Molecular Biological Characterization of a Highly Leukaemogenic Virus Isolated from the Mouse. III. Identity with Mouse Mammary Tumour Virus

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SUMMARY

A highly leukaemogenic virus isolate (DMBA-LV) endogenous to the CFW/D mouse has been found to contain two viral genomes. One was closely related to the type B milk-borne mouse mammary tumour virus (MMTV) and present in tenfold excess over a type C viral genome which was only partially related to xenotropic and polytropic isolates from the CFW/D mouse as well as to the ecotropic Moloney murine leukaemia virus isolate. The thymic lymphoma cell line that produced DMBA-LV expressed high levels of MMTV viral RNA (35S and the 24S envelope mRNA). Both the virus and the virus-producing cell line expressed multiple species of type C viral RNA. Similar species of type C viral RNA were also associated with non-infectious, non-leukaemogenic viral particles present in both normal lymphoid cells and in a MMTV-free thymic lymphoma cell line established from a second chemical carcinogen-induced tumour.

INTRODUCTION

A highly leukaemogenic virus (DBA-LV) which induces thymic lymphomas with an average latent period of 63 days (to death) was isolated from thymic lymphomas induced by 7,12-dimethylbenz[a]anthracene (DMBA) in CFW/D mice (Ball & McCarter, 1971, 1979). This virus is produced by DMBA-LV-induced tumours in the absence of any detectable xenotropic or ecotropic type C viruses that can grow in established tissue culture cell lines. The in vivo leukaemogenic titre of DMBA-LV has been calculated to be a minimum of $1 \times 10^6$ to $5 \times 10^6$ infectious units/ml (Ball, 1979).

There have been numerous reports of the presence of mouse mammary tumour virus (MMTV) in lymphoid tumours. The two most intensively studied cases of MMTV expression in lymphoma cells are those of Vaidya et al. (1980) on the transplanted DBA/2 lymphoma cell line and by Hilkens et al. (1980) on the spontaneous lymphomas in the GR mouse strain. In both studies the lymphoma cells were found to contain large clusters of intracytoplasmic A particles whose further maturation to biologically active MMTV was blocked as a result of a defect in the processing of both the gp52 envelope glycoprotein and gag precursor protein (Nusse et al., 1979; Vaidya et al., 1980).

The DBA/2 tumour cell line produced a low level of particles which, when injected into 4-week-old BALB/c mice, resulted in a low incidence of mammary tumours. The virus was not tested under conditions favourable for leukaemia induction. The virus produced by the DBA/2 lymphoma cell line was also shown to contain a low level of type C viral information whose identity and possible biological activity was not further assessed.

Hilkens et al. (1980) have reported that the appearance of spontaneous lymphomas in GR mice appears to be dependent on the presence of a germinal MMTV provirus at the Mtv-2 locus on chromosome 18 (Michalides et al., 1978). Virtually no thymic lymphomas were observed in...
the strain of GR congenic for Mtv-2; the latter strain has been shown to lack one proviral MMTV copy. However, foster nursing of Mtv-2⁻ mice on GR (Mtv-2⁺) mice restored the incidence of spontaneous lymphoma.

In the present study, the virus recovered from a chemical carcinogen-induced, transplanted thymic lymphoma in CFW/D mice has been shown to be highly leukaemogenic but did not cause mammary tumours. The virus consisted of two unrelated retrovirus genomes, an intact MMTV genome closely related to exogenous milk-borne MMTV isolates and a defective type C genome, partially related to xenotropic, ecotropic and polytropic retrovirus isolates. The type B retrovirus information is present in tenfold excess. The virus-producing tumour cell line contained MMTV RNA corresponding in size to the MMTV genome as well as a 24S RNA species of type C retrovirus RNA detected. The 33S RNA was the most abundant type C viral genome were also present (35S, 33S and 21S). The 33S RNA was the most abundant

METHODS

Mice. CFW/D mice, the principal strain used, were obtained originally from Carworth Farms, New City, N.J., U.S.A. and inbred by strict brother x sister mating for more than 80 generations. Skin grafting and bone marrow transfer experiments indicated that the mice were syngeneic (Ball & Dawson, 1969). BALB/c mice were obtained from the Jackson Laboratories, Bar Harbor, Me., U.S.A. NIH Swiss mice were obtained from the National Institutes of Health, Bethesda, Md., U.S.A.

Tumour induction. The source of DMBA-LV for thymic lymphoma induction was the unconcentrated tissue culture medium in which the virus-producing tumour cell line 485-10 was grown. The virus (0.02 ml) was injected directly into the thymus glands of 1- to 3-day-old mice.

