for the salt concentration (5), this value was found to be 2.35×10^6, a number which represents approximately 80% of the reported molecular weight of MMTV viral RNA (10). These results also suggest that the cDNA probe represents most of the MMTV genome.

Restriction endonuclease digestion of DNA. Restriction endonucleases *Hin*dIII and *Bam*HI were purchased from New England Biolabs, Beverly, Mass., *Eco*RI was purchased from Boehringer Mannheim Corp., West Germany, and *Pst*I was a gift of G. Fey.

DNA (20 μg) was digested with a twofold enzyme excess in the buffers specified by the manufacturers. The digestion mixtures were incubated for 6 h at 37°C and then for an additional 30 min at 37°C with RNase (50 μg/ml) to remove RNA. Finally, the reaction was terminated by the addition of SDS to 0.2%. To ensure complete digestion, in some cases the mixture was

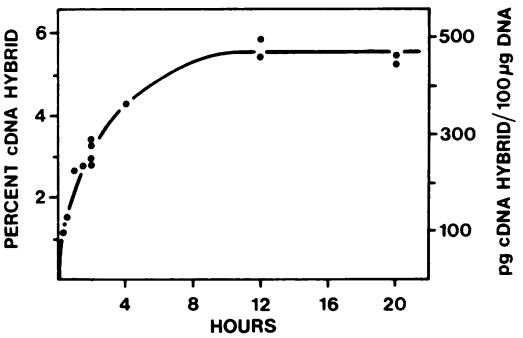

FIG. 1. *Kinetics of hybridization of ^{3}H-labeled MMTV cDNA with C3H liver DNA. C3H liver DNA (15 μg) was hybridized with 1.27 ng of MMTV [^{3}H]-cDNA in 30 μl of hybridization buffer at 69°C. At the indicated times 2-μl samples were diluted into S1 nuclease digestion buffer, and the percent cDNA hybridized was determined as described in the text. Approximately 1% of the cDNA was resistant to digestion with S1 nuclease, and this value was subtracted from each point.*

extracted with chloroform, the DNA was precipitated by the addition of 2 volumes of ethanol, the pellet was resuspended in the appropriate buffer, and the DNA was redigested with a twofold excess of enzyme. After the digestion reaction, each sample of DNA was precipitated by the addition of 2 volumes of ethanol. The precipitates were collected and suspended in 20 μl of 10% Ficoll–0.1% SDS–1 mM EDTA–0.02% bromophenol blue.

Electrophoresis, transfer, and hybridization of enzyme-digested DNA. Restriction enzyme-digested DNA samples were electrophoresed on 0.8 or 1.0% agarose slab gels (slot surface, 5 by 10 mm) made in 36 mM Tris–30 mM sodium phosphate (pH 7.2)–1 mM EDTA at 18 V for 18 h at room temperature. The digested DNA and the molecular weight markers were visualized by ethidium bromide staining and photography under UV light. The DNA fragments in the gel were transferred to a nitrocellulose filter sheet by the blotting technique of Southern (37) as modified by Ketner and Kelly (18). Before hybridization, the membrane filter (Millipore Corp., Bedford, Mass.) was washed twice in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus Denhardt solution (9) at 65°C, with the second wash containing denatured *M. luteus* DNA (20 μg/ml) and 3.3% (vol/vol) phosphate buffer (0.5 M sodium phosphate, 1.5% sodium pyrophosphate [pH 7.3]). The filter was then hybridized by saturating it with a solution of ^{32}P-labeled MMTV cDNA (specific activity, 50×10^6 cpm/μg), SSC, EDTA, SDS, and denatured *M. luteus* DNA so that the final concentrations were, respectively, 25 ng/ml, 4×, 10 mM, 0.1%, and 20 μg/ml. The hybridization mixture and the filter were incubated in a sealable plastic bag between two glass plates for 20 h at 65°C. The filter was then washed at 65°C with 0.1% SDS and 3.3% (vol/vol) phosphate buffer containing 2× SSC, followed by second and third washes in which the 2× SSC was replaced by 1× SSC and then by 0.1× SSC for a total period of 3 h. The dry filter was then

exposed at −70°C to Kodak XR-5 film with an intensifying screen.

Preparation of and hybridization with nick-translated mouse β-globin plasmid DNA. Plasmid pCR1 containing the mouse β-globin structural DNA sequences (34) was provided by B. Mach. The plasmid was nick-translated with [^{32}P]dCTP to a specific activity of 3×10^7 cpm/μg as described previously (46). The filters were prepared and hybridized as described above with the exception that the plasmid DNA was included at a final concentration of 100 ng/ml.

