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Acquisition of Proviral DNA of Mouse Mammary Tumor Virus in Thymic Leukemia Cells from GR Mice

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Male mice of strain GR develop T-cell leukemia at a low frequency late in life. These leukemia cells contain large amounts of mouse mammary tumor virus (MMTV) RNA and MMTV proteins in a precursor form (Nusse et al., J. Virol. 32:251–258, 1979). We used restriction enzyme analysis and molecular hybridization to identify MMTV proviruses in the DNA of these leukemia cells. GR leukemia cells contained additional integrated MMTV proviruses at various sites in the genome. This amplification of MMTV proviruses in GR leukemia cells is not restricted to one particular endogenous MMTV provirus of strain GR. The number and location of the extra MMTV proviruses present in transplants of GR leukemia cells did not change upon serial transplantation of the leukemia cells. Acquisition of MMTV proviruses was also found in a similar leukemia, L1210 of the DBA/2 mouse strain, but not in three other leukemias, SL2 of DBA/2, BW5147 of AKR, and a spontaneous thymoma of BALB/c. The two main classes of MMTV RNA, 35S and 24S, were present in the cytoplasmic RNA of GR leukemia cells, indicating that the aberrant processing of MMTV precursor proteins is not due to anomolously sized RNAs. We could not detect extra RNAs in GR leukemia cells which would represent read-through transcripts of cellular genes adjacent to the extra MMTV proviruses, initiated by a promoter signal in the right MMTV long terminal repeat sequence. These data suggest that acquisition of MMTV proviruses may coincide with the onset of leukemogenesis in GR male mice.

Mouse mammary tumor virus (MMTV) induces mammary tumors in inbred strains of mice. All inbred strains of mice carry proviruses of genetically transmitted, endogenous MMTV variants. In addition, some strains of mice, such as RIII or C3H, contain a milk-transmitted, exogenous MMTV variant. The exogenous MMTVs are usually highly oncogenic. The involvement of endogenous MMTV proviruses in the development of mammary tumors is unclear, except for the endogenous MMTV proviruses associated with mammary tumor induction loci Mtv-1 and Mtv-2. These genes control the development of mammary tumors in the C3Hf and GR mouse strains, respectively (30, 31), and contain one endogenous MMTV provirus (15). The transforming activity of MMTV is restricted to the murine mammary gland, but expression of MMTV in other organs has been observed (for review, see reference 1). High levels of MMTV expression were detected in two other types of tumors, Leydig cell tumors (32) and lymphoid leukemias of mouse strains GR (18) and DBA/2 (28). The expression of MMTV in Leydig cell tumors and lymphoid leukemias appeared to be impaired at various stages of processing of the viral proteins (18, 24, 28). Whether the expression of MMTV in these types of tumors is in any way linked to tumor development remains unknown.

In the present report, we examined the arrangement of MMTV proviruses in lymphoid leukemic cells of strain GR with restriction enzyme analysis and molecular hybridization techniques. GR male mice develop thymic lymphomas around 400 days of age at an incidence of about 20% (11). These leukemia cells are easily transplantable in vivo, and many transplantable lines of leukemia cells have been established. We used these leukemia cells to investigate whether the high level of MMTV expression is correlated with the acquisition of additional MMTV proviruses. Our findings indicate that leukemia cells of strain GR have acquired extra MMTV proviruses which are integrated at various sites of the cellular genome. Serial passaging of these leukemia cells has no apparent effect on the arrangment and number of

MMTV proviruses. These extra MMTV proviruses are present in primary leukemia cells, suggesting that they may be associated with the onset of leukemogenesis in GR mice.

MATERIALS AND METHODS

Leukemia cells, mice, and virus. Spontaneous leukemia cells arise in 1-year-old male GR mice as thymomas which spread through the animal. The leukemia cells are easily transplantable in vivo, and various lines have been obtained from different spontaneous leukemias. The in vivo transplantable lines of GR leukemia cells were maintained as ascites lines, as described previously (10). Two primary thymomas and two transplanted leukemia cell lines at passages 9 and 92 have been used in this study. GR and AKR/Ru mice were obtained from the breeding colony of the Netherlands Cancer Institute, Amsterdam. The transplantable thymic lymphoma SL2 and DBA/2 mice were a kind gift of H. Dullens of the Department of Pathology, University of Utrecht, The Netherlands. The cell line BW5147 was kindly provided by G. Klein, Karolinska Institute, Stockholm, Sweden, and the L1210 cells, a transplantable leukemia cell line in DBA/2 mice, was obtained from I. Steuden, Ludwik Hirzfeld Institute of Immunology and Experimental Therapy, Wroclaw, Poland. MMTV (C3H) was obtained from the Mm5mt/c1 mammary carcinoma cell line (4) and was provided by the Frederick Cancer Center, Bethesda, Md.

Purification of MMTV viral RNA, preparation of cDNA, and extraction of poly(A)-containing RNA. MMTV RNA was isolated from virions purified from the Mm5mt/c1 cell line, and ^{32}P-labeled MMTV cDNA was synthesized as described previously (15). Polyadenylate [poly(A)]-containing RNAs were isolated from spleens and cell pellets with the citric acid RNA isolation procedure as described previously (23). Poly (A)-containing RNA was isolated by passage of total RNA through columns of oligo-deoxythymidylate cellulose.

Digestion of DNA with restriction endonucleases, gel electrophoresis, DNA transfer, and hybridization. Restriction enzymes *Eco*RI, *Hin*dIII, *Pst*I, and *Bam*HI were purchased from Boehringer Mannheim Corp. Twenty micrograms of DNA was digested to completion with an excess of enzyme in the appropriate buffer for 12 h at 37°C. The extraction of the DNA, gel electrophoresis conditions, DNA transfer, and hybridization with ^{32}P-labeled MMTV cDNA were performed as described previously (15).

Poly(A)-containing RNA was electrophoresed on a 1% agarose slab gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled MMTV cDNA as described previously (7).

RESULTS

MMTV proviruses in primary and transplanted leukemia cells of GR mice. The arrangement of MMTV proviruses in the cellular DNA of transplanted leukemia cells of GR mice was studied with restriction enzyme analysis and molecular hybridization techniques. Restriction enzymes which cut MMTV DNA infrequently were used, such as *Eco*RI, which recognizes one site in unintegrated viral DNA of MMTV (GR) (22) and MMTV (C3H) (3) and in integrated MMTV DNAs of BALB/c (2) and GR (5) mice.

