JOURNAL OF VIROLOGY, July 1989, p. 3200–3204
0022-538X/89/073200-05$02.00/0

Vol. 63, No. 7

Absence of Mouse Mammary Tumor Virus Proviral Amplification in Chemically Induced Lymphomas of RF/J Mice

ROBERT KRAFT,[1] SALLY T. ISHIZAKA,[1] SHARON A. OKENQUIST,[1] GEOFFREY CHILDS,[1] FRANK LILLY,[1] AND JACK LENZ[1,2]*

Departments of Molecular Genetics[1] and Microbiology and Immunology,[2] Albert Einstein College of Medicine, Bronx, New York 10461*

Received 24 October 1988/Accepted 16 March 1989

RF/J mice are susceptible to the induction of thymic lymphomas by the carcinogens 3-methylcholanthrene and *N*-methyl-*N*-nitrosourea. Given the association of mouse mammary tumor virus (MMTV) with certain thymomas, we examined genomic DNA from chemically induced lymphomas of RF/J mice for new MMTV proviruses. Of 13 tissue culture lines derived from 3-methylcholanthrene-induced tumors, 5 had acquired new proviruses. MMTV amplification coincided with the appearance of viral mRNAs and proteins. However, no primary tumors or animal-passaged tumors contained new proviruses. These observations indicate that MMTV does not have a role in the tumor induction process, although it may become activated and amplified in tissue culture lines derived from tumors.

RF/J mice exhibit a low incidence of spontaneous lymphomas but are highly susceptible to the induction of lymphomas by the chemical carcinogens 3-methylcholanthrene (MCA) and *N*-methyl-*N*-nitrosourea (MNU) (7, 11, 18, 23). Topical application of MCA at 3 months results in a near 100% incidence of thymic lymphomas by 10 months of age (11). The molecular events responsible for the conversion of a normal cell to a lymphomatous one remain largely unknown. MNU-induced lymphomas frequently contain *ras* genes that have been activated by single-base-pair substitution mutations (15). No evidence for rearrangements of oncogenes was found in MCA-induced lymphomas (4). Karyotypic analysis of MCA-induced tumors showed that they predominantly exhibit a normal diploid complement of chromosomes, with no evidence of chromosome 15 trisomy (13).

One hypothesis for the genesis of chemical lymphomas is that the carcinogen activates an endogenous retrovirus, which then proceeds to induce the tumor. Examination of MCA-induced lymphomas yielded no evidence for the involvement of type C retroviruses (3). However, RF/J mice also inherit the endogenous type B retrovirus, mouse mammary tumor virus (MMTV). In addition to its causative role in the generation of mammary carcinomas in mice, MMTV is associated with various types of T-cell lymphomas. It has been implicated in spontaneous lymphomas that occur in GR mice (24). Passaged tumors and cell lines established from chemically induced thymomas frequently exhibit amplification of MMTV proviruses (8–10), and a highly leukemogenic MMTV has been isolated from a thymic lymphoma cell line that was induced by the polycyclic aromatic hydrocarbon 7,12-dimethylbenz[*a*]anthracene (1, 2, 6). MMTV amplification has also been detected in a kidney adenocarcinoma cell line (12, 29) and a pituitary tumor cell line (27).

Prompted by the evidence implicating MMTV, we undertook a direct investigation of whether this virus is involved in the induction of lymphomas by MCA and MNU in RF/J mice. The approach that we used was to examine primary tumors, animal-passaged tumors, and tissue culture cell lines derived from MCA-induced lymphomas for the presence of MMTV proviruses integrated at novel sites in the cellular genome. DNA isolated from MCA-induced primary thymic lymphomas (3, 4, 14) was digested with *Eco*RI (New England BioLabs, Inc.), which cleaves most MMTV genomes once. Samples (20 μg) of digested genomic DNA were resolved electrophoretically in 0.8% agarose gels and transferred to nitrocellulose as described previously (16). The blots were hybridized to a ^{32}P-labeled probe derived from the full-length MMTV viral genome (22).