Isolation of unintegrated MMTV viral DNA. HTC-M1 cells from a rat hepatoma cell line infected with MMTV(GR) (31) were obtained from K. Yamamoto. The cells were grown in Dulbecco-modified Eagle medium, 10% fetal calf serum, and 10^{-6} dexamethasone. The cells have been shown to contain unintegrated MMTV viral DNA (33) which was isolated as follows. Confluent cells were removed from petri dishes by trypsinization and washed 3 times with cold phosphate-buffered saline. Approximately 10^9 cells were suspended in 50 ml of 10 mM Tris-hydrochloride (pH 8.0)–5 mM EDTA and disrupted with a Potter homogenizer. The nuclei were collected by centrifugation, and the pellet was reextracted in the same buffer. The supernatant fractions containing the cytoplasmic, linear DNA molecules were pooled, and proteinase K and SDS were added to, respectively, 100 μg/ml and 0.5%. After extraction with phenol-chloroform and chloroform, the nucleic acids were precipitated by the addition of ethanol, collected by centrifugation, and dissolved in 2 ml of water. Denatured *M. luteus* DNA (50 μg) was added, and the solution was brought to 0.1 M NaCl–5 mM EDTA–10 mM Tris-hydrochloride (pH 7.5). RNase, incubated for 10 min at 100°C in 50 mM sodium citrate (pH 5.0) to remove DNase, was added to 100 μg/ml, and the RNA was digested for 2 h at 37°C. SDS was added to 0.5%, and the digest was extracted with phenol-chloroform and chloroform. The aqueous phase was applied to a Sephadex G-50 column (0.5 by 40 cm) in 10 mM Tris-hydrochloride (pH 7.5), the excluded volume was pooled, and the nucleic acids were precipitated by the addition of 2 volumes of ethanol. The DNA was collected by centrifugation and suspended in 250 μl of 1 mM EDTA. The content of MMTV-specific sequences was determined by hybridizing increasing amounts of this solution with an excess of ^{3}H-labeled MMTV cDNA under the conditions described above. The hybridization was begun after incubating each reaction for 10 min at 100°C.

RESULTS

MMTV proviral number in mouse DNA. The DNA of all tested inbred strains of mice contains MMTV proviral sequences (22, 25, 35, 44). Before doing the genomic mapping studies of the MMTV proviruses of different mouse strains, we determined, by hybridization, the number of MMTV proviruses endogenous to BALB/c, C3H, and GR mice. BALB/c mice do not transmit MMTV in the milk and have a low incidence of mammary tumor occurrence. C3H mice have a high incidence of early mammary tumor occurrence and transmit viral particles in the milk. In the GR mouse a single dominant gene, the *Mtv-2* locus, controls the high incidence of mammary tumor occurrence (3). This strain is an exception as the endogenous proviruses of other tested mouse strains seem to play no role in early mammary tumor occurrence. We also determined, by hybridization, the number of MMTV proviruses in C3H and GR mammary tumors.

Other investigators have determined the number of MMTV proviruses in organ DNA and mammary tumor DNA isolated from various strains of mice by measuring the kinetics of hybridization of radioactive cDNA to an excess of cellular DNA (22, 25, 44). We employed a cDNA excess hybridization technique developed by Bishop and Freeman (4) to determine the amount of MMTV proviral genes in the DNA isolated from livers and mammary tumors of different mice. With this method both the complexity of the cDNA probe and the complexity of the hybridizable DNA can be determined.

The number of proviral genes in the DNA of each mouse strain was calculated by determining the amount of cDNA hybridized at increasing ratios of cDNA to DNA. This value was represented as picograms of cDNA hybrid per 100 μg of DNA. The plateau value reached when the cDNA-to-provirus ratio was about 10 to 20:1 reflects the amount of provirus in the total cellular DNA. This method has previously been described for determining the number of globin genes in various cellular DNAs (4, 28). The results of such hybridizations are shown in Fig. 2. Knowing the hybridization plateau value and the molecular weight of one genome equivalent of MMTV proviral DNA and correcting for the fact that approximately 80% of the viral RNA complexity is present in the cDNA (as determined above), we were able to calculate the number of MMTV proviral copies. These numbers and the calculations are presented in Table 1. C3H and BALB/c liver DNA and C3H mammary gland DNA contained approximately 7 copies of MMTV proviral DNA per diploid genome, whereas C3H mammary tumor DNA contained 8.5 to 9 copies. GR liver DNA and mammary tumor DNA contained approximately 10 copies of MMTV proviral DNA per diploid genome. These data agree with previously published work which shows that C3H mammary tumors contain increased amounts of MMTV proviral DNA (21, 22, 25).

Restriction enzyme analysis of the location of the endogenous MMTV proviruses. The digestion of the total genomic DNA with restriction enzymes, the separation of the fragments by agarose gel electrophoresis, and the

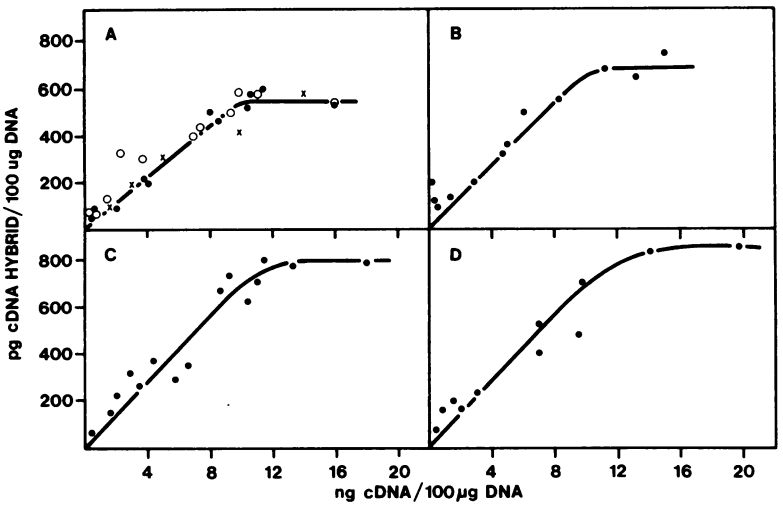

FIG. 2. *Saturation hybridization of MMTV [^{3}H]cDNA with mouse DNA. DNA (1.6 to 10 μg) was lyophilized with amounts of MMTV [^{3}H]cDNA ranging from 30 to 300 pg to achieve the indicated ratios. The nucleic acids were suspended in hybridization buffer at a DNA concentration of 0.5 mg/ml. After incubation at 69°C for 48 h, the entire mixture was diluted into S1 nuclease digestion buffer, and the percent cDNA hybridized was determined. The counts per minute hybridized were converted to picograms of hybrid per 100 μg of input DNA. The DNA was isolated from (A) C3H liver (●), C3H mammary gland (○), BALB/c liver (×); (B) C3H mammary tumor; (C) GR liver; (D) GR mammary tumor.*