To compare the genomic locations of MMTV proviruses in the DNAs of GR leukemia cells with nontumorous tissues, DNAs isolated from leukemia cells and from livers of healthy mice were digested with *Eco*RI, the DNA fragments were fractionated by electrophoresis and transferred to nitrocellulose filters, and proviral DNA-containing fragments were identified. Figure 1, lane 1, shows the *Eco*RI pattern characteristic for GR DNA with 10 main MMTV DNA bands at 22, 16, 11.5, 11.0, 10.0, 8.1, 7.8, 7.0, 6.9, and 6.7 kilobases (kb). The bands in the 7.8- to 8.1-kb and 7.7- to 7.0-kb regions usually appear to be superimposed. The *Eco*RI patterns of four different leukemia DNAs (lanes 2 through 5) all showed extra MMTV-specific bands when compared with that of liver DNA (lane 1). The extra MMTV DNA bands in the different GR leukemia cells all appeared at various locations.

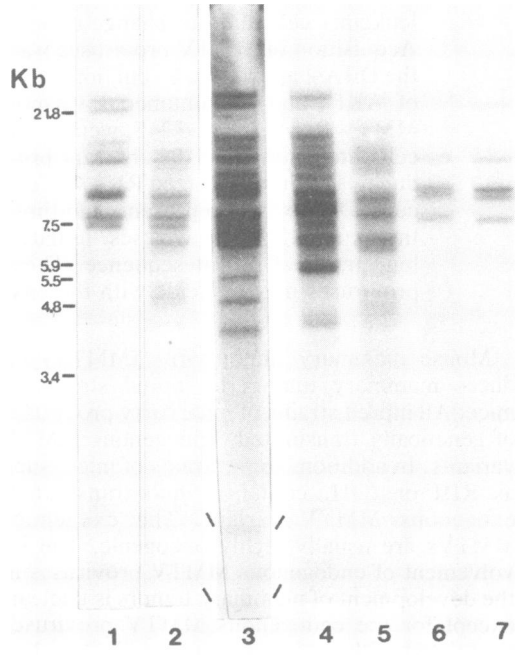

FIG. 1. Presence of extra MMTV proviruses in GR leukemia cells. Twenty micrograms of cellular DNA was digested to completion with *Eco*RI, the DNA fragments were transferred to nitrocellulose sheets, and MMTV DNA-containing cellular DNA fragments were identified by hybridization with MMTV ^{32}P-labeled cDNA as described in the text. *Eco*RI-digested λ DNA was used as a molecular weight marker. Lane 1, GR liver DNA; lanes 2 and 3, DNAs of two different primary GR leukemia cell lines; lanes 4 and 5, DNAs of two different transplanted GR leukemia cell lines at passages 9 and 92, respectively; lane 6, BALB/c liver DNA; lane 7, BALB/c thymoma DNA.

Lanes 6 and 7 in Fig. 1 represent *Eco*RI digestions of BALB/c liver DNA (lane 6) and of DNA from a spontaneous BALB/c thymoma (lane 7) which does not show any extra MMTV DNA-containing fragments. The extra MMTV proviruses in the GR leukemia DNAs are also evident in the digestion patterns with other restriction enzymes, such as *Hin*dIII and *Bam*HI, which recognize one and two cleavage sites, respectively, in the unintegrated MMTV (GR) DNA (22; see Fig. 2). Digestions with *Hin*dIII (Fig. 2, lanes 1 through 5) and with *Bam*HI (lanes 6 through 10) yielded results similar to those obtained with *Eco*RI digestion (Fig. 1, lanes 1 through 5); extra MMTV DNA bands appeared to be present in the DNA of GR leukemia cells compared with control liver DNA. The more intense internal *Bam*HI 1.2-kb MMTV DNA bands in the leukemia DNAs (lanes 7 through 10) confirmed the amplification of MMTV proviruses in the GR leukemia DNAs. The extra MMTV DNA bands shown in Fig. 1 and 2 showed different intensities if the four different leukemias were compared with each other, but also within one particular leukemia DNA. This may be owing to heterogeneity of the tumor cells.

The extra MMTV DNA bands in cellular DNA fragments with bigger sizes than unintegrated MMTV DNA digested by the corresponding restriction enzymes (3, 22) suggest that these extra MMTV DNA fragments are integrated in the cellular DNA and do not result from digestion of unintegrated viral DNA. This was also shown by dividing a GR leukemia cell DNA preparation and treating one half with *Eco*RI and leaving the other half untreated. Both samples were then subjected to gel electrophoresis. Only undigested cellular DNA of >25 kb hybridized with MMTV ^{32}P-labeled cDNA, and no hybridization occurred with DNA fragments in the 9- to 10-kb or lower size ranges (Fig. 3, lane 2). The extra MMTV-specific fragments present in the *Eco*RI digest of this GR leukemia DNA (Fig. 3, lane 3) appear, therefore, to be associated with high-molecular-weight DNA and integrated in the cellular DNA of the leukemia cells.

It has been demonstrated that MMTV-producing tissue culture cell lines contain a large number of MMTV proviruses (6, 22). These extra copies are thought to arise during serial passage of the cells from either new virus infection or reverse transcription of cellular MMTV RNA. To determine whether the number of extra MMTV proviruses in GR leukemia cells would change upon further passage of the cells, DNA was isolated from various passages of a GR leukemia cell line and digested with *Hin*dIII, and the MMTV DNA-containing fragments were identified (Fig. 4). The fifth passage (lane 1)

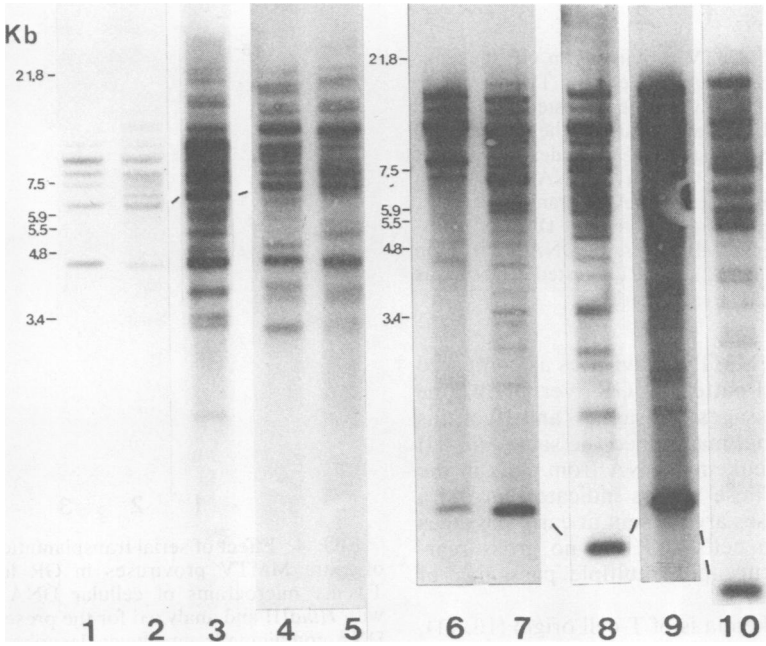

FIG. 2. Extra MMTV proviruses in GR leukemia cells. Twenty micrograms of cellular DNA was digested to completion with either *Hin*dIII (lanes 1 through 5) or *Bam*HI (lanes 6 through 10) and analyzed for the presence of MMTV DNA-containing fragments as described in the legend to Fig. 1. The DNAs are the same as those described in Fig. 1, lanes 1 through 5. *Eco*RI-digested λ DNA was used as a molecular weight marker.

