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Integration of Type B Retroviral DNA in Virus-Induced Primary Murine Thymic Lymphomas

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In previous studies we described the isolation and characterization of a highly leukemogenic virus, DMBA-LV, isolated from a transplanted, chemical carcinogen-induced thymic lymphoma. The virus is composed of a mixture of two unrelated retroviral genomes, one highly related to type B milk-borne mouse mammary tumor virus isolates and the other partially related to type C viral genomes. In the present study, primary thymic lymphomas induced by DMBA-LV in CFW/D, NIH Swiss, C3H/Bi/Ka, and C57BL/Ka mice were assessed for the presence of newly integrated type B retroviral DNA. All 46 primary thymic lymphomas examined contained one to four newly acquired murine mammary tumor virus proviruses. Based on the sizes of provirus-cell DNA junction fragments, the integration of newly acquired murine mammary tumor virus proviruses did not appear to be random.

Murine mammary tumor virus (MMTV), a type B retrovirus, is not found only in the mammary gland and mammary adenocarcinomas; the presence of both mature and immature virions has also been detected in normal lymphoid tissue (29) as well as in a wide range of tumors (7, 13, 14, 32). Furthermore, the replication of MMTV has been shown to occur in organs other than the mammary gland, especially in lymphoid tissues (5) and leukocytes (19). It has been proposed that these latter cells are responsible for viral infection of the mammary gland, infection occurring via the pinocytosis of MMTV-containing leukocyte vesicles by mammary cells (38, 39).

In a recent study (28), leukemic cells from spontaneous thymic lymphomas arising in old male GR mice were found to contain additional integrated MMTV proviruses, which were present in many sites in the genome.

Thymic lymphoma cells can contain high levels of intracytoplasmic A particles, but very few particles appear to be released (23, 37). Particles released by the DBA/2 lymphoma cell line induced a low level of mammary tumors but were not tested under experimental conditions which would have assessed their leukemogenicity (37). A second report (25) that concentrated preparations of intracytoplasmic A particles (prepared from either an in vivo serially transplanted leukemic cell line or from mammary tumors) could induce leukemia was not conclusive. The incidence was low (12%), the latent period was long (300 to 400 days), and extracts of the leukemias induced mammary tumors and not leukemia. Furthermore, the intracytoplasmic A particle preparations were not checked for the presence of type C retroviruses. However, these observations do raise the interesting possibility that MMTV can have oncogenic potential for more than one type of target cell.

In the present study, we used virus DMBA-LV, recovered from a chemical carcinogen-induced thymic lymphoma in CFW/D mice, which has been shown to be highly leukemogenic (4) but did not induce mammary tumors (J. K. Ball, unpublished data). The virus consists of two unrelated retroviral genomes. The major component is a type B retrovirus with a genome highly related (90%) to that of the exogenous milk-borne MMTV isolate from C3H mice. The type B retrovirus is present in 10-fold excess over a defective, noninfectious type C viral genome. No evidence has been obtained that the type C retroviral genome formed a pseudotype with MMTV (3). Strong evidence that the MMTV component of DMBA-LV is the leukemogenic agent has come from in vivo neutralization studies. Thymic lymphoma induction was completely abolished by a monoclonal neutralizing antibody (directed against a gp52 type-specific envelope determinant) but was unaffected by a type C retroviral neutralizing antiserum active against leukemia induction by Moloney murine leukemia virus (J. K. Ball, L. O. Arthur, and G. A. Dekaban, submitted for publication).

In view of the strong evidence that the thymic lymphomas were induced by the type B retrovirus present in DMBA-LV, it was of interest to see whether there was any specificity with respect to MMTV integration in the resulting tumors. Positive results would indicate that a mammalian retrovirus might also induce tumors by the promoter insertion, downstream activation mechanism of leukemogenesis as found for avian leukosis virus-induced tumors in chickens (22). In this report we show that in all 46 DMBA-LV-induced thymic lymphomas examined the apparent nonrandom integration of new MMTV proviruses (one to four copies) could be readily demonstrated.

MATERIALS AND METHODS

Mice. CFW/D mice, originally obtained from Carworth Farms, New City, N.J., and inbred by strict brother × sister mating for more than 80 generations, were used as the principal strain. Skin grafting and bone marrow transfer experiments indicated that the mice were syngeneic (2). NIH Swiss mice were originally obtained as an outbred pregnant female from the National Institutes of Health, Bethesda, Md., and maintained by brother × sister mating for ca. 4 years before use in these experiments. C57BL/Ka and C3H/Bi/Ka mice were kindly provided by H. S. Kaplan. GR mice were obtained from V. Morris, Department of Microbiology, University of Western Ontario, London, Ontario, Canada.

Tumor induction. The source of DMBA-LV for thymic lymphoma induction was the unconcentrated tissue culture medium (Eagle minimal essential medium plus 10% heat-

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inactivated fetal calf serum [Animal Health Laboratories, Toronto, Ontario, Canada]) in which the thymic lymphoma cells had grown. The origin and properties of these cell lines have been described (1, 4).

The virus (0.02 ml) was irradiated with 5,000 rad (4) before being injected directly into the thymuses of mice between 1 and 3 days old. Karyotypic analysis with a chromosomal marker (4; unpublished data) of many tumors induced with such viral preparations (with or without irradiation) has shown that they did not result from tumor cells present in the tissue culture media.

Extraction of high-molecular-weight DNA. Extraction of high-molecular-weight DNA was performed essentially as described previously (8), except that the RNase digestion and the subsequent extraction and dialysis were omitted. The size-selection step was also omitted. All DNA concentrations were determined by the diphenylamine assay (6).

Synthesis of virus-specific DNA probes. ^{32}P-labeled MMTV DNA was prepared by nick translation (33) of molecularly cloned MMTV DNA (27), kindly provided by J. E. Majors. MMTV DNA prepared by this procedure lacked the small *Pst*I fragment (poison sequences) as described previously (27). Two molecularly cloned fragments of the MMTV genome, one corresponding to the long terminal repeat region and one representing the 3′ *env* gene, kindly provided by J. E. Majors and labeled with ^{32}P by nick translation (33) were also used as probes for MMTV. Viral probes were labeled with both [^{32}P]dCTP and [^{32}P]dATP to ensure that the label was distributed in a representative way throughout the viral genome. The specific activity of all probes used was between 1×10^8 and 3×10^8 cpm/μg of DNA.