The normal RF/J mouse genome contains 10 resolvable *Eco*RI fragments that hybridize with MMTV, indicating the presence of at least five endogenous proviruses (Fig. 1A). All nine primary MCA-induced lymphomas that were examined exhibited exclusively the germ line pattern of fragments (Fig. 1A and 1B). No new MMTV proviruses were detected in three X-ray-induced lymphomas or one tumor induced by SL3-3 virus (20) (Fig. 1A). Because passage of Abelson leukemia virus-transformed lymphoma lines in mice was reported to result in the appearance of new MMTV proviruses (9), we tested some MCA lymphomas that had been passaged in mice for the presence of new proviruses. Again, none were detected (Fig. 1B). In addition, none were detected in eight primary MNU-induced lymphomas (5) (Fig. 1C). Thus, none of the primary tumors induced with either agent yielded any evidence of MMTV proviral amplification.

Interpretation of these data to indicate the absence of MMTV amplification presumes that the tumors were monoclonal or at least oligoclonal. If the tumors were polyclonal, new sites of MMTV integration might not be detectable, since they would be unique to particular lineages. To detect clonal events, we chose to examine the lymphoma DNAs for rearrangements of the T-cell receptor B-chain gene (*Tcrb*) constant regions, *C1* and *C2*. During T-cell maturation, the *C1* region of one or both alleles can undergo alterations that result in either its rearrangement or its complete deletion. Detection of specific *C1* rearrangements is diagnostic of clonality.

Lymphoma DNA was digested with *Hin*dIII and analyzed with a probe from the *C2* region. No rearrangements were detected involving the *C2* region, which appeared as a 3-kilobase-pair fragment in genomic digests. However, since all cells contain two copies of *C2* (unless a tumor is aneu-

* Corresponding author.