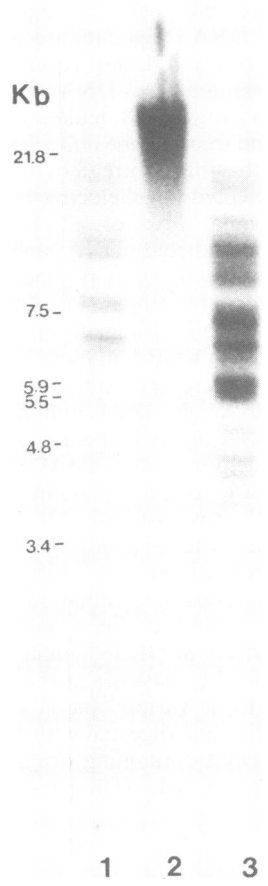

FIG. 3. Extra MMTV proviruses in GR leukemia cells integrated in the cellular genome. Twenty micrograms of cellular DNA was either digested with *Eco*RI or left untreated and analyzed for the presence of MMTV DNA-containing fragments as described in the legend to Fig. 1. Lane 1, GR liver DNA treated with *Eco*RI; lane 2, untreated DNA of transplanted GR leukemia cells; lane 3, *Eco*RI-treated DNA of transplanted GR leukemia cells (the same DNA preparation as that shown in lane 2). *Eco*RI-digested λ DNA was used as a molecular weight marker.

contained extra MMTV proviruses as compared with the *Hin*dIII pattern of GR liver DNA (lane 4). The later passages, passages 8 and 10 of this leukemia cell line, maintained the same *Hin*dIII pattern as the leukemia DNA from cells in the fifth passage. These results indicate that extra MMTV proviruses are present at early passages of the leukemia cells and that no gross rearrangements occur upon multiple passaging of these cells.

Since GR leukemia is of T-cell origin (10, 11), the amplification of MMTV proviruses in leukemia cell DNA may be due to a different pattern of MMTV proviral sequences in the original T-cells. However, the *Eco*RI patterns of GR liver and GR thymus DNA showed no differences (Fig. 5, lanes 1 and 2), indicating that the extra MMTV proviral DNA sequences in GR leukemia cell DNA (Fig. 5, lane 3) are not present in normal T-cells of strain GR. It has not yet been feasible to isolate cells at the preleukemic stage, and therefore, we are unable to determine whether the acquisition of MMTV proviruses is instrumental in the onset of leukemogenesis or whether it is a result of leukemogenic development.

The acquisition of extra MMTV proviruses in GR leukemia DNAs did not coincide by a similar increase of C-type-related proviruses, since hybridization with Moloney leukemia virus and AKR virus-specific cDNAs did not show extra bands in DNAs of early transplants of two different GR leukemia cell lines (A. Berns and W. Quint, unpublished data). We also did not

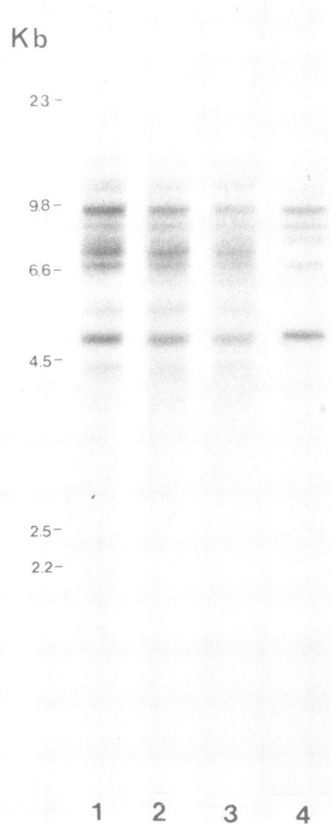

FIG. 4. Effect of serial transplantation on presence of extra MMTV proviruses in GR leukemia cells. Twenty micrograms of cellular DNA was digested with *Hin*dIII and analyzed for the presence of MMTV DNA-containing fragments as described in the legend to Fig. 1. Lanes 1 through 3, DNA of transplants of GR leukemia cell line 16, passages 5, 8, and 10, respectively; lane 4, GR liver DNA. *Hin*dIII-digested λ DNA was used as a molecular weight marker.

observe any change in number or location of the β-globin gene in the four different leukemia cell lines as compared with liver DNA (data not shown).

Two types of MMTV proviruses can be recognized in acquired copies of leukemia cells. The endogenous proviruses of the GR mouse strain can be distinguished into at least two classes which differ from each other in several restriction enzyme recognition sites: the one characterized by Shank et al. (22) and another recently obtained by molecular cloning methods and termed GR-40 (13). This GR-40 provirus is not involved in mammary tumor formation in GR mice. The class characterized by Shank et al. contains 1.6-, 1.3-, 4.1-, and 1.9-kb internal *Pst*I fragments, whereas GR-40 contains 1.0-, 0.6-, 5.4-, and 1.9-kb internal *Pst*I fragments (Fig. 6). We used three molecular probes to characterize the acquired copies which are present in leukemia cell DNA. These probes are MMTV $cDNA_{rep}$, representative of the whole MMTV genome, and two subcloned *Pst*I fragments derived from GR-40. One subclone contains a 5.4-kb internal *Pst*I fragment of GR-40, and the second contains a 1.0-kb *Pst*I fragment positioned within the long terminal repeat (LTR) region of strain GR-40 (13). The analysis of normal and leukemia cell DNA cut with *Pst*I and probed with MMTV $cDNA_{rep}$ is shown in Fig. 7. DNAs of GR liver and thymus (Fig. 7, lanes 1 and 2) contain the *Pst*I fragments (5.4, 4.1, 1.9, 1.6, 1.3 and 1.0 kb) characteristic for the endogenous proviral types shown in Fig. 6 (the 0.6-kb fragment has run off this gel). Leukemia cell DNA (lane 3) cut with *Pst*I shows an increased intensity of hybridization in the 5.4-, 4.1-, 1.9-, and 1.6-kb region of the gel. The 1.0- to 1.3-kb region cannot be clearly interpreted, owing to the resolution of the gel. The increased intensity of hybridization in the 5.4- and 4.1-kb regions, however, suggests that both types of proviruses shown in Fig. 6 are included among the extra copies of MMTV proviral DNA in leukemia cells.