Restriction endonuclease digests of high-molecular-weight DNA. Restriction endonucleases *Eco*RI and *Kpn*I were obtained from Bethesda Research Laboratories, Bethesda, Md., Boehringer Mannheim Biochemicals, Indianapolis, Ind., supplied *Pst*I and *Hin*dIII, and New England Biolabs, Beverly, Mass., supplied *Sac*I. All digestions were done under the conditions recommended by the supplier of the particular enzyme. Digestions of high-molecular-weight cell DNA (or lambda DNA) were done as described previously (10); between 5 and 20 μg of cell DNA was used, and digestion was done with a two- to fourfold excess of restriction endonuclease (i.e., 2 to 4 U of endonuclease per μg of DNA). The completeness of digestion was monitored as described previously (10) and by including a DNA sample, such as normal organ DNA, from GR mice whose restriction pattern was already known. DNA from GR mice was chosen because it contains as an endogenous MMTV provirus an internal fragment which is associated with a high mammary tumor incidence and which, when digested with *Pst*I, yields two fragments of molecular sizes 4.1 and 1.1 kilobases (kb) (11).

Isolation and purification of cytoplasmic unintegrated retroviral DNA. Isolation and purification of cytoplasmic unintegrated retroviral DNA were done by the method of Kung et al. (26).

Southern (DNA) transfers and hybridization conditions. Southern transfer was performed essentially as described previously (35). To rehybridize blots with different DNA probes, the blots were treated as described by Thomas (36).

After separation of the DNA fragments on a 0.7% horizontal agarose gel and transfer to nitrocellulose filters, the filters were prehybridized in a solution containing 50% formamide, 3× SSC (1× SSC: 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll [both from Sigma Chemical Co., St. Louis, Mo.]), 200 μg of yeast RNA per ml (Sigma), and 20 μg of salmon sperm DNA per ml (Sigma) for 16 to 18 h at 42°C. The filters were then hybridized in a solution containing 50% formamide, 3× SSC, 3× Denhardt solution, 200 μg of yeast RNA per ml, 20 μg of salmon sperm DNA per ml, and the appropriate ^{32}P-labeled viral DNA. Hybridizations were carried out at 42°C for 48 h. After hybridization, the filters were washed in decreasing concentrations of SSC (2× SSC for 1 h at room temperature, 0.1× SSC plus 0.1% sodium dodecyl sulfate for 3 h at 50°C, followed by three brief washes in 0.1× SSC). The dried filters were autoradiographed on Du Pont Cronex 4 X-ray film.

All DNA fragment sizes referred to in the text are based on measurements made from the 5′ end of the genome. The restriction map for the unintegrated linear DMBA-LV MMTV DNA with the four restriction endonucleases used in this study is shown in Fig. 1, along with the MMTV map location of the 3′ *env* MMTV DNA probe used.

RESULTS

Infection of lymphoma cells by the MMTV component of DMBA-LV. To study the role of DMBA-LV MMTV in virus-induced thymic lymphomas, it was first necessary to determine whether the DMBA-LV MMTV genome could be distinguished from any endogenous proviral MMTV copies present in the DNA from normal CFW/D tissues and whether virus-induced tumors contained an increase in the number of copies of proviral MMTV DNA. *Pst*I and *Sac*I were chosen for this.

It has previously been shown (11) that the *Pst*I cleavage pattern of unit IV of endogenous MMTV DNA is identical to that of proviruses acquired by infection with milk-borne MMTVs. Both genomes yielded two *Pst*I cleavage fragments. The sizes of these fragments were determined by

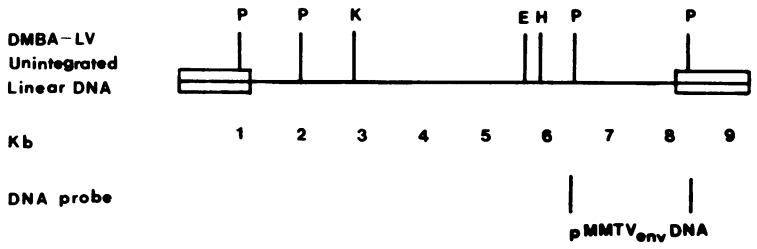

FIG. 1. Restriction map of the unintegrated linear DMBA-LV MMTV provirus showing the cleavage sites for the four restriction endonucleases, *Pst*I (P), *Eco*RI (E), *Hin*dIII (H), and *Kpn*I (K) used in this study. Also shown is the map location of the 3′ *env*-specific DNA probe (pMMTV *env* DNA). Long terminal repeats are indicated by the boxes.

Cohen and Varmus (11) to be 2.5×10^6 and 0.9×10^6 daltons, by Dudley and Risser (16) to be 4.5 and 1.0 kb, and by the present study to be 4.1 and 1.1 kb (Fig. 1 and 2). In strains of mice such as C57BL/Ka, whose endogenous proviruses lack these two unit IV-derived *Pst*I restriction fragments (see Fig. 7A, lane 10), the appearance of these bands in tumor DNA can be used as evidence of infection (see Fig. 7A, lanes 8 and 9). In mouse strains such as NIH Swiss and C3H/Bi/Ka, which contain a provirus with the 4.1-kb fragment, the amplification of this DNA fragment can be taken as evidence of viral infection (see Fig. 7A; compare the 4.1-kb fragment in lanes 1 to 3 with lane 4 and lanes 5 and 6 with lane 7). These latter two strains, although having the 4.1-kb fragment, lack the 1.1-kb fragment (see Fig. 7A; compare lanes 1 and 3 with lane 4 and lanes 5 and 6 with lane 7 at the fragment indicated by the double arrows), as has also been observed in other mouse strains (16). Therefore, the presence of the 1.1-kb fragment and the amplification of the 4.1-kb fragment in the DNA of the tumors induced in these two strains can be taken as evidence of viral infection.