Thymic lymphomas were induced by the carcinogen DMBA as previously described (Ball & McCarter, 1971).

Source of cell lines and virus. The source of all DMBA-LV was unconcentrated tissue culture medium (for biological studies) or concentrated virus (for molecular studies) obtained from the tissue culture medium (Eagle's minimum essential medium plus 10% heat-inactivated foetal calf serum) in which the thymic lymphoma cells were growing. The origin and properties of these cell lines have been described (Ball, 1979; Ball & McCarter, 1979).

MMTV (C,H) was kindly provided by the Viral Resources Center at the Frederick Cancer Research Facility, Frederick, Md., U.S.A.

The thymic lymphoma cell line DMT-10 was established from a DMBA-induced tumour from a CFW/D mouse. The sources of virus (DMT-10V) for biological and molecular studies were the same as those used to obtain DMBA-LV.

The 485-2 and 485-41 cell lines were established from thymic lymphomas induced in 1- to 3-day-old CFW/D mice by the intrathymic injection of virus (DMBA-LV) produced by the 485-10 tumour cell line. The two thymic lymphoma tissue culture cell lines NIH 1T2 and NIH 2T2 were established from tumours induced by the intrathymic injection of DMBA-LV in 1- to 3-day-old NIH Swiss mice.

The established cell line TB was derived from a mixed culture of thymus and bone marrow cells from CFW/D mice (Dekaban et al., 1980). Uninfected NIH 3T3 cells and Moloney murine leukaemia virus (Mo-MuLV) chronically infected JLS-V9 cells (MJD) were obtained from Dr K. Manly, and Dr V. Morris provided a GR mammary tumour cell line. Mink lung cells (CCL64) and MDCK (dog) cells were obtained from the American Type Culture Collection. The MMTV (C,H) tumour cell line was kindly provided by Dr J. Scholm, FG-10 cells, kindly provided by Dr P. J. Fischinger, are permissive for ecotropic and polytropic retroviruses (Fischinger et al., 1978).

The effect of dexamethasone stimulation on virus production was carried out by treating semi-confluent virus-infected cell cultures with 10⁻⁶ m-dexamethasone for 24 h prior to collecting a 24 h tissue culture harvest.

The original source of all Moloney viruses used was a tumour tissue extract (a gift from Dr J. B. Moloney) from a BALB/c mouse tumour induced by the Moloney murine sarcoma–leukaemia virus complex. Mo-MuLV was cloned free of the sarcoma virus (Mo-MuSV) as previously described (Ball et al., 1979).

Endogenous virus (xenotropic and VL30S) present in the TB cell line was induced with iododeoxyuridine (I UdR) (Sigma) as previously described (Ball et al., 1979).

Polytropic recombinant retroviruses were prepared by one of us (J. A. McCarter, unpublished results). These viruses (Pt-MuLV) are recombinant MuLVs derived from endogenous xenotropic or polytropic sequences of the CFW/D mouse and the ts, mutant of TB-derived Mo-MuLV. The recombinant polytropic viruses are no longer ts, and have acquired a xenotropic host range. The Pt-MuLVs also differ in nucleotide sequence homology from the parental ts TB Mo-MuLV by 15%. These Pt-MuLVs induced a low level of leukaemia when injected into newborn CFW/D mice.

Extraction and purification of viral RNA. Viral RNA was extracted according to previously published procedures (Ball et al., 1979).
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Extraction of total cellular RNA. To obtain a preparation of total cellular RNA, more than 10⁹ cells or approximately 200 mg of minced tissue were washed three times in phosphate-buffered saline. After the final wash the cells or tissue were resuspended in 5 vol. RNA alkaline buffer (0-025 M-EDTA pH 8.0, 0-075 M-NaCl, 0.5 % SDS, 5 mg/ml heparin, 0-1 % diethyl pyrocarbonate) (all volumes are with respect to the volume of cell pellet or weight of tissue). Next, 5 vol. alkaline buffer-saturated phenol were added and the mixture was homogenized in a Brinkman Polytron at full speed for 20 to 30 s. The homogenate was poured into a 30 ml Corex tube and centrifuged in a Sorvall SS34 rotor at 10000 rev/min for 10 min at 4 °C. The aqueous phase was removed from the protein interface and extracted with 5 vol. phenol: chloroform: isomyl alcohol (50:48:2). The phases were separated as above. The aqueous phase was removed and precipitated with 1/4 vol. 2 M-NaCl and 2-5 vol. 95 % ethanol overnight. The RNA precipitate was pelleted as above and separated as above. The aqueous phase was removed and precipitated with 2.5 vol. 95% ethanol overnight. The RNA precipitate was pelleted at 10000 rev/min for 30 min. The EDTA–sodium acetate precipitation was performed twice more, first for 30 min and then for 2 h. After the final precipitation, the RNA pellet was resuspended in 0-5 ml of alkaline buffer and digested with 25 µg/ml proteinase K (Boehringer Mannheim) for 30 min, the enzyme having been self-digested for 1 h at 37 °C before use. This was then extracted twice with 1 ml phenol: chloroform: isomyl alcohol (50:48:2). The final aqueous phase was made 0.25 M-NaCl and precipitated with 2.5 vol. 95% ethanol overnight. The RNA precipitate was pelleted at 10000 rev/min for 30 min. The concentration of RNA was determined by its absorbance at 260 nm.