To strengthen this conclusion, the 5.4-kb *Pst*I fragment of GR-40 DNA was used as a hybridization probe in the analysis shown in Fig. 8. Lanes 5 and 6 show the hybridization of the 5.4-kb fragment to *Eco*RI-cut leukemia cell and liver DNA. As expected, all of the DNA fragments recognized by MMTV $cDNA_{rep}$ (Fig. 3, lane 3) hybridized with this DNA fragment. The intensity of the bands which correspond to fragments containing right-hand (3′) MMTV proviral DNA information is less because only 0.9 kb of these fragments are represented in the 5.4-kb probe (see Fig. 6). Lanes 1 and 2 show the results obtained with *Pst*I-digested DNAs. *Pst*I-digested leukemia cell DNA shows an increased hybridization signal in the 5.4- and 4.1-kb region as compared with liver DNA. The *Pst*I fragment of 1.3 kb, which shares homology with the 5.4-kb fragment of GR-40 DNA, also shows an increased amount of hybridization. This result supports the conclusion drawn from Fig. 7; i.e., proviral DNA characterized by both the 5.4- and 4.1-kb internal *Pst*I fragment is increased in copy number in leukemia cell DNA.

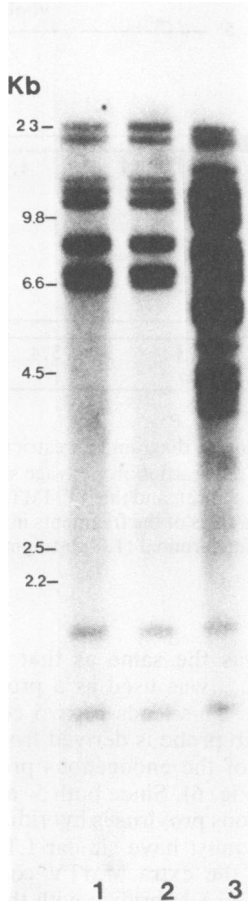

FIG. 5. Absence of extra MMTV proviruses in thymocytes of GR leukemia cells. Twenty micrograms of cellular DNA was digested with *Eco*RI and analyzed for the presence of MMTV DNA-containing fragments as described in the legend to Fig. 1. Lane 1, GR liver DNA; lane 2, GR thymus DNA (a pool of thymuses from young male mice was used for the DNA extraction); lane 3, DNA of transplanted GR leukemia cells. *Hin*dIII-digested λ DNA was used as a molecular weight marker.

Finally, we used as a hybridization probe the 1.0-kb *Pst*I fragment located in the LTR region of GR-40 (Fig. 6) to analyze the sequences of the acquired MMTV copies in leukemia cell DNA. Lanes 7 and 8 of Fig. 8 show the hybridization of the 1.0-kb probe to *Eco*RI-digested leukemia cell and liver DNA. For both DNAs, the hybridiza-

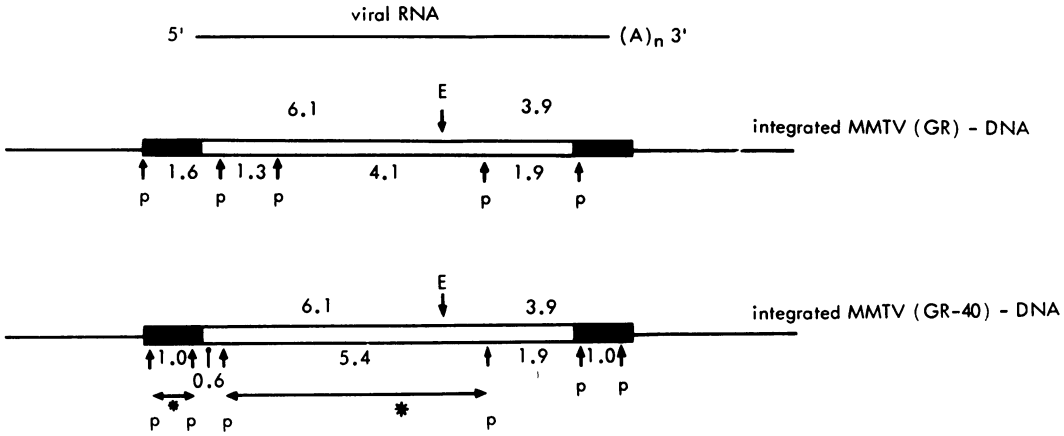

FIG. 6. Schematic diagram of restriction enzyme cleavage sites in MMTV proviral DNAs in GR DNA. Arrows indicate the restriction cleavage sites of *Eco*RI (E) and *Pst*I (p). The cleavage map of MMTV (GR) DNA is from Shank et al. (22), and that of MMTV (GR-40) DNA is from Hynes et al. (13). The numbers between the arrows indicate the sizes of the fragments in kb $\times 10^{-3}$. The indicated *Pst*I fragments of MMTV (GR-40) DNA were used as probes for terminal (1.0-kb fragment) and internal (5.4-kb fragment) MMTV DNA sequences.

tion pattern was the same as that seen when MMTV $cDNA_{rep}$ was used as a probe (Fig. 3, lanes 1 and 3). This leads to two conclusions. First, the 1.0-kb probe is derived from the LTR region of one of the endogenous proviruses of GR mice (13; Fig. 6). Since both 5' and 3' sides of the endogenous proviruses hybridize with this probe, all five must have similar LTR regions. Second, since the extra MMTV copies in the leukemia cell DNA hybridize with the 5.4-kb as well as with the 1.0-kb probe, these extra proviruses also contain LTR-specific DNA. This conclusion was further investigated by digesting both DNAs with *Pst*I and then hybridizing them with the 1.0-kb probe. There were a number of additional *Pst*I fragments in the range of 2 to 7 kb in the leukemia cell DNA (Fig. 8, lane 3) as compared with liver DNA (lane 4). These fragments probably represent the right-hand (3') joints of acquired MMTV proviral DNA and flanking host sequences. The *Pst*I fragments of 1.6 and 1.0 kb indicative for the ends of the two MMTV proviral classes (see Fig. 6) do not seem to increase in hybridization intensity in leukemia cell DNA as compared with liver DNA (lane 3 versus lane 4). Thus, although the internal *Pst*I fragments of the two proviral classes are overrepresented in leukemia cell DNA, their corresponding ends, the 1.6- and 1.0-kb *Pst*I fragments, appear to be present in similar amounts in liver and leukemia cell DNA. Instead, a new band between 1.0 and 1.3 kb is present in leukemia cell DNA. This result suggests that the terminal sequences of the acquired MMTV proviruses in leukemia cell DNA do not correspond to either class of endogenous proviral DNA but contain a new *Pst*I fragment not previously present in liver DNA.