Digestion of DNA from normal thymuses of CFW/D mice with *Pst*I (Fig. 2, lane C) yielded a restriction pattern similar to that found for DNA from normal tissues of the C57BL/Ka mouse strain (which lacks the 4.1- and 1.1-kb *Pst*I fragments)

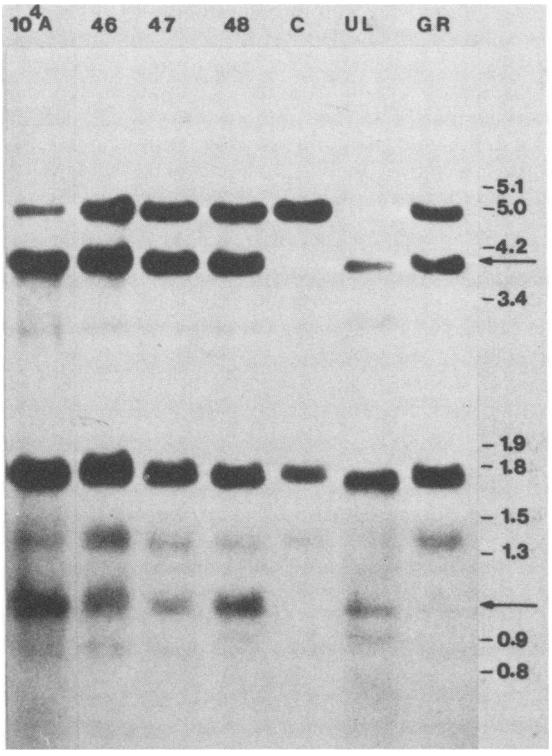

FIG. 2. *Pst*I digestion of DNA (10 μg per sample) from normal and tumor tissues from CFW/D mice. Digested DNA was size-fractionated on a 0.7% agarose gel, transferred to nitrocellulose paper, and hybridized with 2×10^6 to 3×10^6 cpm of nick-translated, ^{32}P-labeled pMMTV DNA. Lanes contained (left to right) DNA from DMBA-LV-induced primary tumors 10^4A, 46, 47, and 48, normal thymus DNA (lane C), unintegrated linear MMTV DNA isolated from the virus-producing tumor cell line 485-10 (lane UL), and DNA from normal kidneys from the GR mouse strain (lane GR). The molecular sizes shown on the right (in kb) are based on DNA molecular weight marker III (Boehringer Mannheim). The positions of the 4.1- and 1.1-kb fragments are indicated by arrows.

but different from that of the DNA from normal tissues of GR mice, which have been shown (11) to contain an endogenous MMTV provirus containing the unique unit IV *Pst*I fragments (Fig. 2, lane GR). Since CFW/D mice lack an endogenous provirus containing the unit IV MMTV DNA, the appearance of these *Pst*I digestion products in the DNA from virus-induced primary thymic lymphomas (Fig. 2, lanes 1 to 4) indicated that the tumor cells had been infected by an exogenous type of MMTV present in DMBA-LV. *Pst*I digestion of the unintegrated linear DNA present in the virus-producing tumor cell line corresponding to the DMBA-LV MMTV did in fact yield the two unique unit IV fragments (Fig. 2, lane UL). If an exogenous type of MMTV is present in CFW/D mice, it could not be detected in normal lymphoid tissues of mice 3 days to 8 weeks old. Furthermore, if CFW/D mice are infected with an exogenous MMTV, then the infected cells must represent less than 1% of the total cell population in any organ examined (the limit of detection of exogenous viral sequences by the experimental procedure used; J. K. Ball, unpublished data).

The appearance of the 4.1- and 1.1-kb *Pst*I fragments in the DNA from all 39 individual primary thymic lymphomas examined (data not shown) suggested that there had been an infection by the exogenous MMTV component of DMBA-LV and that the DMBA-LV MMTV component contained *Pst*I cleavage fragments indentical to those present in MMTV from C3H and GR mice [MMTV(C3H) and MMTV(GR)]. Digestion of both exogenous and endogenous MMTV proviruses with *Sac*I released a large internal 7.7-kb fragment. The amplification of this 7.7-kb fragment in the DNA of all 39 primary thymic lymphomas (data not shown) confirmed that infection by DMBA-LV MMTV had occurred.

The 39 primary thymic lymphomas induced in CFW/D mice by DMBA-LV were examined for the presence of unintegrated linear MMTV DNA. This was done by Southern analyses of the uncut DNA from each of the tumors. The DNA fragment longer than 22.1 kb represents MMTV-containing uncleaved tumor DNA, whereas the MMTV-containing DNA fragment which always migrated as a 9.3-kb fragment corresponds to unintegrated linear MMTV DNA (Fig. 3). The variation in the amounts of unintegrated linear MMTV DNA present in different tumors (Fig. 3) can contribute to the differences in the intensity of the restriction fragments. Circular forms of MMTV DNA were not detected in any of the 39 tumors induced in CFW/D mice or in the two virus-induced tumor cell lines. The reason for this is not known, but similar findings have also been reported (24) for Moloney murine leukemia virus-induced thymic lymphomas. The absence of circular forms of MMTV DNA may indicate that significant reinfection of the tumor cells did not occur. The unintegrated linear DNA may result from intracellular transcription of viral RNA in intracytoplasmic A particles known to be present in DMBA-LV-induced tumors (3).