Extraction and purification of poly(A) cellular RNA for Northern transfers. To obtain undegraded total poly(A) cellular RNA, the procedure of Strohman et al. (1977) was followed with the modification that 7 M-instead of 6 M-guanidinium hydrochloride was used.

Poly(A)-containing viral RNA was isolated as described by Wang et al. (1975) with the modification that NaCl was used in the binding buffer, instead of LiCl (Ball et al., 1979).

Northern RNA transfer. The electrophoresis and transfer of RNA to nitrocellulose was performed essentially as described by Thomas (1980). The viral DNA probes used to detect viral RNA were prepared using molecularly cloned Mo-MuLV (Bems et al., 1980) or MMTV (Majors & Varumus, 1981). The probes were labelled with [32P]dATP and [32P]dCTP by nick translation as described by Rigby et al. (1977).

Synthesis of virus-specific cDNA probes. The viral cDNA probe corresponding to the NIH 3T3 (MJD) Mo-MuLV was synthesized using detergent-disrupted virions as described by Rothenberg & Baltimore (1976). The cDNA probe representing the IUDR-induced virus from TB cells was prepared as described by Besmer et al. (1979) using detergent-disrupted virions in the presence of calf thymus primer (Taylor et al., 1976). DNA complementary to DMBA-LV was synthesized using detergent-disrupted virions in the presence of calf thymus primer DNA or with purified DMBA-LV viral RNA, calf thymus primer DNA and purified avian myeloblastosis virus (AMV) reverse transcriptase (a gift from Life Sciences Inc., St Petersburg Fla., U.S.A.) according to the method of Taylor et al. (1976) as previously described (Ball et al., 1979). Either method provided a cDNA that gave indistinguishable results. All other cDNAs were synthesized using purified viral RNA, calf thymus primer DNA and purified AMV reverse transcriptase (Taylor et al., 1976) as previously described (Ball et al., 1979). The representative nature of viral probes made using the calf thymus primer procedure was confirmed by showing that the MMTV 32P-labelled cDNA detected all the internal FstI fragments, which had the correct molecular weights characteristic of the intact MMTV genome when analysed using the Southern blot procedure. Since all other cDNA probes were made in an identical manner, they were assumed also to be representative. The self-priming activity of calf thymus DNA was always less than 5 %. The env gene-specific DNA was prepared (G. A. Dekaban, unpublished data) as a subclone in pBR325 of an EcoRI–BglII-derived fragment from the λ clone of a BALB/c xenotropic virus (Roblin et al., 1978) kindly provided by Drs R. Mural and J. I. Hale.

All DNA viral probes had a specific activity of 1 × 10⁸ to 2 × 10⁸ ct/min/µg for 32P-labelled probes and 1 × 10⁷ ct/min/µg for 3H-labelled probes.

**Nucleic acid hybridizations.** Each viral 3H-labelled cDNA (approx. 1000 ct/min per hybridization) was hybridized to excess vRNA in 2 × saline sodium citrate plus 0-1 % SDS, 1 mg/ml yeast RNA and 100 µg/ml calf thymus DNA. All hybridizations were carried out to a C t value of 20 mol/s at a RNA:DNA ratio of 400:1, at 68 °C. The hybridization values reported represent the maximum extent of hybridization as determined in a C t analysis. The extent of hybridization was assessed by measuring resistance to S1 nuclease as described by Leong et al. (1972). All the numerical values given in the text (except those recorded in Fig. 7) for the final extent of hybridization in heterologous reactions are values which have been normalized to the maximum extent of hybridization between a cDNA and its RNA homologue. Hybridizations to total cell RNA were performed at a cellular RNA concentration of 4 mg/ml with approximately 2000 ct/min of 3H-labelled cDNA in the presence of 0-01 M-Tris–HCl pH 7-4, 2 mM-EDTA at a NaCl concentration of 0-9 M. Hybridizations were carried out at 68 °C to C t values in excess of 1 × 10⁶. The extent of hybridization was assessed by measuring resistance to S1 nuclease. The number of copies of viral RNA found in tumour cells and normal tissue was calculated according to the method of Hayward et al. (1981).
RESULTS