Expression of MMTV RNA in GR leukemia cells. GR leukemia cells express high levels of MMTV proteins. The processing of the MMTV gag precursor into internal MMTV proteins appears, however, to be inhibited at the step of phosphorylation (18). We wished to see whether this could be owing to the synthesis of aberrantly sized MMTV RNA molecules and to investigate the possibility that integration of extra MMTV proviruses may lead to transcription of genes juxtaposed to these extra MMTV proviruses. The transcription of juxtaposed genes may be enabled by the viral promoter located in the 3' LTR. Poly(A)$^+$ RNA from GR leukemia cells and from spleens of healthy GR mice was therefore separated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with three different MMTV DNA probes: a total MMTV ^{32}P-labeled cDNA representative for the whole MMTV RNA genome, the 1.6-kb *Pst*I fragment of MMTV (GR) DNA representative for the whole LTR region (see Fig. 6), and a probe specific for the U5 part of the LTR region. The latter probe contains 80 nucleotides from the 3' end of the U5 part of the LTR plus approximately 150 nucleotides of the adjacent 5' end of MMTV DNA (14) (Fig. 9). All three probes detected a 35S and a 24S MMTV RNA class in the poly(A)$^+$ RNA from GR leukemia cells, suggesting that the aberrant processing of the MMTV gag precursor in GR leukemia cells as observed by Nusse et al. (18) is not due to major deletions in the MMTV RNA.

Using LTR- and U5 LTR-specific probes, we

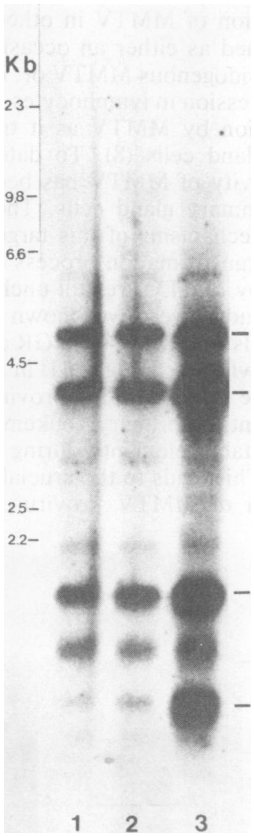

FIG. 7. Amplification of particular *Pst*I MMTV DNA fragments in GR leukemia cells. Twenty micrograms of cellular DNA was digested with *Pst*I and analyzed for the presence of MMTV DNA-containing fragments. Lane 1, GR liver DNA; lane 2, GR thymus DNA; lane 3, DNA of transplanted GR leukemia cells. The bars at the right mark the amplified MMTV DNA-containing bands. *Hin*dIII-digested λ DNA was used as a molecular weight marker.

did not detect extra RNAs hybridizing besides the 35S and 24S RNAs (Fig. 9, lanes 2 and 3), nor were any detected at longer exposure times, indicating that there were no transcripts of cellular genes juxtaposed with extra MMTV proviruses initiated by a promoter in the right MMTV LTR, as occurs in avian leukosis virus- induced chicken leukemia (16, 19).

Additional MMTV proviruses in leukemia DNAs of other mouse strains. The finding of extra MMTV proviruses in GR leukemia cell DNA is surprising in view of the fact that MMTV has no known leukemogenic activity. We therefore examined two other leukemia DNAs for the presence of extra MMTV proviruses: the transplantable thymic lymphoma SL2 of mouse strain DBA/2 (3a, 33) and the cell line BW5147 derived from a spontaneous AKR/J thymic lymphoma (12).

No extra MMTV proviral sequences were detected in either of these two T-cell leukemias (data not shown). However, another B-cell leukemia, L1210, showed an amplification of MMTV proviruses. The transplantable L1210 leukemia cell line is derived from a chemically induced B-cell leukemia in mouse strain DBA/2. It contains MMTV antigens associated with the mammary leukemia antigen (17, 27). This mammary leukemia antigen contains incompletely processed MMTV precursor proteins (34). DNA from L1210 cells and DBA/2 mouse livers as a control was digested with *Eco*RI and *Hin*dIII, and the MMTV DNA-containing fragments were identified (Fig. 10). The *Eco*RI and *Hin*dIII MMTV DNA patterns of L1210 DNA (Fig. 10, lanes 2 and 4) contained many more bands than were present in the control, DBA/2 liver DNA

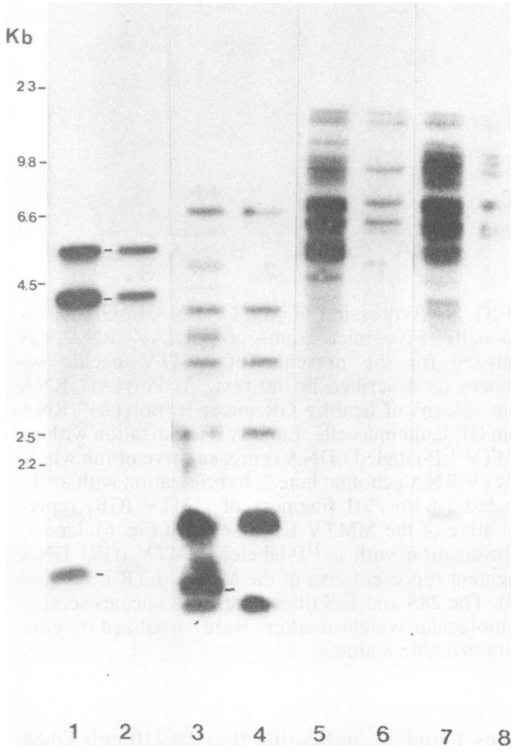

FIG. 8. Identification of amplified *Pst*I MMTV DNA fragments in GR leukemia DNA. Twenty micrograms of cellular DNA was digested with either *Pst*I (lanes 1 through 4) or *Eco*RI (lanes 5 through 8) and hybridized to the 5.4-kb internal *Pst*I MMTV DNA fragment of MMTV (GR-40) DNA (lanes 1, 2, 5, and 6) or the 1.0-kb terminal *Pst*I MMTV DNA fragment of MMTV (GR-40) DNA (lanes 3, 4, 7, and 8). The *Pst*I MMTV (GR-40) DNA fragments were nick-translated with ^{32}P. The bars in lanes 1 through 4 mark the main amplified MMTV DNA fragments. *Hin*dIII-digested λ DNA was used as a molecular weight marker.

