Alterations in the U₃ Region of the Long Terminal Repeat of an Infectious Thymotropic Type B Retrovirus

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We isolated and characterized a type B thymotropic retrovirus (DMBA-LV) which is highly related to mouse mammary tumor virus (MMTV) isolates and which induces T-cell thymomas with a high incidence and a very short latent period. Regions of nonhomology between the DMBA-LV genome and the MMTV genome were identified by heteroduplex mapping and nucleotide sequence studies. In the electron microscope heteroduplex mapping studies the *Eco*RI-generated 5' and 3' fragments of the DMBA-LV genome were compared with the corresponding fragments of the MMTV (C3H and GR) genome isolated from mammary tumors. The results indicated that DMBA-LV contained a region of nonhomologous nucleotide sequences in the 3' half of the U₃ region of the long terminal repeat (LTR). Nucleotide sequence studies confirmed these results and showed that in this region 440 nucleotides of the MMTV (C3H) sequences were deleted and substituted with a segment of 122 nucleotides. This substituted segment in the form of a tandem repeat structure contained nucleotide sequences derived exclusively from sequences which flanked the substitution loop. The distal glucocorticoid regulatory element was unaltered, and two additional copies of the distal glucocorticoid regulatory element-binding site were present in the substituted region. The restriction endonuclease map of the reconstructed molecular clone of DMBA-LV was identical to that corresponding to unintegrated linear DMBA-LV DNA present in DMBA-LV-induced tumor cell lines. Since the nucleotide sequences of the LTRs present in four different DMBA-LV proviral copies isolated from a single thymoma were identical, we concluded that they were derived from the same parental virus and that this type B retrovirus containing an alteration in the U₃ region of its LTR could induce thymic lymphomas. Thus, DMBA-LV represents the first example of a productively replicating type B retrovirus that contains an LTR modified in the U₃ region and that has target cell and disease specificity for T cells.

Type B retroviruses have been associated primarily with the induction of mammary adenocarcinomas, implying a strict target cell specificity for mouse mammary tumor virus (MMTV). This class of retroviruses does not contain an oncogene and hence induces tumors after a long latent period. However, there is extensive literature documenting the presence of type B retroviral information in both normal lymphoid tissue and lymphoid tumors.

In 1971 we first reported (5) the isolation of a highly leukemogenic retrovirus (DMBA-LV) from a dimethylbenz (α)-anthracene-induced thymic lymphoma. Further characterization (1, 6) of this retrovirus suggested that it was a type B retrovirus. In liquid hybridization studies we determined that the genome of DMBA-LV was highly related (90%) to that of a mammary tumor-inducing isolate of MMTV (C3H) (4). Further, the proteins of DMBA-LV were also highly related to those of MMTV (16), and the leukemogenicity of DMBA-LV was completely neutralized by a monoclonal antibody against MMTV type B envelope glycoprotein (2). The virus did not induce mammary tumors (2). In restriction endonuclease mapping studies we showed that the DMBA-LV genome could be clearly distinguished from endogenous MMTV proviral copies and that all DMBA-LV-induced thymomas contained multiple DMBA-LV proviral copies (15). The virus was also shown to have specificity for T cells (3). Primary tumors induced by DMBA-LV as well as tissue

culture cell lines derived from them replicated and released a biologically active, thymotropic type B retrovirus (3).

In the early 1980s Hilkins et al. (26) and Michalides et al. (35) reported the appearance of spontaneous lymphomas in old GR male mice, and Vaidya et al. (45) showed that lymphoma cell lines of DBA origin contained type B retroviral information. More recently it has been shown that C57Bl/6 T-cell lymphoma cell lines contain amplified copies of endogenous type B proviral genomes (20, 21). The association of a type B retrovirus with kidney adenocarcinomas has also been reported (23).

Since in all of the above-mentioned studies it was shown that the nonmammary tumors contained multiple new type B proviral copies, it was tempting to try to correlate the different biological phenomena associated with the presence of these proviral copies in different tissues with the genomic structure of these copies. In all cases examined (lymphoid tumors in GR mice [34, 36]; lymphoid tumor cell lines [20, 21]; and kidney adenocarcinomas [12, 48]), structural alterations were detected in the U₃ region of the long terminal repeat (LTR) of the new proviral copies. The alterations consisted of either the deletion of approximately 350 to 450 nucleotides or the deletion and substitution of new sequences. The substituted segments were composed of sequences flanking either or both ends of the deleted region. The alteration in the U₃ region of the LTR of the type B proviral copy present in the kidney adenocarcinoma cells was unique in that the deleted region was small (113 base pairs [bp]), and the 91 bp which constituted the substituted

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segment were of unknown origin (48). None of the lymphoid tumors or cell lines containing the additional MMTV proviral copies with structural alterations in their LTRs produced infectious virus. This has made it impossible to directly relate the alteration in LTR structure to a change in disease tropism.