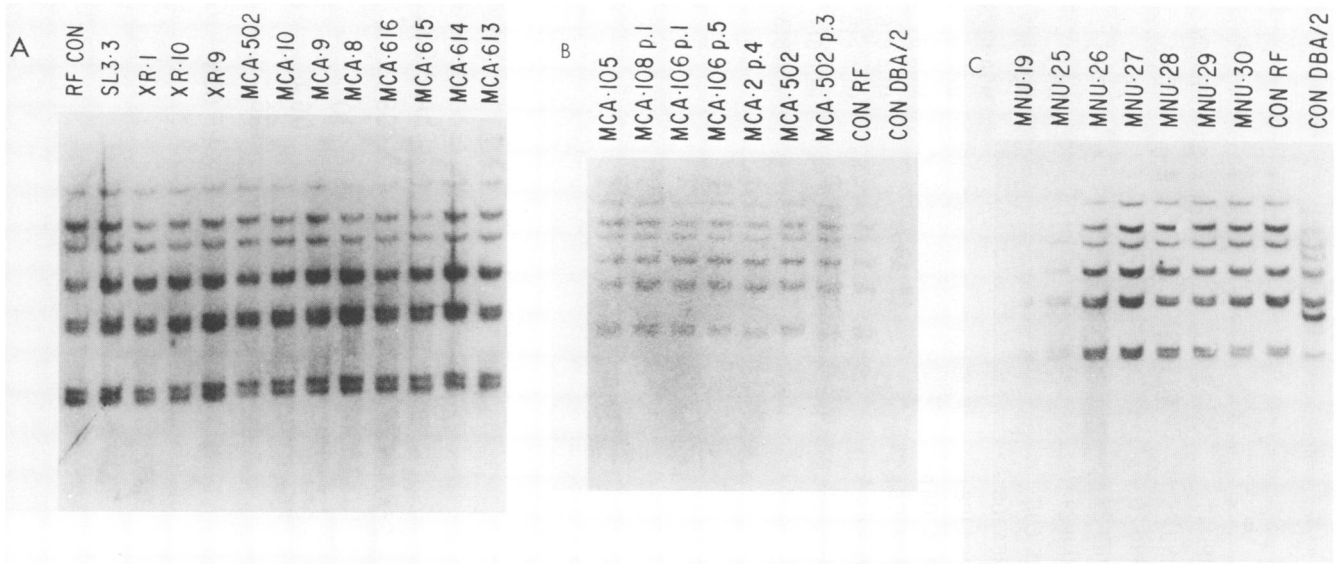

FIG. 1. Hybridization of the full-length MMTV probe to *Eco*RI-digested RF/J mouse DNAs. Normal RF/J liver DNA (lanes CON RF) has 10 *Eco*RI fragments that hybridize (note that the second and third bands from the origin resolve poorly). DBA/2J liver DNA (lanes CON DBA/2) is included as a size marker. The DBA/2J fragments were reported to be 16.7, 15.0, 11.7, 10.0, 9.0, 8.3, 7.8, 6.7, 6.5, 5.8, 4.5, and 1.4 kilobase pairs (21). (A) DNAs from eight primary MCA-induced lymphomas (lanes labeled MCA), three primary X-ray-induced tumors (lanes labeled XR), one SL3-3 virus primary lymphoma (lane SL3-3), and one control sample (lane RF CON) were analyzed. The numbers in the names of the samples refer to specific individual mice. (B) DNAs from two primary MCA-induced lymphomas (lanes MCA-105 and MCA-502) are shown. Also analyzed were DNAs from four syngeneic animal-passaged tumors (MCA-108, MCA-106, MCA-2, and MCA-502). The serial passage numbers at the time the samples were collected are indicated. (C) DNAs from eight primary MNU-induced lymphomas are shown.

ploid for the chromosome containing this locus), this approach provided an internal control for normalizing the relative amounts of each DNA. The *C2* probe also hybridized with *C1* sequences. Rearrangements of *C1* could be identified as novel fragments, whereas deletions were evident as decreases in hybridization of the germ line *C1* fragment without the appearance of new fragments. Since tumor tissue may also contain nonlymphomatous cells, the relative intensities of *C1* fragments on the blot were not necessarily integral numbers.

Comparison of tumor DNA with normal liver DNA (Fig. 2, lane RF CON) showed that all of the tumors lacked at least one of the 9.5-kilobase-pair germ line *C1* fragments and most lacked both. The small amount of this fragment seen in many of the samples was presumably due to the presence of nontumor cells. Half of the tumors lacked both germ line fragments and did not show any new bands. This indicated that these samples either were polyclonal or were monoclonal and had both *Tcrb* alleles rearranged to the *C2* region. The rest of the tumors had novel *C1* fragments. Most of these had one or two. This was consistent with the predominance of one or two cell lineages in these tumors. One lymphoma (Fig. 2A, lane MCA-614) clearly showed three new bands, indicating that this tumor most probably was either oligoclonal or triploid for the *Tcrb*-containing chromosome.

We conclude that clonal events could be detected in at least half and probably more of the lymphomas. The absence of new MMTV proviruses was consistent with a lack of infection of the prelymphomatous cell rather than an inability to detect clonally restricted viral integration sites. Thus, these results argue that MMTV does not play a causative role in the generation of MCA- and MNU-induced lymphomas.

We also tested tissue culture cell lines established from MCA-induced thymic lymphomas for the presence of new MMTV proviruses. Unlike the primary tumors, a total of 5 of 13 lines that were examined had novel MMTV proviruses. Three lines with new proviruses are shown in Fig. 3. Each had acquired several new genomes. Lines 106 and 106A were independent clones derived from the same original tumor. They clearly had viral genomes integrated at different locations.

This observation, coupled with the fact that new MMTV proviruses were detected only in tissue culture cell lines, argued that proviral amplification in these lymphomas occurred exclusively during tissue culture passage. We did not attempt to elucidate the origin of the new proviruses. Comparisons of viral restriction enzyme site polymorphisms in other lymphomas that contain new proviruses have shown that these may originate from endogenous MMTV genomes (24). Presumably, the new proviruses that we detected were also derived from one or more germ line proviruses. However, it is unclear how endogenous MMTV became activated during passage of the cells.