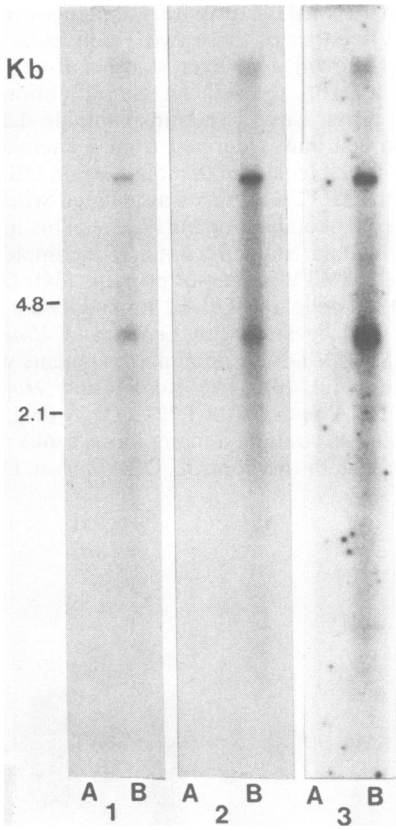

FIG. 9. Expression of MMTV RNA in GR leukemia cells. Five micrograms of poly(A)$^+$ RNA was analyzed for the presence of MMTV-specific sequences as described in the text. A, Poly(A)$^+$ RNA from spleens of healthy GR mice; B, poly(A)$^+$ RNA from GR leukemia cells. Lane 1, Hybridization with an MMTV ^{32}P-labeled cDNA representative of the whole MMTV RNA genome; lane 2, hybridization with a ^{32}P-labeled 1.6-kb *Pst*I fragment of MMTV (GR) representative of the MMTV LTR (see also Fig. 6); lane 3, hybridization with a ^{32}P-labeled MMTV (GR) DNA fragment representative of the MMTV LTR U5 region (14). The 28S and 18S ribosomal RNA species serving as molecular weight markers were visualized by ethidium bromide staining.

(lanes 1 and 3), indicating that L1210 cell DNA had acquired MMTV proviruses which also appear to be integrated (data not shown). The phenomenon of acquisition of MMTV proviruses in leukemia cells is therefore not restricted to the GR leukemia system but can also be detected in the chemically induced B-cell leukemia cell line L1210 of DBA/2 mice.

DISCUSSION

Murine mammary gland cells have always been considered the target tissue for MMTV. The expression of MMTV in other tissues has been explained as either an occasional expression of the endogenous MMTV or, in the case of MMTV expression in lymphocytes, as the result of an infection by MMTV as it travels to the mammary gland cells (8). To date, the transforming activity of MMTV has been described only in mammary gland cells. The molecular-biological mechanisms of this target specificity and of the transformation process of mammary gland cells by MMTV are still unclear.

In this study, we have shown that thymic leukemia cells of mouse strain GR contain extra MMTV proviruses integrated in the cellular genome. The extra MMTV proviruses are already present in primary leukemia cells and behave as stable elements during serial transplantation. This leads to the crucial question: Is amplification of MMTV proviruses in thymic

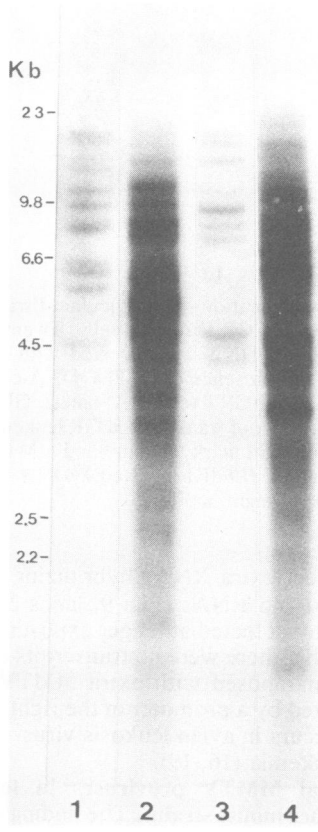

FIG. 10. Demonstration of extra MMTV proviruses in L1210 leukemia cells. Twenty micrograms of cellular DNA was digested with either *Eco*RI (lanes 1 and 2) or *Hin*dIII (lanes 3 and 4) and analyzed for the presence of MMTV DNA-containing fragments as described in the legend to Fig. 1. Lanes 1 and 3, DBA/2 mouse liver DNA; lanes 2 and 4, L1210 leukemia cell DNA. *Hin*dIII-digested λ DNA was used as a molecular weight marker.

leukemia cells of strain GR associated with the onset of leukemogenesis?

The involvement of retroviruses in originating spontaneous tumors which are nonvirally induced appears to be, in general, difficult to approach. One may search for a close correlation between tumor development and expression of the retrovirus or for permanent molecular-biological events, such as integration of proviruses in the DNA of tumor cells. These are, however, no definite proofs for a causative relationship, which can be obtained only by inducing tumors upon infection of susceptible animals with the particular retroviruses. No evidence as yet exists that MMTV can induce leukemia upon infection of mice. The finding of additional MMTV proviruses in the DNA of leukemia cells and their absence in normal thymocytes may suggest that this acquisition of MMTV proviruses and the onset of leukemia are coinciding events, but it does not provide any proof for a causative relationship between them. Such an acquisition could have been the result of chromosomal rearrangements or translocations or both. Spira et al. (25) have reported a trisomy of chromosome 15 in murine T-cell leukemias. Such a trisomy cannot be held responsible for the many additional copies of MMTV proviruses in GR leukemia cells since chromosomal amplification would result in more intense bands but not in extra bands at different genomic locations, as observed in GR leukemia DNAs. In addition, analysis of *Pst*I-digested liver and leukemia cell DNA hybridized with the 1.0-kb LTR-specific probe (Fig. 8, lanes 3 and 4) showed that the DNA fragments representing the joint between proviral and cellular DNA did not have an increased hybridization intensity in leukemia cell DNA. Thus, the region of the chromosome containing the endogenous MMTV proviruses has not been amplified in leukemia cell DNA. Moreover, a chromosomal analysis of GR leukemia cells did not show any gross rearrangements (Hilkens, unpublished data).