TABLE 1. *Amount of MMTV-specific DNA in mouse tissues as determined by hybridization with MMTV cDNA*

Mouse strain	Tissue	pg of cDNA hybrid per 100 μg of DNA	MMTV proviral gene no. per diploid genome
C3H	Liver	550	6.9[a], 6.3[b]
	Mammary gland	550	6.9, 6.0[b]
	Mammary tumor	680	8.6
BALB/c	Liver	550	6.9
GR	Liver	790	10.0
	Mammary tumor	860	10.7

[a] Determined as follows: the molecular weight of one proviral equivalent is 6.0×10^6 and one mouse cell has DNA with a molecular weight of 3×10^{12}; therefore, one MMTV genome is 2×10^{-4}% of the total genome. 100 μg of DNA containing one MMTV genome has 200 pg of gene but only 100 pg of + strand. A plateau value of 550 pg of cDNA hybrid per 100 μg of DNA is equivalent to 687 pg of gene per 100 μg of DNA as the cDNA probe represents approximately 80% of the viral genome. 687 pg of gene is equivalent to approximately 6.9 copies of MMTV per diploid cell.

[b] Determined from the kinetics of MMTV cDNA hybridization as described in the text and in Fig. 2.

transfer of the DNA to a nitrocellulose filter (the Southern DNA filter transfer technique [37]) followed by hybridization with radioactive cDNA allowed an analysis of the restriction enzyme sites in proviral DNA and the flanking host DNA sequences. We used these techniques to analyze the location of the MMTV provirus in DNA isolated from the livers and mammary tumors of different inbred strains of mice. The restriction enzyme cutting sites of the MMTV viral DNA have been determined. Unintegrated viral DNA has been isolated from rat hepatoma cells infected with MMTV and digested with various restriction enzymes (36; N. E. Hynes, B. Groner, H. Diggelmann, R. Van Nie, and R. Michalides, Cold Spring Harbor Symp. Quant. Biol., in press). *Eco*RI and *Hin*dIII cut the provirus once close to the center of the genome, whereas *Bam*HI cuts it twice and yields an internal fragment of approximately 1.0 kilobases (kb) along with two end fragments. Upon digestion of the cellular DNA with *Eco*RI or with *Hin*dIII, each provirus yields two fragments whose molecular weights correspond to the distance between the restriction enzyme site on the provirus and the nearest site in the cellular DNA. This is also the case for digestion with *Bam*HI. An additional fragment of approximately 1.0 kb containing only proviral DNA may also arise from a *Bam*HI digestion of the endogenous proviruses.

For analyzing the genomic locations of the endogenous MMTV proviruses, DNA isolated from the liver, an organ which contains only the endogenous proviruses, was digested with *Eco*RI, *Hin*dIII, and *Bam*HI. The DNA fragments resulting from the digestion of the liver

DNA of various strains of mice were fractionated by electrophoresis and transferred to nitrocellulose filters, and provirus-containing fragments were identified. The results of this analysis are shown in Fig. 3.

For facilitating the comparison, the sizes of the MMTV-containing *Eco*RI fragments of the different strains are listed in Table 2. Such a comparison reveals that among the seven different strains, the BALB/c, CBA, and C57BL strains had almost identical *Eco*RI fragments, the C3H strain shared fragments with the BALB/c mouse and the DBA mouse, and the DNA from the A mouse also had fragments common to the BALB/c mouse DNA and to the DNA of other examined mice. The similarity in the *Eco*RI patterns of the CBA, BALB/c, and C57BL DNA led us to digest these DNAs with another enzyme. Lanes 2 and 4 of Fig. 3 reveal that the MMTV-containing *Hin*dIII fragments of CBA and BALB/c DNA were identical, whereas the C57BL DNA yielded the same pattern with two additional bands. The pattern of MMTV-containing *Eco*RI fragments arising from the DNA of the GR mouse appeared to be different from that of the other strains tested. Seven of nine fragments identified in Fig. 3 and listed in Table 2 did not correspond to those found in either the DBA DNA or BALB/c DNA. The limits of resolution of the method do not allow us at this point to rule out the presence of additional *Eco*RI fragments, especially those of the size of about 7 kb. This leaves the possibility that additional *Eco*RI fragments of similar mo-