Characterization of new DMBA-LV MMTV integration sites in CFW/D thymic lymphomas. The presence of unintegrated linear MMTV DNA in the tumors raised the question of whether the presence and amplification of internal *Pst*I and *Sac*I cleavage fragments observed resulted solely from the unintegrated linear DNA or whether the integration of DMBA-LV MMTV had also occurred. To examine this question further, tumor DNA was digested with three different restriction endonucleases, each of which had been shown to cleave the oncogenic MMTV(C3H) and MMTV(GR) genomes only once (20). Because digestion with

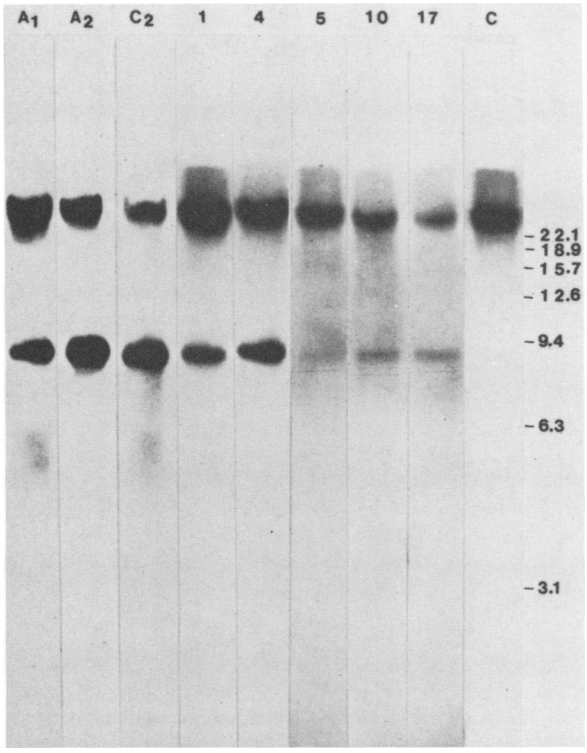

FIG. 3. Unintegrated linear MMTV DNA present in eight DMBA-LV-induced primary thymic lymphomas in CFW/D mice. Uncut high-molecular-weight DNAs (10 μg per sample) from tumors A_1, A_2, C_2, 1, 4, 5, 10, and 17 are shown. The last lane (C) contained uncut DNA from a normal thymus. After size fractionation and Southern transfer the blot was hybridized with 2×10^6 to 3×10^6 cpm of ^{32}P-labeled pMMTV DNA. The molecular sizes (shown on the right in kb) are based on DNA molecular weight marker IV (Boehringer Mannheim).

these enzymes results in DNA-containing fragments consisting of a portion of the MMTV genome plus adjacent cellular sequences, they can be used to locate the position and number of newly acquired MMTV proviruses in MMTV-induced tumors. This experimental approach was possible because digestion of DNA from DMBA-LV-induced tumors yielded *Pst*I and *Sac*I fragments which were of the same size classes as those generated by the digestion of unintegrated linear MMTV DNA (Fig. 2; unpublished data), which has been shown to be colinear with MMTV viral RNA (11, 20, 34).

To assess the number of endogenous proviral MMTV copies in the DNA of normal CFW/D mouse tissues, DNA was extracted from thymus tissue of normal CFW/D mice and digested with a number of restriction endonucleases known to cleave the MMTV genome at a single site. The results (Fig. 4) indicated that cleavage of normal thymus DNA with *Eco*RI (Fig. 4A), *Kpn*I (Fig. 4B), or *Hin*dIII (Fig. 4C) yielded six MMTV-containing DNA fragments (lane 2 of each panel) when DNA representing the complete MMTV genome was used as a hybridization probe. The MMTV-containing DNA fragment of 11.2 kb (Fig. 4B, lanes 2 and 4) derived from normal thymus DNA after cleavage with *Kpn*I consisted of two fragments of identical size, one detected with pMMTV *env* DNA (Fig. 4, lane 4) and one detected with pMMTV *gag* DNA (data not shown). Thus, there are three endogenous MMTV proviruses present in CFW/D mice. Hybridization of the same blots with a DNA probe representative of the 3′ *env* region of the genome yielded the fragments shown in the fourth lane of each of the panels. It should be noted that when this DNA probe was used after *Eco*RI digestion, the 6.1-kb endogenously derived MMTV-containing fragment (panel A, lane 4) was virtually the same size as the 5′ *Eco*RI fragment derived from unintegrated linear MMTV DNA (Fig. 4A, lane 1). Thus, the *Eco*RI fragment of 6.1 kb generated from all the tumor DNAs after *Eco*RI digestion and hybridization with pMMTV *env* DNA represents an endogenous MMTV-derived fragment. Hybridization of *Eco*RI-cleaved unintegrated linear MMTV DNA with pMMTV *env* DNA yielded a single 3.6-kb fragment (Fig. 4A, lane 3). A similar situation was found after digestion with *Kpn*I (Fig. 4B, lanes 1 to 4). The largest fragment derived from unintegrated linear MMTV DNA (6.2 kb) and originating from the 3′ *env* region of the genome (Fig. 4B, lane 3) migrated as a doublet, with the smallest fragment (6.3 kb) derived from endogenous MMTV provirus when hybridized with pMMTV DNA (Fig. 4B, lane 2). Digestion of normal DNA and unintegrated linear MMTV DNA with *Hin*dIII (Fig. 4C, lanes 1 to 4) yielded MMTV-containing DNA fragments of sufficiently different size classes as to clearly indicate their origin.

A total of 39 primary thymic lymphomas induced in CFW/D mice by DMBA-LV were examined for the presence of newly acquired proviral MMTV DNA. To ensure that MMTV-containing DNA fragments of a similar size class were derived from the same end of the MMTV genome, the hybridization probe used was one representing the 3′ end of the viral genome (27). In all of the primary tumors examined, new MMTV DNA-containing fragments (in addition to those representing the three endogenous MMTV proviruses) were detected. In virtually all tumors, an MMTV DNA-containing fragment derived from unintegrated linear MMTV DNA was detected (see Fig. 5A to C for the fragment indicated by the arrow and the corresponding fragments in Fig. 4A to C, lanes 3).