Nucleotide sequence homology to type C ecotropic retroviruses

To determine the nucleotide sequence homology between DMBA-LV and a representative ecotropic virus, Mo-MuLV grown on NIH 3T3 was chosen as the prototype virus. The Mo-MuLV was chosen because of the availability of extensively characterized representative cDNA probes (Ball et al., 1979; Dekaban et al., 1980). Mo-MuLV grown on NIH 3T3 cells was used so that it would be free of any endogenous MuLV sequences characteristic of the CFW/D mouse (the strain of origin of DMBA-LV). Furthermore, NIH 3T3 cell DNA does not contain any endogenous ecotropic MuLV proviral DNA and therefore the only ecotropic MuLV released by Mo-MuLV-infected NIH 3T3 cells was Mo-MuLV (Chattopadyhay et al., 1980; Chan et al., 1980). Less than 0.1% VL30S RNA contaminated NIH 3T3 Mo-MuLV (Dekaban et al., 1980).

The ability of DMBA-LV vRNA to protect NIH 3T3 Mo-MuLV cDNA is shown in Fig. 1. There are two important features to note. First, the DMBA-LV vRNA protected only 43% (after normalization) of the NIH 3T3 Mo-MuLV cDNA, indicating either that they are not closely related or that the DMBA-LV type C vRNA is subgenomic in size. Secondly, the type C nucleotide sequences were not the most abundant set of sequences present in DMBA-LV because, if they had been, the 1/2 C, t values of the homologous Mo-MuLV C, t curve and the DMBA-LV–Mo-MuLV C, t curve should have been essentially the same, but they were not (1/2
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Ct of homologous Mo-MuLV hybridization = 2.0 × 10⁻²; 1/2 Ct of DMBA-LV–Mo-MuLV hybridization = 7.3 × 10⁻¹). Thus, the nucleotide sequence complexity of DMBA-LV was greater than that normally attributed to a homologous retrovirus species. The type C nucleotide sequences detected by an ecotropic cDNA probe represented only approximately 1/10 of the total DMBA-LV vRNA. Identical results were obtained with Mo-MuLV grown on TB cells (data not shown).

To determine whether the partial homology with Mo-MuLV was the result of a defective or deleted type C genome in DMBA-LV, two experiments were carried out. First, using cDNA probes specific for the env and gag-pol regions of the Mo-MuLV genome (Ball, 1979; Dekaban et al., 1980) it was found that DMBA-LV contained nucleotide sequences which were only 43% and 36%, respectively, related to the corresponding regions represented in the above two viral probes (data not shown). That the incomplete homology observed was not the result of DMBA-LV being composed of a deleted type C viral genome was confirmed in a second experiment in which the size of vRNA present in DMBA-LV was determined. Two size classes of type C viral genomes were detected (Fig. 2). DMBA-LV contained RNA of a size class corresponding to a complete viral genome (35S or 3.0 × 10⁶ daltons) as well as a subgenomic RNA size class (33S or 2.4 × 10⁶ daltons). The genetic relatedness of these two vRNA species is unknown.

Nucleotide sequence homology to type C xenotropic and polytropic retroviruses

The low nucleotide sequence homology between DMBA-LV and type C ecotropic viruses suggested that other classes of type C viral information [such as xenotropic (Xe) or polytropic (Pt)] may be present. The data of Fig. 1 (b) indicate that DMBA-LV vRNA protected the TB Xe cDNA by 59.9%. The 1/2 Ct value (3.0 × 10⁻¹) and the shape of the Ct curve (Fig. 3) are very similar to the 1/2 Ct and shape of the Ct curve of the DMBA-LV vRNA–Mo-MuLV cDNA...
hybridization (Fig. 1a), suggesting that a common set of nucleotide sequences was being detected. However, because the homology is incomplete, neither of the two species of type C vRNA detected in DMBA-LV can represent the complete TB-derived Xe viral genome. However, the increased protection of the TB Xe cDNA by DMBA-LV does indicate that the type C vRNA in DMBA-LV vRNA is more xenotropic than ecotropic in nature.