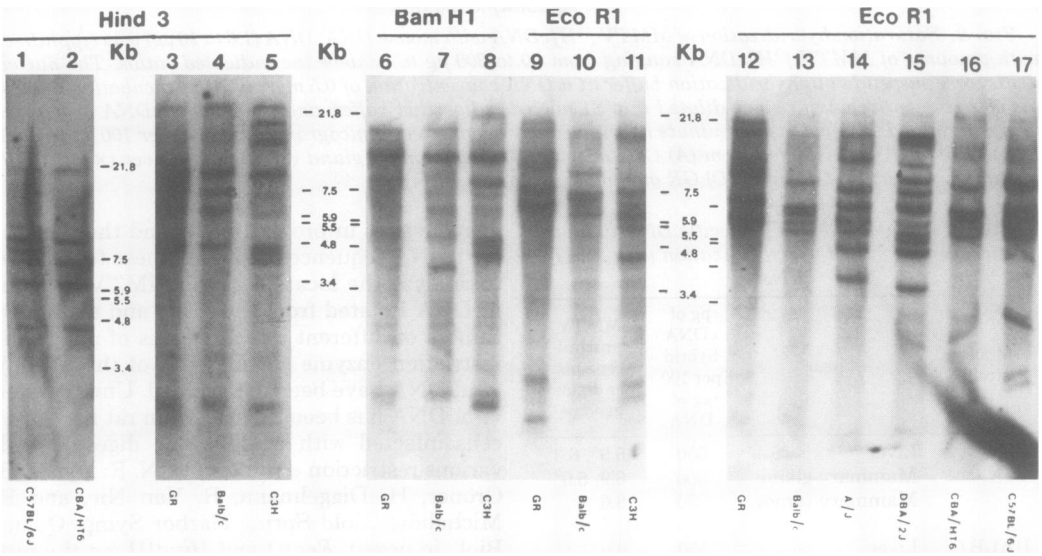

FIG. 3. *Analysis of the endogenous MMTV provirus in the DNA of different strains of mice. DNA was isolated from the livers of the indicated strains of mice and digested with different restriction endonucleases. The samples were electrophoresed in an 0.8% agarose gel, transferred to a nitrocellulose filter, and annealed with ^{32}P-labeled MMTV cDNA as described in the text. The markers indicate the position in this and in subsequent figures of the fragments of EcoRI-cut lambda phage DNA detected by staining with ethidium bromide.*

TABLE 2. *MMTV-containing DNA fragments[a] resulting from the restriction enzyme digestion of the liver DNA of different mouse strains*

Strain	Enzyme	Kb of fragments
DBA/2J	*Eco*RI	14.5 13.5 11.5 9.7 8.7 7.7 7.4 6.7 6.2 5.9 5.4 4.2
BALB/c	*Eco*RI	14.5 9.6 7.5 7.15 6.7 6.1
C3H/HeJ	*Eco*RI	14.5 7.5 6.7 6.2 5.9 5.4 4.2
CBA/H-T6	*Eco*RI	9.6 7.5 7.15 6.7 6.1
C57BL/6J	*Eco*RI	10.3 9.6 7.7 7.5 7.15 6.7 6.1
A/J	*Eco*RI	14.5 8.85 7.7 6.7 6.1 5.4 4.3
GR	*Eco*RI	22.8 16.0 11.5 11.0 10.0 8.1 7.8 6.9 6.7
C3H/HeJ	*Hin*dIII	22.0 16.5 12.0 8.2 7.0 6.0 4.3 4.15 3.7
C3H/HeJ	*Bam*HI	21.8 10.5 7.7 7.0 6.1 4.8 4.6 4.2

[a] Only the fragments larger than 3 kb are listed.

lecular weight were present in the DNA of GR and DBA mice or of BALB/c mice.

The results can best be interpreted in light of the genetic origins of the different mice. As previously noted, the C3H and CBA strains can be traced to the cross of a BALB/c mouse with a DBA mouse. Each MMTV-containing *Eco*RI band was correlated with a band present in one of the parents. The DNA from A mice, which are related to the BALB/c strain, also had MMTV bands in common with the BALB/c DNA as well as with the DNA of DBA and C3H mice. The DNA from C57BL mice, which can also be traced back to related stocks, had, with the exception of two additional bands, the same pattern of MMTV-containing fragments as the BALB/c mouse. The results suggest that in mice of related genetic origins the patterns of MMTV-containing *Eco*RI fragments are similar, suggesting that the proviruses are located in similar genomic environments. The pattern of fragments arising from the GR mouse, which is genetically unrelated to the others, appeared different. In addition, the patterns of MMTV-containing *Eco*RI fragments arising from two other European bred strains (RIII and O20) have also been identified and differ from each other and from the GR strain (Hynes et al., in press). This suggests that the MMTV proviruses in these strains are located in different genomic environments.

The interpretation of the preceding results depends upon two conditions. One condition is that the integrated viral DNA is colinear with the unintegrated species. This can be tested at least for newly integrated milk virus-specific proviral DNA. The results suggesting that this is true are presented below. The second condition is that whereas the restriction enzyme digestion pattern of the viral DNA synthesized from milk-borne MMTV is known (36), the restriction enzyme digestion pattern for the MMTV proviruses endogenous to different mouse strains is not known. At the present time this problem is difficult to solve as there is no readily available source of viral RNA or DNA from strains, such as BALB/c or C57BL/6J, with a low incidence of mammary tumor occurrence. Some evidence for the relatedness of the endogenous proviruses can be obtained from the results of the *Bam*HI digestion patterns. Figure 3, lanes 6 through 8, shows that at least some of the proviruses endogenous to GR, BALB/c, and C3H/HeJ mice contained the internal *Bam*HI fragment of approximately 1.0 kb which also arose from a digestion of the isolated viral DNA. However, another smaller fragment also arose from the *Bam*HI digestion of GR as well as C3H DNA. In addition, Cohen et al. (7) have published a pattern of MMTV-containing fragments resulting from the digestion of the BALB/c DNA with *Bam*HI in which the 1.0-kb fragment is absent. These results suggest that these may be endogenous proviruses with different *Bam*HI sites. These results will be further discussed below.