In addition to these shared DNA fragments, certain tumors appeared to share other restriction endonuclease fragments of a similar molecular weight when digested with *Eco*RI (Fig. 5A), *Kpn*I (Fig. 5B), or *Hin*dIII (Fig. 5C). It was necessary to assume that the MMTV-cell DNA fragments generated by each of the three different restriction endonucleases used would not necessarily be the same size. Therefore, if tumors shared *Eco*RI fragments of the same size class, we would expect these tumors to have *Kpn*I-generated fragments with similar sizes (but different from those found by *Eco*RI cleavage). Similarly, the same tumors should have *Hin*dIII fragments of the same size, but these would most likely differ in size from those detected after *Eco*RI or *Kpn*I digestion. Tumors which share *Eco*RI fragments of the same size class (for example, tumors 6 and 9 share a common size fragment), *Kpn*I fragments of a common size class, and a common size *Hin*dIII fragment are shown in Fig. 5. A comparison of the fragment sizes generated from the DNA of tumors 14 and 16 with the same three enzymes again indicated that certain tumors shared the same size of MMTV-containing DNA fragments.

To determine how many tumors shared certain virus-cell DNA fragments of a common size class, each of the 39 tumors was digested with *Eco*RI, and those MMTV-containing fragments of size classes which could be unambigously resolved by gel electrophoresis were determined. Table 1 lists all the tumors which shared at least two fragments of a particular size class. Excluded from the data are fragments

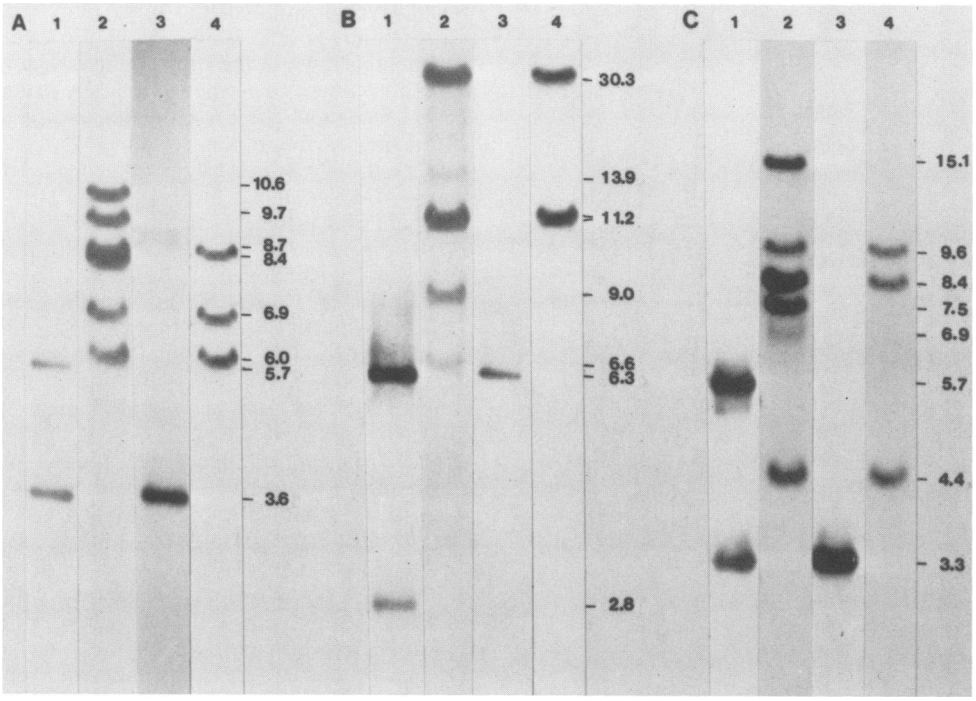

FIG. 4. Digestion of cell DNA (10 μg per sample) from normal thymuses from 4-week-old CFW/D mice and unintegrated linear MMTV DNA (1 μg) by *Eco*RI (A), *Kpn*I (B), and *Hin*dIII (C). Digested DNA was size-fractionated on a 0.7% agarose gel, transferred to nitrocellulose paper, and hybridized with 2×10^6 to 3×10^6 cpm of ^{32}P-labeled pMMTV DNA (lanes 1 and 2, each panel) or 6×10^6 to 8×10^6 cpm of ^{32}P-labeled pMMTV *env* DNA (lanes 3 and 4, each panel). The molecular sizes (in kb) of the resulting MMTV-containing DNA fragments derived from endogenous MMTV proviruses or unintegrated linear MMTV DNA are shown on the right and are based on DNA molecular weight markers III and IV (Boehringer Mannheim).

of size classes which overlapped or which were not clearly separated from fragments derived from the endogenous MMTV proviruses. From the results it is clear that fragments of certain size classes were present in more than one tumor. This was confirmed by pooling 0.2 μg of DNA from all 39 tumors and showing (Fig. 6) that digestion with three different restriction endonucleases resulted in the appearance of approximately six to eight new MMTV-containing fragments.

We attempted to look for the integration of new type C retroviral genomes in the same primary tumors. Although we used three different viral probes (one representing the Moloney murine leukemia virus genome, one representing the retroviral type C long terminal repeat, and one representing