To test whether DMBA-LV contained nucleotide sequences characteristic of a recombinant Pt-MuLV, hybridization experiments were carried out using the Pt-MuLV strain 4506 isolated from the CFW/D mouse. The results are shown in Fig. 1 (c). DMBA-LV contains nucleotide sequences which are 63.9% homologous to nucleotide sequences present in Pt-MuLV 4506. This level of hybridization differs from that found between Mo-MuLV (the parental Mo-MuLV used to generate ts1) and DMBA-LV, suggesting that those sequences acquired by ts1 which resulted in its polytropic properties (as expressed by the isolate Pt-MuLV 4506) could be present in DMBA-LV. There was no nucleotide sequence homology between DMBA-LV and v-mos-specific information (data not shown).

The level of VL30S RNA (Howk et al., 1978) present in DMBA-LV 70S RNA was 0.001% (data not shown).

Nucleotide sequence homology to type B retroviruses

From previously published data on DMBA-LV (Ball & McCarter, 1979) there were two observations that suggested that additional types of virions may be involved. First, electron micrographs of thin sections through tumour cells producing DMBA-LV showed large clusters of intracytoplasmic type A particles. Second, SDS–polyacrylamide gel electrophoresis of [3H]leucine-labelled DMBA-LV showed a protein profile much more characteristic of type B retroviruses (MMTV) than of type C virions. These observations suggested that DMBA-LV might be a mixture of type B and type C retroviruses. To explore this possibility further, molecular hybridizations were carried out. The data of Fig. 2(a) showed that DMBA-LV vRNA was able to protect MMTV (C3H) by 90%, indicating that the MMTV present in DMBA-LV is more related to milk-borne MMTV than to endogenous MMTV [the latter being only 70 to 75% related to milk-borne MMTV (Michalides & Schlom, 1975; Ringold et al., 1976)]. From the 1/2 C1t value (3.0 x 10^-2) the type B nucleotide sequences can be calculated to be present in at least a tenfold excess over the type C sequences.

The reciprocal hybridization experiment (Fig. 2b) again demonstrated that MMTV (C3H) vRNA is capable of protecting 70% of the DMBA-LV cDNA. The 20% discrepancy in the level of hybridization is due to the demonstrated presence of type C vRNA nucleotide sequences
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(present at levels of 15 to 30%, depending on the class of type C retrovirus used for detection) in DMBA-LV.

There was no nucleotide sequence homology between the two viral isolates from Mus cervicolor, M432 and the type B MMTV (Callahan et al., 1976; Schlom et al., 1978) and DMBA-LV in reciprocal hybridization assays (data not shown).

**Expression of retrovirus information in thymic lymphoma cell lines and normal CFW/D lymphoid tissues**

Total cellular RNA was isolated from the 485-10 tumour cell line and hybridized to cDNA probes representative of DMBA-LV, MMTV (C3H), TB Xe, Pt-MuLV 4506 and VL30S RNA. The results are shown in Fig. 3. The major species of cellular vRNA was homologous (93%) to MMTV (C3H). The minor species of vRNA was homologous to the various type C viral cDNA probes and the levels of hybridization attained were in close agreement with those found for DMBA-LV vRNA.

In Table 1 are given the levels of vRNA present in the total cellular RNA of normal thymus from the CFW/D mouse. There appeared to be no MMTV sequences detectable but there were detectable levels of type C viral RNA nucleotide sequences. The extent of homology and the 1/2 C1t values for the expression of these type C viral sequences were similar to those found in the 485-10 tumour cell line. Similar results were obtained for the two DMBA-LV-induced tumour cell lines from NIH Swiss mice.

An analysis of the size classes of retrovirus RNAs present in tumour cell lines established from DMBA-LV thymic lymphomas in CFW/D and NIH Swiss mice indicated that MMTV viral RNA was expressed in the tumour cell lines. Two DMBA-LV MMTV vRNA species (35S and 24S) were present in all thymic lymphoma cell lines (Fig. 2c) and these two RNA species migrated in parallel with the 35S and 24S MMTV cellular vRNA species present in MMTV (GR) tumour cells (Robertson & Varmus, 1979). There was no expression of MMTV vRNA in normal tissue of CFW/D mice (Fig. 4).