Analysis of the location of the β-globin gene in different strains of mice. In the preceding section we analyzed the genomic locations of the MMTV proviruses in a number of different mice. We extended this comparison to another gene, that coding for β-globin. Liver DNA isolated from the different mice was digested with *Eco*RI, an enzyme which does not cut within the coding or intervening sequences of the β-globin gene (34, 42), and, following gel electrophoresis, the β-globin-specific fragments were identified. The results are shown in Fig. 4. C3H/HeJ, BALB/c, A/J, CBA/H-T6, and DBA/2J mice exhibit the hemoglobin diffuse gene product (38) and contain two linked β-globin genes (29). Lane 3 shows the two β-globin-containing restriction fragments of the BALB/c DNA which migrate at approximately 14 and 7 kb, as previously identified (42). This pattern appears identical to that obtained with DNA isolated from the livers of CBA/H-T6, A/J,

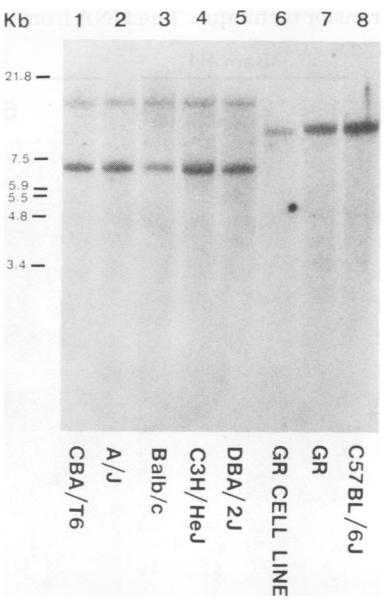

FIG. 4. *Analysis of the β-globin gene in the DNA of different strains of mice. DNA was isolated from the livers of the indicated strains of mice, digested with EcoRI, and analyzed as described in the legend to Fig. 3, except that the filters were annealed with* ^{32}*P-labeled nick-translated pCR1 plasmid DNA containing β-globin-specific sequences. The sizes of the fragments in lanes 1 through 5 are 14.0 and 7.0 kb; in lanes 6 through 8 the fragment is 10 kb.*

C3H/HeJ and DBA/2J mice (Fig. 4; lanes 1, 2, 4, and 5). The C57BL/6J and GR mouse strains contain only one β-globin chain (38). Lanes 7 and 8 show that the β-globin gene from both strains was contained in an *Eco*RI DNA fragment of approximately 10 kb. Our results suggest that in strains of different genetic origins such as GR and C57BL/6J the macroenvironment of the β-globin gene is similar.

Analysis of MMTV proviral integration sites in mammary tumor DNA. C3H mice transmit MMTV in the milk and have a high incidence of early mammary tumor occurrence. BALB/c mice transmit no milk-borne MMTV and have a low incidence of tumor formation. However, when BALB/c mice are foster nursed on C3H mothers (BALB/cfC3H), their mammary tumor occurrence becomes high. The hybridization of mammary tumor DNA has demonstrated that there is an increase in the amount of MMTV proviruses. The extra copies are assumed to derive from the exogenous virus acquired via the milk. It may be possible that mammary tumor formation is related to the site of integration of the exogenous provirus in the cellular DNA. One can partially characterize the integration sites by analyzing the DNA isolated from different mammary tumors with the DNA filter transfer technique. The DNA from individual C3H mammary tumors was isolated and digested with different restriction enzymes. The fragments containing MMTV-specific sequences were detected, and the results are shown in Fig. 5. It can be seen that the DNA of individual C3H mammary tumors contained not only the restriction fragments present in the liver DNA, but for each tumor it was possible to identify additional MMTV fragments. These new bands are indicated by the lines on the left side of the individual lanes. It was not possible in all cases to identify new MMTV-specific bands from the digestion with one enzyme. This might have been due to the coelectrophoresis of tumor-specific bands with those deriving from the endogenous proviral sequences. In the *Eco*RI digest of tumor 3 DNA (lane 6) no new bands were seen. However, a new band was evident when the same DNA was digested with *Bam*HI (lane 2). If one copy of the provirus was additionally integrated in the mammary tumor DNA, two new MMTV-specific bands were expected. We observed this for the *Eco*RI digestion of tumor 1 DNA (lane 8) and for the *Hin*dIII digestion of tumor 4 DNA (lane 11). Other tumor-specific bands in tumors 2, 3, and 5 might have been masked by the endogenous proviruses.

The DNA isolated from four separate tumors removed from one BALB/cfC3H mouse was di-