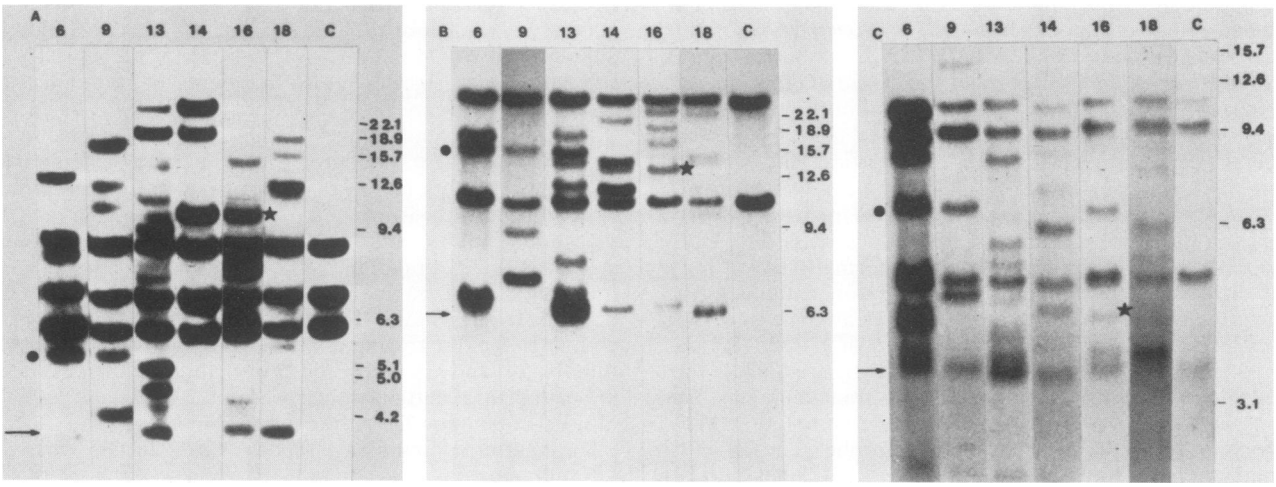

FIG. 5. Digestion of representative primary thymic lymphomas from CFW/D mice with *Eco*RI (A), *Kpn*I (B), and *Hin*dIII (C). Lanes contained DNA from tumors 6, 9, 13, 14, 16, and 18 and normal thymus DNA (lanes C). The Southern blots were hybridized with ^{32}P-labeled pMMTV *env* DNA. The molecular sizes (in kb) shown on the right of each panel are based on DNA molecular weight markers III and IV (Boehringer Mannheim). Arrows, MMTV DNA fragments derived from the 3′ end of the unintegrated linear MMTV genome; ●, MMTV-containing DNA fragment of a common size class from tumors 6 and 9; ★, fragment of a common size class in tumors 14 and 16.

TABLE 1. Summary of data from 39 primary thymic lymphomas sharing 3′ *env* MMTV-cell DNA fragments of a common size class[a] after cleavage with *Eco*RI

Fragment size (kb)	No. of tumors sharing fragment
6.8	5
7.8	9
8.5	4
9.8	8
12.5	8
15.5	3
18.0	2

[a] Only MMTV-containing DNA fragments of size classes clearly distinguishable from those originating from endogenous MMTV proviruses and from unintegrated linear MMTV DNA are included in the data.

the xenotropic-polytropic [recombinant] *env* region of the genome), we were unable to detect integration of any new type C retroviral proviruses (J. K. Ball et al., unpublished data).

Analyses of thymic lymphomas induced by DMBA-LV in other mouse strains. Since DMBA-LV was capable of inducing thymic lymphomas in NIH Swiss, C3H/Bi/Ka, and C57BL/Ka mice (4), it was of interest to see whether newly integrated DMBA-LV MMTV proviruses were present in the DNA of virus-induced primary tumors in these mouse strains. *Pst*I was used to cleave DNAs from thymic lymphomas and normal kidney and thymus tissue from these three strains of mice (Fig. 7A). The NIH Swiss and C3H/Bi/Ka mouse strains had the 4.1-kb fragment but lacked the 1.1-kb MMTV DNA fragment as a part of their endogenous MMTV proviruses (Fig. 7A, lanes 4 and 7). However, in each of the lymphomas there was a clear amplification of the 4.1-kb tumor-specific fragment (Fig. 7A, lanes 1 to 3, 5, and 6), and the 1.1-kb fragment was present. The C57BL/Ka normal mouse DNA lacked the 4.1- and 1.1-kb fragments, but both fragments were clearly detectable in the lymphoma cell DNAs (Fig. 7A, lanes 8 and 9). Furthermore, hybridization with an MMTV long terminal repeat DNA probe (Fig. 7B) confirmed the presence of the 1.1-kb fragment in the tumors and also showed that the DMBA-LV-induced thymic lymphomas contained new MMTV proviruses. These results were confirmed by showing that when the same normal and tumor cell DNAs were digested with *Sac*I there was a marked amplification of the 7.7-kb MMTV DNA fragment (data not shown).

Digestion of the normal and tumor cell DNAs from DMBA-LV-induced thymic lymphomas in NIH Swiss, C3H/Bi/Ka, and C57BL/Ka mice with *Eco*RI and *Hin*dIII (Fig. 8) indicated that integration of newly acquired MMTV proviruses had occurred. Since only two or three tumors per strain were examined, it was not possible to determine how

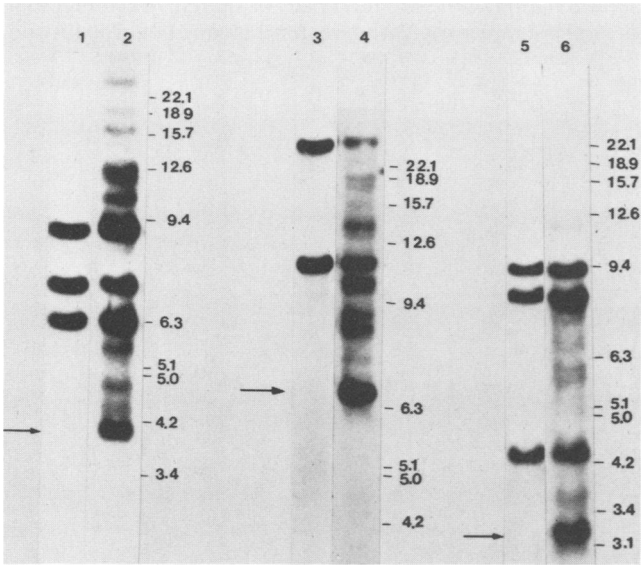

FIG. 6. Discrete new MMTV-containing fragments in the pooled DNAs (0.2 μg per tumor) from 39 DMBA-LV-induced primary thymic lymphomas (from CFW/D mice). Pooled DNAs (lanes 2, 4, and 6) and normal thymus DNA (lanes 1, 3, and 5) were digested with *Eco*RI (lanes 1 and 2), *Kpn*I (lanes 3 and 4), or *Hin*dIII (lanes 5 and 6). After size fractionation and Southern transfer, the blots were hybridized with 6×10^6 to 8×10^6 cpm of pMMTV *env* DNA. Molecular sizes (in kb) shown on the right are based on DNA molecular weight markers III and IV (Boehringer Mannheim). Arrows, MMTV DNA fragments derived from the 3′ end of the unintegrated linear MMTV genome.