GR mice have five endogenous MMTV proviruses; one of them becomes expressed in complete virus particles in mouse milk and is associated with the mammary tumor induction locus Mtv-2 (15). The extra MMTV proviruses in GR leukemia cells may be derived from the milk-transmitted MMTV, but they may also be DNA copies from the other endogenous MMTV proviruses. The finding of extra MMTV proviruses in GR leukemia cells may therefore be the result of an early infection of preleukemia cells by milk-transmitted MMTV, or it may be due to integration of MMTV DNA copies from activated endogenous MMTV proviruses without prior viral infection of the cells. To determine which one of these endogenous proviruses is responsible for the extra copies found in leukemia cell DNA, we used probes specific for internal regions and termini of MMTV proviral DNA. From the data shown in Fig. 8, we concluded that at least two types of endogenous proviruses of strain GR are included among the extra copies found in leukemia cell DNA. It seems that the termini of the extra proviruses contain a *Pst*I fragment of approximately 1.3 kb which is not present in liver DNA. One possible explanation for this result is that both types of viral DNA shown in Fig. 6 are present in leukemia cells, but upon integration into genomic DNA, a small deletion of DNA close to the termini has occurred. For example, if 300 bases of DNA encompassing the second *Pst*I site shown for GR-40 DNA were lost, the new terminal fragment of approximately 1.3 kb which we observed in the *Pst*I digest of leukemia cell DNA (Fig. 8, lane 3) could be generated from both types of endogenous proviruses. To verify this hypothesis, it will be necessary to isolate and sequence the ends of the leukemia cell-specific MMTV proviruses. Variation in LTR sequences of acquired proviruses has also been observed in Moloney murine leukemia virus-induced leukemia cells (29).

The finding that leukemia cells of GR mice contain extra MMTV proviruses is unprecedented. Acquisition of proviruses is usually due to an infection of the cells by a retrovirus, followed by viral DNA synthesis and integration. In GR leukemia cells, very little mature MMTV has been observed, either in in vivo transplants or in in vitro-maintained leukemia cells (18). The extra MMTV proviruses are, then, most likely derived from DNA transcripts of MMTV RNA molecules present in preleukemia cells. A similar result has been observed in MMTV-infected rat hepatoma cells, where unintegrated viral DNA can be found. Since these cells produce no virus, the viral DNA was assumed to arise from reverse transcription of MMTV RNA (20, 21). However, in these rat hepatoma cells, most of the MMTV DNA molecules do not become integrated, whereas the extra MMTV DNA molecules in the leukemia cell DNA appear to be integrated in the cellular genome at various locations. This conclusion arises from the result that DNAs from different leukemia cell lines gave different patterns of MMTV-specific fragments when digested with three different restriction enzymes. In general, proviruses resulting from exogenous viral infection integrate at random sites of the cellular genome, as has been reported for both MMTV and murine leukemia RNA tumor viruses (3, 6, 26). This, then, also holds true for the amplified MMTV proviruses in GR leukemia cells.

Owing to the large number of extra bands, it is

not possible to identify extra MMTV proviruses integrated at similar cellular sites in the four different GR leukemia cell lines. The presence of a promoter sequence in the LTR region of the integrated provirus could lead to continuous expression of the gene downstream from the proviral promoter, as appears to be the case in avian leukosis virus-induced leukemia cells in chickens (16). The insertion of new MMTV proviral DNA in GR leukemia cells does not, however, result in such a transcription of juxtaposed genes under the direction of the added promoter signals, since no other bands than the viral 35S and 24S RNAs were detected with LTR- and U5 LTR-specific MMTV probes (Fig. 9). It is still possible that such a transcript would be of the same size as the viral RNAs and thereby comigrate with them and remain undetected. Also, transcription of juxtaposed genes may not be derived from the extra MMTV LTR promoter signal but from disturbing the genetic surrounding by inserting the extra MMTV proviral DNA. These transcripts would not be detected by LTR-specific probes but by probes specific for juxtaposed cellular sequences (19).

We did not observe an extensive acquisition of MMTV proviruses in other T-cell leukemias, such as SL/2 from DBA/2 mice or BW5147 from AKR mice. These T-cell leukemias express no MMTV RNA. Interestingly, the MLA leukemia line from DBA/2 mice, which expresses large amounts of MMTV RNA, did not show any rearrangements of MMTV proviruses, as has been reported (28). The L1210 B-cell leukemia from DBA/2 mice, however, also demonstrated an acquisition of MMTV proviruses in leukemia DNA. L1210 leukemia cells resemble GR leukemia cells in having incompletely processed MMTV proteins and no synthesis of complete virus particles. The most likely explanation for the presence of extra MMTV proviruses in the GR and L1210 leukemia systems is that expression of endogenous MMTV proviruses leads to an accumulation of MMTV RNA, resulting in integration of MMTV viral DNA without prior viral infection of the cell. Whether this integration occurs at the onset of leukemia development and thereby provides a marker for T-cell leukemia development in GR male mice remains a challenging question.

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LITERATURE CITED

1. **Bentvelzen, P., and J. Hilgers.** 1980. The murine mammary tumor virus, p. 311–355. *In* G. Klein (ed.), Viral oncology. Raven Press, New York.
2. **Cohen, J. C., J. E. Majors, and H. E. Varmus.** 1979. Organization of mouse mammary tumor virus-specific DNA endogenous to BALB/c mice. J. Virol. **32**:483–496.
3. **Cohen, J. C., P. R. Shank, V. L. Morris, R. Cardiff, and H. E. Varmus.** 1979. Integration of the DNA of mouse mammary tumor virus in virus infected, normal and neoplastic tissue of the mouse. Cell **16**:333–345.

3a. **Dullens, H. F. J., J. Hilgers, B. J. Spit, E. de Heer, R. A. Weijer, C. D. H. van Basten, and W. den Otter.** 1982. Staging, growth, properties, and metastatic behaviour of transplantable murine thymic lymphomas. Int. J. Tissue React. **4**:15–25.