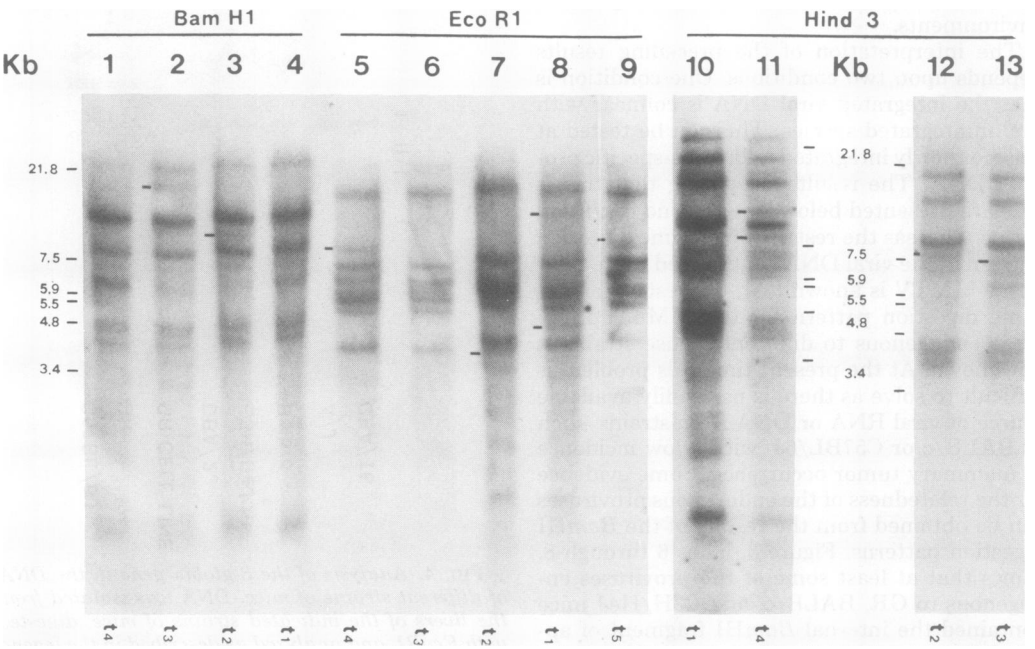

FIG. 5. *Analysis of MMTV proviral sequences in the DNA of individual C3H mammary tumors. DNA was isolated from five individual C3H mammary tumors (t1 through t5), digested with different restriction endonucleases, and analyzed as described in the legend to Fig. 3. The markers to the left of lanes 2, 3, 5, 7, 8, 9, 11, 12, and 13 indicate the position of tumor-specific MMTV-containing fragments identified in the DNA isolated from the different tumors.*

gested with *Eco*RI, and the fragments containing MMTV-specific sequences were detected. These results are shown in Fig. 6. In the scheme to the right, the bands arising from the endogenous proviruses are indicated by solid lines, whereas the tumor-specific fragments are indicated by dotted lines. As was the case for the C3H tumors, each BALB/cfC3H mammary tumor contained additional MMTV proviral copies.

It should be noted that the BALB/cfC3H DNA was obtained from the BALB/c colony kept in the Netherlands Cancer Institute, whereas the BALB/c DNA used for lanes 10 and 13 of Fig. 3 was isolated from mice of the colony of the Swiss Institute for Experimental Cancer Research. The MMTV DNA fragment of 6.7 kb arising from the Swiss BALB/c colony was missing from the DNA of the Amsterdam BALB/c colony. Cohen et al. (7) have also obtained a pattern of MMTV-containing *Eco*RI DNA fragments generated from BALB/c DNA which is missing the 6.7-kb band and resembles the patterns shown in Fig. 6. Possible explanations for these results could be that the proviral loci in the original BALB/c stock were heterozygous or that the Amsterdam colony of mice lost proviral sequences or that the BALB/c colony in Switzerland gained an MMTV provirus. A similar observation has also been reported by Cohen and Varmus (8), who compared the endogenous

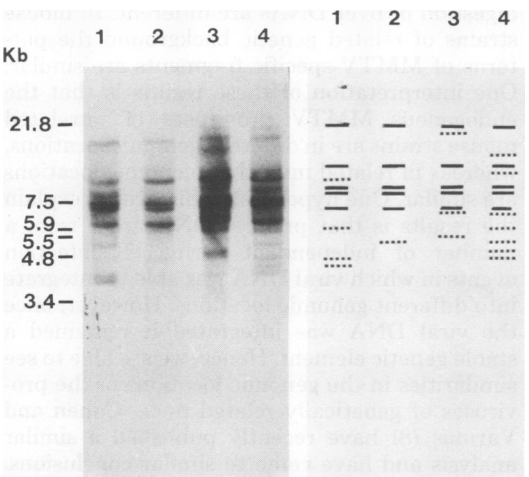

FIG. 6. *Analysis of MMTV proviral sequences in the DNA of individual BALB/cfC3H mammary tumors. DNA isolated from four individual mammary tumors removed from one mouse was digested with EcoRI and analyzed as described in the legend to Fig. 3. In the schematic diagram on the right, the bands arising from endogenous proviruses are indicated by solid lines, whereas the additional tumor-specific fragments are indicated by dotted lines.*

MMTV proviruses in different colonies of C3H/St mice.

The results shown in Fig. 5 and 6 can be summarized as follows. First, although each tumor contained at least one additional copy of MMTV proviral DNA, the extra bands in the different tumor DNAs did not coelectrophorese. This suggests that exogenous proviral DNA can integrate into different sites in mammary gland cell DNA. Second, in most cases the additional bands in the tumor DNAs were clearly visible, although they were not of the same intensity as those specific for the endogenous proviruses. Therefore, it can be concluded that a high percentage of the cells in each tumor contained the additional MMTV proviruses integrated into the same site in the genomic DNA. The results also suggest that each tumor originated from a small number of cells or from one single MMTV-infected cell.

A comparison of the genomic locations of the newly acquired proviral copies in mammary tumor DNA depends upon the assumption that the integrated proviral DNA is colinear with the unintegrated viral DNA. We tested this by digesting the DNA isolated from a C3H mammary tumor containing additional MMTV proviral sequences with *Pst*I, an enzyme which cleaves the proviral genome in five places to yield six fragments of DNA which have been orientated with respect to the viral RNA genome (36). One end fragment could not be detected because one enzyme recognition site was too close to the end of the proviral DNA. If the newly integrated MMTV is colinear with the unintegrated viral DNA, then the digestion of mammary tumor DNA with *Pst*I should yield bands which correspond to the internal *Pst*I cutting sites, whereas the fragment which is joined to the cellular DNA should have an altered molecular weight.