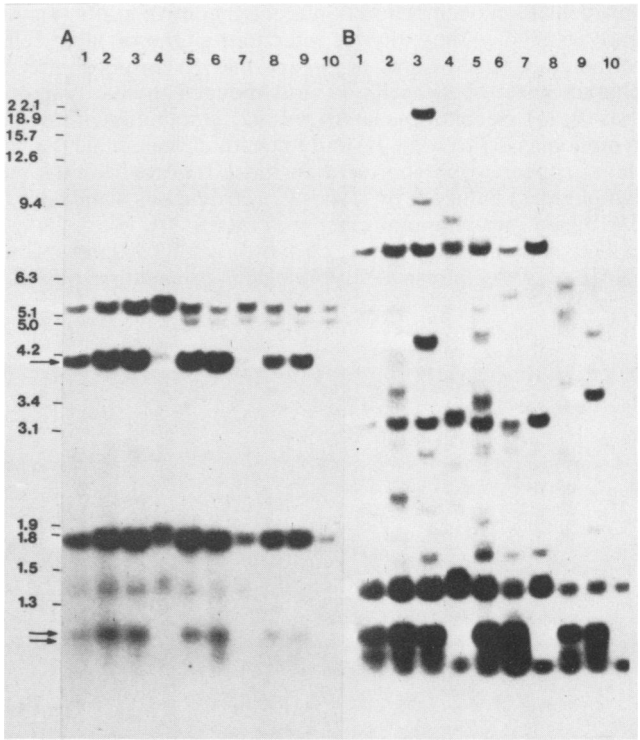

FIG. 7. *Pst*I digestion of 7.5 μg of DNA from normal tissue and DMBA-LV-induced primary thymic lymphomas from NIH Swiss, C3H/Bi/Ka, and C57BL/Ka mice. After size fractionation and Southern transfer, the blot was hybridized first with 2×10^6 to 3×10^6 cpm of ^{32}P-labeled pMMTV DNA (A) and then with 6×10^6 to 8×10^6 cpm of pMMTV long terminal repeat (B). Lanes: 1 to 3, DNA from NIH Swiss lymphomas SpC5, SpC6, and SpC7, respectively; 4, normal thymus DNA from NIH Swiss mice; 5 and 6, DNA from C3H/Bi/Ka thymic lymphomas 1 and 2, respectively; 7, normal C3H/Bi/Ka thymus DNA; 8 and 9, DNA from C57BL/Ka thymic lymphomas 1 and 2, respectively; 10, normal C57BL/Ka thymus DNA. Molecular sizes (in kb) shown are based on DNA molecular weight markers III and IV (Boehringer Mannheim). The positions of the 4.1- and 1.1-kb fragments are indicated by arrows.

many virus-cell DNA junction fragments of specific size classes were shared by more than one tumor. However, of the ca. 20 new MMTV-cell DNA junction fragments detected after cleavage with *Eco*RI in the seven tumors from the three different strains, 10 shared a common size with *Eco*RI-generated, MMTV-containing fragments derived from tumors induced by DMBA-LV in CFW/D mice. The tumors induced in the three strains also had common MMTV-containing DNA fragments (less than 4.1 kb in size) which most likely arose from the unintegrated linear DNA which was detected in every lymphoma of each of the three mouse strains examined.

DISCUSSION

DMBA-LV is a highly leukemogenic virus which contains two unrelated viral genomes, an exogenous MMTV related to the MMTV(C3H) isolate and an apparently biologically defective type C retroviral genome. Furthermore, there are several features in the development of thymic lymphomas induced by DMBA-LV which differ from those described for the induction of thymic lymphomas by type C retroviruses. First, unlike Moloney murine leukemia virus (1) or radiation leukemia virus (21), DMBA-LV is not thymolytic. In fact, intrathymic injection of DMBA-LV resulted in an earlier repopulation of intrarenal thymic grafts by host bone marrow cells than occurred in noninfected control grafts (4). As early as 30 days post-thymic infection, all the dividing cells in the graft were of host origin and had 41 chromosomes, a characteristic of the cells of virus-induced thymic lymphomas (9, 15). Second, the latent periods to detection of thymic lymphomas (35 to 45 days) and to death of the animal (ca. 60 days) appear to be shorter than those reported for thymic lymphomas induced by type C retroviruses, and third, DMBA-LV was nontumorigenic in rats.

The separation of the retroviral genomes present in DMBA-LV by physical or biological procedures has not been possible. Therefore, in an attempt to understand which of the viral genomes is responsible for the biological properties of DMBA-LV, in vivo infection was assessed by looking for the presence, in the DNA of tumor cells, of newly integrated proviral MMTV DNA corresponding to the MMTV genome in DMBA-LV.

On the basis of the data presented, it is clear that the MMTV genome present in DMBA-LV was present in the DNA of all 46 thymic lymphomas examined. Using the restriction endonuclease *Pst*I, we could clearly distinguish between fragments derived from the three endogenous MMTV proviruses present in CFW/D mice and fragments derived from the exogenous MMTV present in DMBA-LV. Two new tumor-specific unit IV *Pst*I fragments of 4.1 and 1.1 kb were present in the DNA of all 39 thymic lymphomas induced in CFW/D mice. These two tumor-specific fragments were identical in size to the tumor-specific DNA bands found in mammary tumors induced by the highly tumorigenic milk-borne MMTV variants (11, 12). Digestion with *Sac*I also revealed the marked amplification of one internal MMTV fragment in the DNAs of all the DMBA-LV-induced lymphomas examined. This fragment is also found in mammary tumors induced by milk-borne MMTV (11, 17).