To investigate the type C viral expression in the tumour cell line 485-10, poly(A)-containing RNA was analysed using the Northern blot transfer technique and hybridization with viral probes representing both the intact Mo-MuLV genome and the env gene characteristic of recombinant and xenotropic viruses. While 35S and 33S RNAs were detected using a probe representative of the Mo-MuLV genome, no RNA of a size class (21S) corresponding to that of the env mRNA was detected (Fig. 5). However, using a DNA probe corresponding to the env region of the genome, a low level of 21S RNA was detected in the poly(A) RNA fraction from DMBA-LV-producing cells (Fig. 6). A similar size class of RNA was also detected in the cell RNA from a second DMBA-LV-induced thymic lymphoma cell line (485-41) as well as in the RNA from the chemical carcinogen-induced thymic lymphoma cell line DMT-10. On long exposure of the X-ray films a trace amount (not apparent in Fig. 6) of 35S and 33S RNAs was detected in both DMBA-LV and 485-41 cells.

It is not clear how the different species of type C vRNA are related to one another. The same molecular weight species were also detected in the poly(A)-containing RNA from normal lymphoid tissues (Fig. 4b).

The type C env DNA did not detect any 24S RNA. This would appear to rule out the possibility that the MMTV env mRNA represented a recombinant gene carrying nucleotide sequences homologous to type C retrovirus sequences.

**Characterization of virus produced by DMT-10 cells**

As noted above, we examined the type C retrovirus information in a thymic lymphoma cell line (DMT-10) established directly from a primary DMBA-induced tumour. The DMT-10 cells released viral activity detectable by reverse transcriptase assay. The activity found represented 25 to 35% of the reverse transcriptase activity released from a comparable number of 485-10 cells. However, the virus was not infectious in vitro (Table 2a) nor did intrathymic injection of undiluted virus into newborn CFW/D mice result in tumour induction (Table 2b).
Table 1. Levels of types B and C vRNAs in different lymphoma cell lines as compared to normal tissue

<table>
<thead>
<tr>
<th>Source of cell RNA</th>
<th>cDNA probe</th>
<th>IUdR-TB Xe</th>
<th>MMTV</th>
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<tr>
<td></td>
<td>% Hybridized</td>
<td>1/2 C\textsubscript{t}</td>
<td>% Total cell RNA</td>
</tr>
<tr>
<td>CFW/D 485-10</td>
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<tr>
<td>NIH IT2</td>
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<td>NIH 2T2</td>
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<td>CFW/D normal thymus*</td>
<td>56</td>
<td>3.4 \times 10^2</td>
<td>0.1</td>
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</table>

* Taken from 7-day-old mice.
† These values were not determined because the hybridization reaction was less than 5%, as measured by a C\textsubscript{t} of 1 \times 10^4 to 2 \times 10^4.

Table 2. In vitro infectivity of DMT-10 virus (a) and tumour induction in vivo by DMT-10 virus (b)

<table>
<thead>
<tr>
<th>(a)</th>
<th>Cell line</th>
<th>Permissive for</th>
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<th>Control</th>
<th>Dexamethasone</th>
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<tr>
<td></td>
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<td>32</td>
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<td></td>
<td>CCL64</td>
<td>Xenotropic</td>
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<th>(b)</th>
<th>Test virus</th>
<th>No. of mice injected</th>
<th>No. of mice with tumours</th>
<th>Average latent period (days)</th>
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<tr>
<td>DMBA-LV*</td>
<td>42</td>
<td>40</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>DMT-10† Expt. 1</td>
<td>16</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Expt. 2</td>
<td>42</td>
<td>0</td>
<td>-</td>
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* 0.02 or † 0.04 ml of an 18-h undiluted tissue culture harvest from 60 × 10^6 cells injected intrathymically into 24- to 48-h-old mice.
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Fig. 4. Detection and size analysis of types B and C vRNA in normal CFW/D tissues. Poly(A)⁺ RNA was extracted from young CFW/D mice: normal, 3-day-old, thymus (lanes 1 and 6); 4-week-old, bone marrow (lanes 2 and 7); 4-week-old, thymus (lanes 3 and 8) and from 485-2 lymphoma cells (lanes 4 and 5). RNA (5 to 6 μg) was denatured in glyoxal, size-fractionated on a 1% agarose gel, transferred and hybridized with either ³²P-labelled molecularly cloned MMTV DNA (a) or ³²P-labelled molecularly cloned Mo-MuLV DNA (b). RNA mol. wt. markers were papaya mosaic virus 32S RNA, TB cell ribosomal RNA and phage Qβ 25S RNA.