The DNA from a C3H mammary tumor and a C3H liver was digested with *Pst*I, and the fragments of DNA-containing MMTV sequences were identified. These were compared to fragments arising from a *Pst*I digestion of unintegrated viral DNA isolated from MMTV-infected cells. The results are shown in Fig. 7. C3H mammary tumor DNA (lane 2) yielded four fragments of 4.0, 1.7, 1.2, and 0.9 kb which arose from internal *Pst*I cutting sites on the viral DNA, whereas the 1.1-kb fragment arising from the 3′ end of the unintegrated MMTV viral DNA was not evident (compare lanes 1 and 2). This suggests that the newly acquired mammary tumor-specific provirus is attached to the cellular DNA somewhere within this end fragment. Other more detailed restriction enzyme mapping experiments of newly integrated mammary tu-

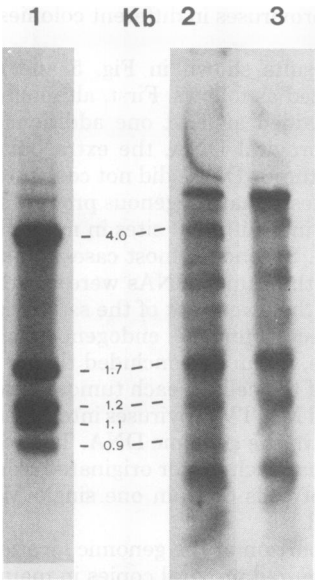

FIG. 7. *Analysis of MMTV(GR) viral DNA and C3H DNA digested with PstI. Linear viral DNA (lane 1), C3H mammary tumor DNA (lane 2), and C3H liver DNA (lane 3) were digested with PstI and analyzed as described in the legend to Fig. 3, except that a 1.0% agarose gel was used. The markers in the center indicate the sizes of the fragments resulting from the PstI digestion of MMTV(GR) viral DNA.*

mor-specific proviruses (7) show that the provirus is attached to the cellular DNA within a region a few hundred base pairs from the ends of the unintegrated viral DNA. These results suggest that newly acquired proviruses are colinearly integrated.

Lane 2 reveals that C3H mammary tumor DNA contained two additional fragments at 5.2 and 0.7 kb which strongly hybridized with MMTV. These fragments arose from the endogenous proviruses as they were also present in C3H liver DNA (lane 3). The intensity of these fragments suggests that they may have arisen from internal *Pst*I sites on the C3H endogenous proviruses. This leads to the conclusion that C3H endogenous proviruses have different *Pst*I cutting sites than does viral DNA arising from a milk-borne viral infection. In a recent publication (8), Cohen and Varmus have come to a similar conclusion from a study of the endogenous proviruses of the C3H strain and related strains.

Digestion with *Pst*I yielded fragments comprised of viral and cellular DNA and therefore should be useful in characterizing the genomic location of proviral DNA. However, the bands representing these fragments will be weaker than those representing the internal proviral fragments as they arise from only one provirus. In Fig. 7, lanes 2 and 3, it is possible to see faint bands which might represent the end fragments, but digestion with *Eco*RI, *Hin*dIII, or *Bam*HI yielded patterns which are more useful for characterizing the genomic location of proviral DNA.

DISCUSSION

In this paper we presented the results of studies of the MMTV proviral number and its organization within the cellular DNA isolated from organs and mammary tumors of different strains of mice.

All strains of mice contain MMTV-specific sequences in their genomic DNA (22, 25, 35, 44). By a cDNA excess hybridization technique (4), we determined that C3H liver DNA, C3H mammary gland DNA, and BALB/c liver DNA contain approximately 7 copies each of MMTV per diploid genome, whereas GR liver DNA contains approximately 10. These numbers are in close agreement with published values (22, 25). As previously observed (22, 25), we found that C3H mammary tumor DNA contains additional copies of the MMTV provirus.

We used the DNA filter transfer technique (37) to compare the locations of the MMTV proviruses endogenous to different mouse strains. The results reveal that in mouse strains of unrelated origins, such as BALB/c, DBA/H-T6, and GR, the patterns of MMTV-containing restriction enzyme fragments arising from the digestion of liver DNAs are different. In mouse strains of related genetic background the patterns of MMTV-specific fragments are similar. One interpretation of these results is that the endogenous MMTV proviruses of unrelated mouse strains are in different genomic locations, whereas in related mice their genomic locations are similar. One hypothesis which could explain the results is that proviral DNA arose from a number of independent germ cell infection events in which viral DNA was able to integrate into different genomic locations. However, once the viral DNA was integrated it remained a stable genetic element. Hence, we are able to see similarities in the genomic locations of the proviruses of genetically related mice. Cohen and Varmus (8) have recently published a similar analysis and have come to similar conclusions. They also saw that different inbred mouse strains resulting from the Bagg albino × DBA mating carried MMTV proviruses in similar genomic locations. In addition, they determined that the genomic locations of MMTV proviruses in feral mice are different, results which can be compared to our findings on inbred mouse strains which are genetically unrelated. In an-