Digestion of thymic lymphoma cell DNA with *Eco*RI, *Kpn*I, or *Hin*dIII yielded information on the number and location of the sites of the new MMTV retroviral proviruses. New DMBA-LV proviral MMTV integration sites were detected in the DNA from all 46 thymic lymphomas examined. Although there was no evidence of a single specific integration site for DMBA-LV MMTV, there was evidence suggesting that there may be a small number of preferred integration sites since a large number of thymic lymphomas contained provirus-cell DNA junction fragments of identical sizes. Although these types of analyses do not permit a determination of the exact number of shared viral integration sites, the number is clearly greater than one but probably not

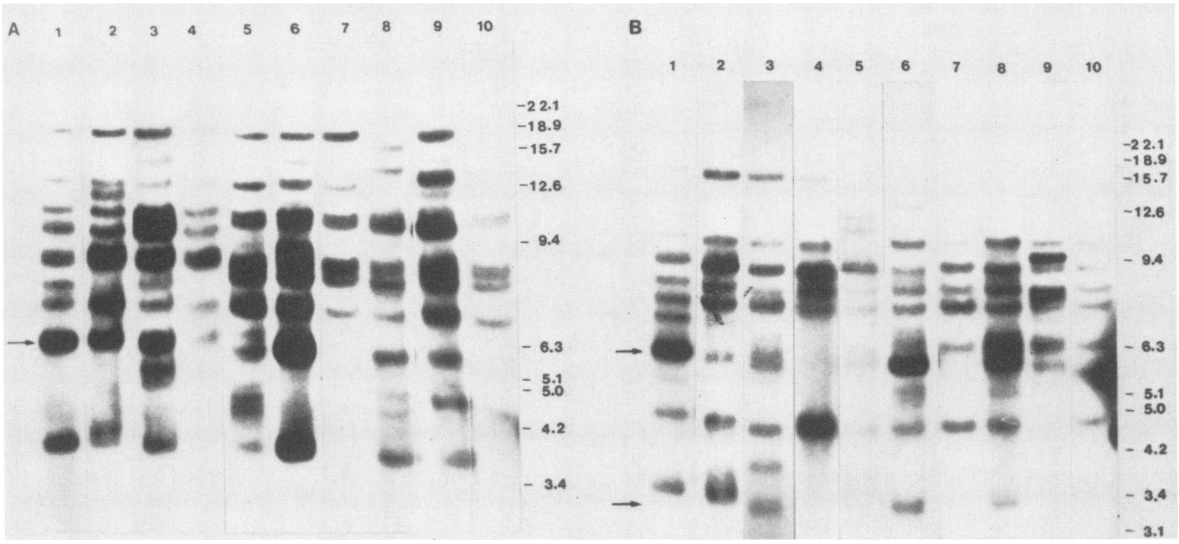

FIG. 8. *Eco*RI and *Hin*dIII digestion of 7.5 μg of DNA from normal tissues and DMBA-LV-induced thymic lymphomas from three strains of mice. Lanes: 1 to 4, DNA from NIH Swiss tumors SpC5, SpC6, SpC7, and normal thymus, respectively; 5 to 7, DNA from C3H/Bi/Ka tumors 1 and 2 and normal thymus, respectively; 8 and 9, DNA from C57BL/Ka tumors 1 and 2, respectively; 10, normal C57BL/Ka thymus DNA. The blot was hybridized with 2×10^6 to 3×10^6 cpm of ^{32}P-labeled pMMTV DNA after Southern transfer. Molecular sizes (in kb) shown are based on DNA molecular weight markers III and IV (Boehringer Mannheim). The arrow on the left of panel A indicates the larger MMTV DNA fragment derived from unintegrated linear DNA (the duration of the gel run was such that the smaller-size fragment [2.80 kb] ran off the gel). The arrows on the left of panel B indicate the two MMTV DNA fragments derived from unintegrated linear MMTV DNA.

in excess of five to eight, as indicated from the maximum number of fragments detected in each of the groups of pooled tumors (Fig. 6) after digestion with each of three different restriction endonucleases.

In virus-induced mammary tumors it has been found that there are at least two large regions of cellular DNA in which new MMTV proviruses are integrated (30, 31). For one of these regions, defined as *int*1 (30), it appears that there are five different sites around which new MMTV proviruses cluster. Our finding that there appears to be a small number of apparently shared integration sites for DMBA-LV MMTV is therefore not unique. What appears to be different in our system is that the nonrandom integration events are directly detectable in the virus-induced thymomas by use of a viral DNA probe. This may suggest that the region in which DMBA-LV MMTV integrates is smaller (perhaps because of the different target cell[s] involved), so that the chance of directly detecting these sites with a viral DNA probe is more probable. Until cloning and characterization of the cellular flanking sequences are complete, it will not be known whether the apparently nonrandom integration of DMBA-LV MMTV implies that the virus is able to promote an oncogene downstream from the site of integration (18, 22) or whether the virus has integrated into a region of the genome containing enhancer sequences (30).

The results of the present study are in contrast to those reported (28) for the presence of new MMTV proviruses in spontaneous thymic lymphomas appearing in 18- to 24-month-old male GR mice. In these studies the integration of many new copies of MMTV proviruses was detected. No apparent selectivity in the site of integration was found. Recently it was reported that certain transplanted T-cell lymphomas contained amplified and rearranged MMTV proviruses (16). Interestingly, in the independently transplanted T-cell tumor lines there were multiple new sites containing MMTV proviruses; these sites were highly conserved within a given mouse strain. It is not known whether any of the common MMTV integration sites found in the transplanted cell lines share homology with the apparently common integration sites detected in the primary T-cell thymic lymphomas described in the present study.

The apparent ability of DMBA-LV MMTV to induce leukemia, as well as the fact that mouse strains with a low incidence of mammary tumors contain small numbers of endogenous MMTV proviruses (which can be distinguished from exogenous, highly tumorigenic proviruses), provides a unique model system with which to study the mechanism(s) involved in the induction of thymic lymphomas. Such studies of leukemogenic type C retroviruses in mice have been made extremely difficult because of the presence of so many copies (15 to 25) of endogenous type C proviruses.

Thus, on the basis of all our available data (3; this report), as well as our finding that the leukemogenicity of DMBA-LV can be completely neutralized by a monoclonal antibody directed against the major glycoprotein (gp52) of MMTV(C3H) (Ball et al., submitted), we conclude that a retrovirus highly related to a tumorigenic type B retrovirus can induce leukemia. More precise information on the distribution and characterization of the genome integration sites for DMBA-LV MMTV will come from the studies in progress with molecularly cloned MMTV-cell DNA junction fragments.

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