Several experiments were performed to investigate the mechanism by which MMTV proviral amplification occurred. RNA was isolated from several lymphoma lines as previously described (3, 4) and tested by Northern (RNA) blotting with the MMTV probe (Fig. 4). Thymus and spleen RNAs from age-matched, untreated RF/J mice showed one or more species of MMTV-related RNA. Four cell lines were examined that did not contain any new proviruses. Two of these (303 and 412) did not express detectable levels of MMTV sequences. The other two lines without additional proviruses (103 and 210) expressed an RNA that was slightly larger than viral *env* mRNA. We did not investigate the nature of this species. Three lines that did have new MMTV

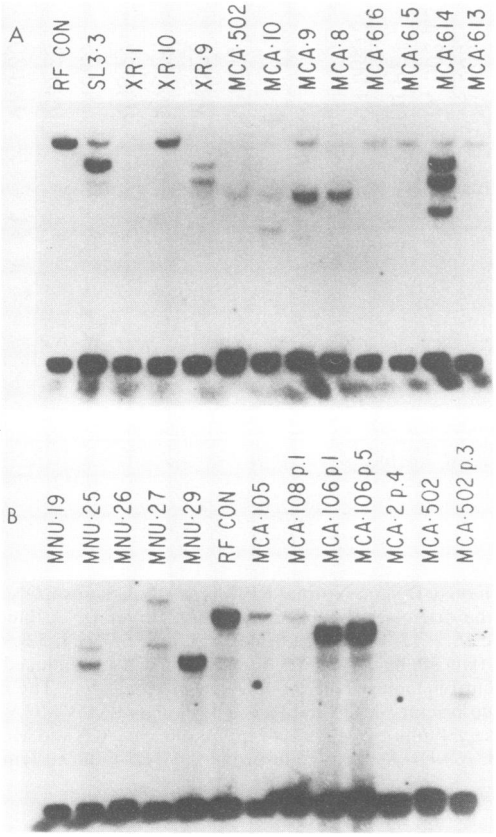

FIG. 2. T-cell receptor B-chain rearrangements in chemically induced lymphomas. DNAs that were tested for MMTV rearrangements in Fig. 1 were digested with *Hin*dIII and hybridized with a *Tcrb C2* probe. The band of high mobility is generated by hybridization to the *C2* region. The other bands are derived from the *C1* region. (A) The same MCA-, X-ray-, and virus-induced lymphomas that were tested in Fig. 1A were analyzed. (B) The same MNU- and MCA-induced tumors that were tested in Fig. 1B and 1C were analyzed. Insufficient DNA was available from MNU tumors 28 and 30 to perform this analysis. RF CON is from liver cells of an untreated mouse.

proviruses (106, 106A, and 409) also expressed the same-sized RNA. In addition, these lines expressed RNAs of 8 and 3 kilobases. These are the sizes characteristic of the full-length genome and *env* mRNAs of MMTV in lymphoma cells (19, 25). Thus, proviral amplification in MCA lymphoma cell lines was correlated with the presence of the RNAs necessary for viral replication.

Expression of viral antigens was investigated by using an antibody generated against disrupted MMTV particles (Research Resources Division, National Cancer Institute). Antibody binding was detected by indirect immunofluorescence with fluorescein isothyocyanate-coupled rabbit anti-goat immunoglobulin G (Organon Teknika) by using a FACS II fluorescence-activated cell sorter (Becton Dickinson & Co.). The cell lines that did not express any RNAs (303 and 412 [Fig. 5]) gave the lowest signal. The above background fluorescence levels may indicate that there is a low level of cross-reactivity between the antiserum and cellular proteins. Immunoprecipitation (17) of [^{3}H]leucine-labeled 412 cells yielded no detectable proteins (data not shown). The lines that expressed a single RNA species (103 and 210) exhibited higher levels of viral-antigen synthesis (Fig. 5). The lines that

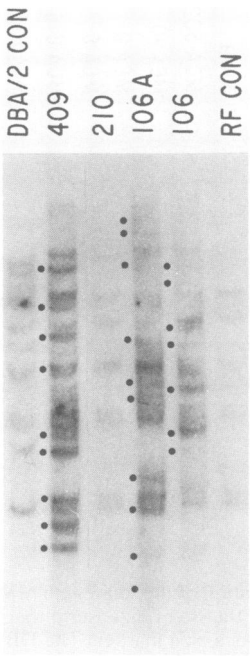

FIG. 3. MMTV proviruses in tissue culture cell lines established from MCA-induced lymphomas. DNAs were digested with *Eco*RI and hybridized with the MMTV probe. Four lines are shown; three (lines 409, 106, and 106A) have new proviruses, whereas one (line 210) does not. Dots to the left of the lanes show the positions of new fragments that hybridize to MMTV. Normal DNA from a DBA/2 and an RF mouse (lanes DBA/2 CON and RF CON) are also shown. Sizes of the DBA/2 fragments are listed in Fig. 1.