other publication (24), the authors determined that the MMTV proviruses endogenous to the A/HeJ mouse are located on at least three separate chromosomal pairs. This result also favors the idea that separate viral germ line infection events led to proviral DNA. An alternative interpretation of the differences seen in the patterns of MMTV-containing fragments in, for example, the GR and C57BL DNA is that the sequences surrounding the proviruses in the different mice have diverged. To analyze this possibility we used the Southern DNA filter transfer technique (37) to analyze the β-globin gene in the different mouse strains. C57BL/6J and GR mice have the *Hb-S* locus and code for only one β-globin protein. Both strains contain the β-globin gene within a 10-kb *Eco*RI fragment of DNA. Recently it has been reported that the C57BL/10 mouse has its β-globin gene within a 10-kb *Eco*RI fragment (45). Upon further analysis by RPC5 chromatography, this fragment was found to be composed of two different 10-kb pieces which could be differentiated by digestion with restriction enzymes. Whether the 10-kb band arising from the digestion of GR DNA with *Eco*RI also contains two separate fragments is not known. The β-globin gene in these two mouse strains of different genetic origins appears to be in a similar macroenvironment. The same analysis has been carried out for the α-amylase gene. A recombinant plasmid containing α-amylase mRNA-specific sequences recognizes at least four genomic DNA *Eco*RI fragments. The DNA from the seven different mouse strains which we analyzed gave rise to the same pattern of α-amylase-containing *Eco*RI fragments (B. Groner, N. E. Hynes, and U. Schibler, unpublished observations). These results suggest that these structural genes in the different mouse strains are similarly organized, whereas MMTV proviral DNA is located in different genomic sites. This could reflect separate germ line integration events into a relatively similar background of structural genes in the different strains. The validity of this idea awaits further comparative studies with probes specific for other structural genes.

One difficulty in comparing the endogenous proviruses of different strains is that the restriction enzyme sites in these proviruses have not been mapped and may vary from the pattern determined for isolated viral DNA. For most of the mouse strains it appears that the endogenous proviruses never yield viral particles. Thus, purified genomic DNA may be the best substrate for such an analysis. Previous publications suggest that there is a high degree of cross hybridization between the endogenous MMTV proviruses of different strains (20, 30). However, RNase T_1 analyses (11) and hybridization analyses (20) have shown that differences do exist. As pointed out in the results, we have evidence that at least some of the restriction enzyme sites of endogenous proviruses correspond to sites in isolated viral DNA. The viral internal *Bam*HI fragment of 1.0 kb also appears in a *Bam*HI digestion of liver DNA from C3H, BALB/c, and GR mice. However, the digestion of C3H liver DNA with *Pst*I reveals that only two of the four internal *Pst*I fragments obtained from a digest of viral DNA are identical. This result suggests that some of the restriction enzyme sites of the endogenous proviruses vary from those determined for isolated viral DNA.

An additional observation which we have made in the course of these comparative studies is that DNA isolated from different colonies of BALB/c mice yields different numbers of MMTV-containing *Eco*RI fragments. There is one additional fragment present in the DNA of the Swiss colony. We attempted to analyze this difference further by digesting the DNA with *Pst*I. This enzyme has been used in this work (Fig. 7) and in others (7, 8) to distinguish endogenous MMTV proviruses from those corresponding to the exogenous milk-borne virus. Upon digestion with *Pst*I, DNA isolated from mice of both colonies gave rise to the same pattern (data not shown), which has previously been published (8). If the Swiss BALB/c colony has acquired a new MMTV provirus, it cannot be distinguished from the others by *Pst*I and therefore does not correspond to the BALB/cfC3H tumor-specific viral variant.

With the Southern blotting technique we analyzed the DNA of mammary tumors taken from C3H mice or from BALB/c mice foster nursed on C3H mothers. The animals received exogenous, milk-borne MMTV, and we determined that each tumor contained at least one additional copy of MMTV proviral DNA. It is interesting to note that tumors from BALB/cfC3H animals contain higher amounts of newly acquired proviruses than those isolated from C3H animals. We analyzed a number of C3H mammary tumors and have detected a maximum of one new proviral copy, whereas one of the BALB/cfC3H tumor DNAs shown in Fig. 6 contained at least four new proviral copies. Other investigators (7) who looked at BALB/cfC3H tumors also found high amounts of newly integrated MMTV proviruses. Perhaps C3H mice are more resistant to the C3H virus than are BALB/c mice. Different types of experiments have led others to suggest this (13).

The comparison of the pattern of MMTV-

containing restriction enzyme fragments obtained with different tumor DNAs allows the following conclusions to be made. First, the pattern of the extra MMTV-containing DNA bands is different in each tumor, suggesting that the exogenous viral DNA can integrate at different sites in the host genome. Second, the fact that extra bands of MMTV-containing DNA are evident suggests that a high percentage of the tumor cells contained the exogenous viral DNA integrated in the same site and that the tumor may have arisen from one cell or from a small number of infected mammary gland cells.

In two recent publications (7, 32), other investigators have reported that MMTV proviral DNA can integrate into a number of genomic sites. In one case (7), the integration sites of newly acquired, tumor-specific proviruses were compared. In the other report (32), the genomic locations of the proviral DNA in an MMTV-infected rat cell line were determined. The exogenous infection of cells with other retroviruses, including murine leukemia virus (39), spleen necrosis virus (17), and avian sarcoma virus (14), has yielded results which suggest that the proviral DNA specific for these viruses can integrate into a number of genomic sites.

The different patterns of MMTV-containing sequences in various mouse strains and the different proviral integration sites found in individual mammary tumor DNAs suggest that there are multiple sites on the cellular genome available for proviral DNA integration. Conclusive evidence as to whether the different MMTV integration sites share common features can only be drawn from a sequence analysis of the genomic DNA flanking the provirus. Such an analysis may help to clarify the mode of proviral DNA integration and may reveal sequences related to the control of MMTV proviral expression.

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