A molecular biological characterization of the nucleotide sequences present in DMT-10 virus and cells indicated that DMT-10 did contain and release type C retrovirus information. The hybridization results, obtained with the same cDNA probes as those used to characterize the nucleotide sequences present in DMBA-LV, are shown in Fig. 7(a, d). From the final extent of hybridization, as well as from the 1/2 Ct values, it would appear that the type C retrovirus information in DMT-10 is highly related, if not identical, to the type C information present in DMBA-LV. However, no type B retrovirus RNA was expressed in DMT-10 cells nor was any detected in DMT-10-derived virus (Fig. 7c).

DISCUSSION

From the results of our molecular hybridization analyses, it is clear that DMBA-LV contained both type C and type B retrovirus genomes. There was nearly complete nucleotide sequence homology between the milk-borne MMTV (C₃H) and the type B sequences found in DMBA-LV (90%) and that found between MMTV (C₃H) and type B RNA present in DMBA-LV-producing tumour cells (93%). The 10% non-homology found between the genomes of DMBA-LV MMTV and MMTV (C₃H) may be significant because the restriction endonuclease cleavage patterns of the unintegrated linear DMBA-LV MMTV DNA, generated by at least two enzymes (PstI and BamHI), differ from those reported for MMTV (C₃H) (J. K. Ball, unpublished results).

The type C sequences of DMBA-LV were related by only 43% to the ecotropic Mo-MuLV. The type C nucleotide sequences found in DMBA-LV were more related to the polytropic and xenotropic classes of type C retroviruses. DMBA-LV vRNA protected 59.5% of the IUdR-induced TB Xe cDNA, compared with a 43% homology found with ecotropic Mo-MuLV. DMBA-LV vRNA protected recombinant Pt-MuLV 4506 cDNA more (63.9%) than it did the ecotropic Mo-MuLV from which Pt-MuLV 4506 was partly derived. It is possible that DMBA-
LV contains polytropic sequences of the type implicated in leukemogenesis (Hartley et al., 1977; Fischinger et al., 1978). However, if these sequences are present in DMBA-LV they do not appear to be expressed as infectious type C retrovirus (J. K. Ball et al., unpublished results).

When the 485-10 tumour cell line was examined for the expression of type B retrovirus information, it was found that there was expression of all the MMTV information necessary for the translation of the gag and pol proteins and of the 24S envelope mRNA necessary for translation of the envelope glycoproteins. The levels of DMBA-LV MMTV vRNA were very high at nearly 6000 copies/cell. Unlike MMTV-producing mammary tumour cell lines, this high level of MMTV vRNA was maintained in the absence of glucocorticoid hormone stimulation (Ringold et al., 1975; Scolnick et al., 1976; Young et al., 1977; Vaidya et al., 1980).

Multiple species of type C-specific vRNA were found in the tumour cell lines. Based on the intensities of the vRNA bands as detected in Northern RNA filter transfer and hybridization experiments, it would appear that in the 485-10 cells the 33S species was present in excess over the 35S RNA, whereas in the virus both species were present in approximately equal concentrations. The type C sequences, present at 340 copies/cell, were 18 times less abundant in
Fig. 7. $C_t$ analysis of nucleotide sequences in DMT-10 virus and cells. (a) DMT-10 viral RNA hybridized to IUdR-VL30S cDNA ($\bullet$) and TB Xe (MDCK) cDNA ($\bigcirc$) and (b) to Pt-MuLV 4506 cDNA ($\bullet$) and Mo-MuLV cDNA ($\bigcirc$). (c) DMT-10 viral RNA hybridized to DMBA-LV cDNA ($\bullet$) and MMTV ($C_3H$) cDNA ($\bigcirc$). (d) Total cell RNA from DMT-10 cells hybridized to IUdR-TB Xe (MDCK) cDNA ($\bullet$) and MMTV ($C_3H$) cDNA ($\bigcirc$).

485-10 cell RNA than were MMTV sequences. The relationship between genome-size 35S vRNA and all the subgenomic species present is not clear. The 21S type C RNA detected by the $env$ probe probably represents the spliced mRNA for the gp70 and p15E envelope proteins as shown by others (van Zaane et al., 1977; Fan & Verma, 1978). The other subgenomic vRNAs could have arisen through aberrant splicing mechanisms or they could represent transcription products of deleted endogenous retrovirus genomes. Whatever their origin, the concentration and species of type C vRNA seen in the two DMBA-LV-induced tumour cell lines (485-10 and 485-41) appear to be similar to the type C retrovirus information detected in DMT-10 tumour cells and in normal thymus and bone marrow cells. The virus released by DMT-10 cells also contains type C retrovirus sequences which appear to be identical to those present in DMBA-LV. Intrathymic injection of DMT-10 virus has never induced any neoplastic disease.