expressed full-length plus *env* RNAs and had new MMTV proviruses (409 and 106) exhibited the highest levels of viral-antigen expression (Fig. 5). As a control, we determined the fluorescence of the MMTV particle-producing murine mammary carcinoma cell line, MM5MI, grown in 10 μM dexamethasone. This line yielded a level of antigen similar to that seen in line 409 (data not shown). Immuno-

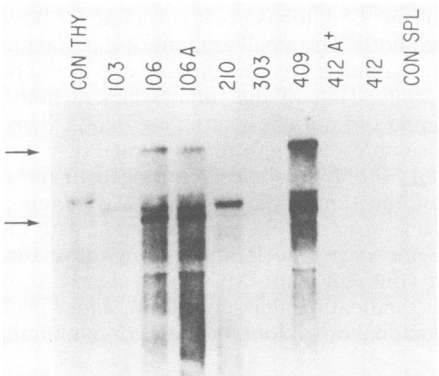

FIG. 4. MMTV RNAs in cell lines from MCA-induced lymphomas. Northern blots of total RNA isolated from lymphoma cell lines were hybridized with the MMTV probe. RNAs isolated from the thymus (lane CON THY) and spleen (lane CON SPL) of a 6-month-old RF/J mouse were also tested. In one case, 5 μg of poly(A)-containing RNA was also tested (lane 412 A^+). Arrows denote the positions of the full-length viral genomic and envelope subgenomic RNAs.

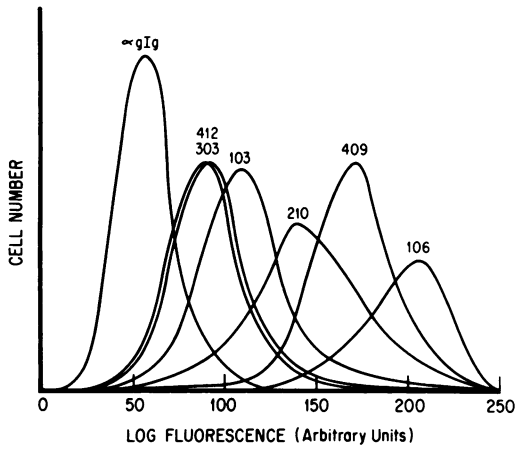

FIG. 5. Fluorescence-activated cell sorter analysis of MMTV antigen expression in cell lines from MCA-induced lymphomas. Names of the cell lines are indicated above the fluorescence peak for each.

precipitation of line 106 cells metabolically labeled with [^{3}H]leucine showed that these cells produced viral envelope and capsid proteins (data not shown).

The production of viral mRNAs and antigens is consistent with the possibility that the new MMTV proviruses were derived by MMTV replication. However, several reports have noted that production of mature MMTV viral proteins and virion particles is blocked in particular lymphoma lines (9, 26, 28). This occurs even when abundant viral mRNA synthesis can be detected (9). We have not undertaken a careful analysis of viral protein synthesis, maturation, or particle assembly. Thus, it remains possible that the new proviruses were generated by a mechanism distinct from the standard viral replicative cycle. Whatever the process of proviral amplification is, it appears to be confined to tissue-culture-passaged cells in the MCA-induced lymphoma system. Although it does not appear to play a role in the generation of the primary tumors, it may have a role in the growth of these cells in culture.

We thank J. Chinsky, M. Goodenow, and U. Dasgupta for assistance with these experiments. We thank J. Berman, O. Prakash, and N. Sarkar for providing probes used in these studies. We thank J. Racevskis and P. Etkind for the MM5MI cell line and D. Lombardi for typing the manuscript.

This work was supported by Public Health Service program project grant CA31855 from the National Institutes of Health. R.K. was supported by Public Health Service training grant CA09060 from the National Institutes of Health. S.T.I. is a Special Fellow of the Leukemia Society of America. G.C. was supported by an Irma T. Hirschl Career Scientist Award. J.L. was supported by an American Cancer Society Junior Faculty Research Award.

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