4. **Fine, D. L., H. K. Plowman, S. P. Kelly, L. O. Arthur, and E. A. Hillman.** 1974. Enhanced production of mouse mammary tumor virus in desamethasone-treated 5-iododeoxyuridine stimulated mammary tumor cells in culture. J. Natl. Cancer Inst. **52**:1881–1886.
5. **Groner, B., E. Buetti, H. Diggelman, and N. E. Hynes.** 1980. Characterization of endogenous and exogenous mouse mammary tumor virus proviral DNA with site-specific molecular clones. J. Virol. **36**:734–745.
6. **Groner, B. and N. E. Hynes.** 1980. Number and location of mouse mammary tumor virus proviral DNA in mouse DNA of normal tissue and of mammary tumors. J. Virol. **33**:1013–1025.
7. **Groner, B., N. E. Hynes, and H. Diggelman.** 1979. Identification of mouse mammary tumor virus specific mRNA. J. Virol. **30**:417–420.
8. **Hageman, P., J. Calafat, and J. H. Daams.** 1972. The mouse mammary tumor viruses, p. 283–300. *In* P. Emmelot and P. Bentvelzen (ed.), RNA viruses and host genome in oncogenesis. North Holland Publishing Co., Amsterdam.
9. **Hayward, W. C., B. G. Neel, and S. M. Astrin.** 1981. Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature (London) **290**:475–480.
10. **Hilgers, J., W. J. van Blitterwijk, W. S. Bont, G. J. Theuns, R. Nusse, J. Haverman, and P. Emmelot.** 1975. Distribution and antibody-induced redistribution of a mammary tumor virus-induced and a normal antigen on the surface of mouse leukemia cells. J. Natl. Cancer Inst. **54**:1335–1342.
11. **Hilkens, J., J. Hilgers, R. Michalides, B. van der Zeijst, A. Colombatti, M. Van der Valk, N. Hynes, and B. Groner.** 1980. Analysis of murine retroviral genes and genes for viral membrane receptors by somatic cell genetics, p. 1033–1048. *In* M. Essex, G. Todaro, and H. Zur Hausen (ed.), Viruses in naturally occurring cancers. Cold Spring Harbor Conference on Cell Proliferation, 7. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York.
12. **Hyman, R., and V. Stallings.** 1974. Complementation patterns of Thy-1 variants and evidence that antigen loss variants "pre-exist" in the parental population. J. Natl. Cancer Inst. **52**:429–436.
13. **Hynes, N., N. Kennedy, U. Rahmsdorf, and B. Groner.** 1981. Hormone responsive expression of an endogenous proviral gene of mouse mammary tumor virus after molecular cloning and gene transfer into cultured cells. Proc. Natl. Acad. Sci. U.S.A. **78**:2038–2042.
14. **Kennedy, N., G. Knedlitschek, B. Groner, N. E. Hynes, P. Herrlich, R. Michalides, and A. A. Van Ooyen.** 1982. Long terminal repeats of endogenous mouse mammary tumor virus contain a long open reading frame which extends into adjacent sequences. Nature (London) **295**:622–624.
15. **Michalides, R., R. van Nie, R. Nusse, N. Hynes, and B. Groner.** 1981. Mammary tumor induction loci in GR and DBAf mice contain one provirus of the mouse mammary tumor virus. Cell **23**:165–173.
16. **Neel, B. G., W. S. Hayward, H. L. Robinson, J. Fang, and**

S. M. Astrin. 1981. Avian leukeosis virus-induced tumors have common proviral integration sites and synthesize discrete new RNAs: oncogenesis by promoter insertion. Cell 23:323–334.
17. Nowinski, R. C., L. J. Old, D. H. Moore, G. Geering, and E. A. Boyse. 1967. A soluble antigen of the mammary tumor virus. Virology 31:1–14.
18. Nusse, R., L. Van der Ploeg, L. Van Duyn, R. Michalides, and J. Hilgers. 1979. Impaired maturation of mouse mammary tumor virus precursor polypeptides in lymphoid leukemia cells, producing intracytoplasmic A particles and no extra cellular B type virions. J. Virol. 32:251–258.
19. Payne, G. S., S. A. Courtneidge, L. B. Crittenden, A. M. Fadly, J. H. Bishop, and H. E. Varmus. 1981. Analysis of avian leukosis virus DNA and RNA in Bursal tumors: viral gene expression is not required for maintenance of the tumor state. Cell 23:311–322.
20. Ringold, G. J., K. R. Yamamoto, G. M. Tomkins, J. M. Bishop, and H. E. Varmus. 1975. Dexamethasone-mediated induction of mouse mammary tumor virus RNA: a system for studying glucocorticoid action. Cell 6:299–305.
21. Ringold, G. M., P. R. Shank, and K. R. Yamamoto. 1978. Production of unintegrated mouse mammary tumor virus DNA in infected rat hepatoma cells is a secondary action of dexamethasone. J. Virol. 26:93–101.
22. Shank, P. R., J. C. Cohen, H. E. Varmus, K. R. Yamamoto, and G. M. Ringold. 1978. Mapping of linear and circular forms of mouse mammary tumor virus DNA with restriction endonucleases: evidence for a large specific deletion occurring at high frequency during circularization. Proc. Natl. Acad. Sci. U.S.A. 75:2112–2116.
23. Shibler, U., M. Tosi, A. D. Pittet, L. Fabiani, and P. K. Wellauer. 1980. Tissue-specific expression of mouse α-amylase genes. J. Mol. Biol. 142:93–116.
24. Smit, G. 1978. Evidence for precursor-product relationship between intracytoplasmic A particles and mouse mammary tumor virus cores. J. Gen. Virol. 41:193–200.
25. Spira, J., F. Wiener, S. Ohno, and G. Klein. 1979. Is trisomy cause or consequence of murine T cell leukemia development? Studies on Robertsonian translocation mice. Proc. Natl. Acad. Sci. U.S.A. 76:6619–6621.
26. Steffen, D., and R. A. Weinberg. 1978. The integrated genome of murine leukemia virus. Cell 15:1003–1010.
27. Stück, B., E. A. Boyse, L. J. Old, and E. A. Carswell. 1964. ML: a new antigen found in leukemias and mammary tumor of the mouse. Nature (London) 203:1033–1034.
28. Validya, A. B., C. A. Long, J. B. Sheffield, A. Tamura, and H. Tanaka. 1980. Murine mammary tumor virus deficient in the major glycoprotein: biochemical and biological studies in virions produced by a lymphoma cell line. Virology 104:279–293.
29. Van der Putten, H., W. Quint, J. Van Raaij, E. Robanus Maandag, I. M. Verma, and A. Berns. 1981. M-MuLV-induced leukemogenesis: integration and structure of recombinant proviruses in tumors. Cell 24:729–739.
30. Van Nie, R., and A. A. Verstraeten. 1975. Studies of genetic transmission of mammary tumor virus by C3Hf mice. Int. J. Cancer 16:922–932.
31. Van Nie, R., A. A. Verstraten, and J. D. De Moes. 1977. Genetic transmission of mammary tumor virus by GR mice. Int. J. Cancer 19:383–390.
32. Varmus, H. E., N. Quintrell, E. Medeiros, R. C. Nowinski, and N. H. Sarkar. 1973. Transcription of mouse MTV genes in tissues from high and low tumor incidence mouse strains. J. Mol. Biol. 79:663–679.
33. Wolf, A., R. Barfoot, and R. A. Johnson. 1972. Xenogeneic recognition of tumor specific plasma membrane antigens derived from mouse lymphoma cells. Immunology 22:485–491.
34. Zak-Nymark, T., J. Steuden, and C. Radzikowski. 1978. Mammary leukemia (ML) antigen isolated from L1210 leukemia cells. Int. J. Cancer 21:490–495.