The type C vRNA present in all the tumour cell lines examined, as well as in normal lymphoid tissue, is most likely a constituent of the type C particles seen budding in normal lymphoid tissues of the CFW/D mouse (Frei et al., 1973). The vRNA present in these particles, like that present in DMBA-LV and DMT-10, appears not to be infectious since sensitive co-cultivation procedures used for the detection of type C retroviruses were consistently negative for both the type C particles present in normal lymphoid tissues (J. A. McCarter, unpublished results), those in DMBA-LV (Ball & McCarter, 1979) and those in DMT-10 (this paper). Using the same co-cultivation procedures, infectious virus could consistently be recovered from normal lymphoid tissues of other mouse strains (Ball & McCarter, 1979). Thus, it appears that the lymphoid tissues of CFW/D mice constitutively express defective type C retrovirus information and these type C sequences will be expressed in all CFW/D cell lines including those established from lymphoid tumours.

Earlier work (Ball & McCarter, 1979; and data not shown) showed that both rare type C and abundant intracytoplasmic A particles could be detected in the same 485-10 lymphoma cell. It was possible then that type C viral RNA and proteins could form a pseudotype virus employing the type B envelope gp52 and gp37 to form the envelope. Such a possibility had to be considered
since a type B–C pseudotype retrovirus [Kirsten MuSV(MMTV)] has been constructed in vitro (Schochetman et al., 1979).

Assuming that the type C genome is responsible for leukaemia induction, then its infectivity would be dependent on its ability to utilize MMTV envelope glycoproteins to form a pseudotype. It was possible to test for the presence of pseudotypes by taking advantage of the fact that although MMTV can infect murine cells, it is unable to complete the replication cycle due to the presence of an intracytoplasmic restriction mechanism (Závada et al., 1977). A pseudotype type C viral genome with an MMTV envelope should then gain entry into a murine cell and go through a normal replication cycle. MMTV, on the other hand, will be restricted from growth and thus any virus production would be due to the DMBA-LV type C viral genome. The results of such experiments were all negative for virus production (Ball & McCarter, 1979; and data not shown). Furthermore, no evidence was obtained for recombination between retrovirus types B and C in the env region of the genome because no 24S RNA, of a size class corresponding to the MMTV env mRNA, was detected using a type C env-specific DNA probe. However, the available data do not permit us to rule out recombination in the gag-pol regions of the genome.

MMTV can be forced to infect heterologous cell lines (Vaidya et al., 1976; Ringold et al., 1977; Howard et al., 1977). However, when DMBA-LV with a high in vivo leukaemogenic titre was used to infect mink cells (one of the cell lines known to be permissive to MMTV infection), a productive infection (as assayed by reverse transcriptase) could not be detected even after prolonged passage of cells or after stimulation with dexamethasone. Furthermore, using more sensitive co-cultivation procedures for infecting cells, no productive infections were obtained (Ball & McCarter, 1979). The reason for this apparent inability to infect mink cells is not clear at present. It may be because DMBA-LV MMTV represents a different variant of MMTV which cannot infect mink cells and exhibits a tropism specific for thymus cells, as is the case with one other leukaemogenic retrovirus (Decleve et al., 1974). Also, while DMBA-LV is capable of inducing leukaemia, it has not induced mammary tumours (J. K. Ball, unpublished results) under the appropriate experimental conditions in CFW/D mice (strain of origin of the virus) or in BALB/c mice, a strain permissive for mammary tumour induction.

In summary, DMBA-LV, a highly leukaemogenic retrovirus isolate was found to be a mixture of types B and C retrovirus genomes. The type B genome, present in tenfold excess over the type C information, represents a complete genome with a high level of nucleotide sequence homology with the exogenous, type B milk-borne virus of C3H origin. The type C information appears not to form infectious pseudotypes with MMTV and, because of its non-infectious nature in vitro, is most likely defective and probably represents constitutively expressed endogenous retrovirus sequences present in the CFW/D mouse. This raises the question whether it is the MMTV or the type C component of DMBA-LV that is leukaemogenic. Molecular cloning experiments currently under way should permit a direct assessment of the ability of the MMTV component, free of type C information, to induce leukaemia.

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A leukaemogenic virus closely related to MMTV 2189


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