In this report we show that in four different DMBA-LV provirus-cell DNA junction fragments isolated from a single primary thymic lymphoma, the LTRs had an identical alteration in the U₃ region. We therefore conclude that DMBA-LV represents the first example of a productively replicating type B retrovirus that contains an LTR modified in the U₃ region and that has disease specificity for T cells.

MATERIALS AND METHODS

Virus and tumor induction. DMBA-LV was obtained from the thymic lymphoma cell line 485-10 (16). The tumor from which integrated DMBA-LV proviral copies were molecularly cloned was induced in CFW/D mice by the intrathymic injection of 0.03 ml of a cell-free preparation of the tissue culture medium from the 485-10 cell line. This cell line was grown in McCoy 5A medium plus 10% heat-inactivated fetal calf serum and antibiotics.

Isolation and purification of cytoplasmic unintegrated DMBA-LV DNA. We previously showed that the majority of all primary tumors induced by DMBA-LV contained high levels of unintegrated linear viral DNA. To obtain a restriction endonuclease map of the DMBA-LV genome, we isolated the unintegrated linear viral DNA from the virus-producing tumor cell line 485-10 by the method of Kung et al. (29). Additional purification included centrifugation through a sucrose gradient to remove any contaminating high-molecular-weight cell DNA (32).

Molecular cloning of integrated proviral copies. High-molecular-weight DNA was prepared from a primary tumor containing four DMBA-LV proviral copies. The DNA was digested with *EcoRI* (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), a restriction endonuclease which cuts the viral genome once in the *pol* gene. The cleaved DNA was size fractionated on a 10 to 40% sucrose gradient (in 1 M NaCl–20 mM Tris chloride [pH 8.0]–5 mM EDTA), and fractions containing the appropriate size classes of DNA fragments were ligated (T4 ligase; New England Biolabs, Inc., Beverly, Mass.) to purified Charon 4A *EcoRI* arms, packaged in vitro into phage particles (using Packagene reagents; Promega Biotech), and used to infect the LE392 strain of *Escherichia coli*. The lambda phage library was screened for the presence of MMTV-containing information. Of a total of 6×10^5 plaques screened with a ³²P-labeled pMMTVrep DNA (kindly provided by J. Majors), 9 were found to contain MMTV-host cell DNA junction fragments larger than the *EcoRI*-generated fragments derived from the endogenous type B proviruses of normal CFW/D mouse tissues (15). The exogenous DMBA-LV origin of viral information in the selected fragments was also confirmed by digestion with restriction endonucleases whose cleavage sites in DMBA-LV differ from those present in the endogenous type B proviruses of CFW/D mice.

From the nine original plaques containing DMBA-LV DNA information, two were selected to reconstruct a complete molecular clone of the viral genome. For reconstruction of the complete DMBA-LV genome the majority of the cellular flanking sequences were first removed by restriction endonuclease digestion, leaving 0.4 kilobases (kb) at the 5' end and 2.5 kb at the 3' end. The 3' virus-cell DNA fragment

was subcloned into pBR322 at the *EcoRI*-*HindIII* sites to generate the recombinant plasmid pB6-3'VC, and the 5' virus-cell DNA fragment was subcloned into pUC12 at the *EcoRI*-*HindIII* sites to generate the recombinant plasmid p2-5'VC. The 5' and 3' viral sequences were isolated free of the vector and ligated together at the *EcoRI* site to form the viral genome, which was then ligated into pUC12 at the *HindIII* site to generate a molecular clone of the viral genome.

Restriction endonuclease mapping. Restriction endonucleases *EcoRI*, *XbaI*, and *KpnI* were purchased from Bethesda Research Laboratories). All other enzymes were purchased from Boehringer Mannheim Biochemicals, Quebec, Quebec, Canada. Restriction endonuclease digestions, agarose gel electrophoresis, and Southern transfers and hybridizations were carried out as previously described (16, 44). Molecular clones of the MMTV genome, LTR, and *gag-pol* and *env* genes were kindly provided by J. Majors. A molecular clone representing the 1.1-kb *PstI* fragment from the beginning of the *gag* gene (derived from an endogenous BALB/c MMTV provirus) was kindly provided by D. Gray (24). Nitrocellulose filters were processed as previously described (16).

Heteroduplex mapping. Recombinant plasmid p2-5'VC was used to compare the 5' half of DMBA-LV with a recombinant plasmid containing the complete unintegrated provirus of MMTV (GR) (7). Recombinant plasmid p2-5'VC contains a 5.5-kb *HindIII*-*EcoRI* insert consisting of 500 bp of flanking mouse DNA, the 5' LTR, the entire *gag* gene, and *pol* sequences up to the *EcoRI* site. It was linearized by *EcoRI* for heteroduplex analysis. The complete circularly permuted provirus of MMTV (GR) was excised from its vector by *EcoRI*, and the linear 9-kb fragment was used for heteroduplex analysis.

Recombinant plasmid pB6-3'VC was used to compare the 3' half of the type B provirus from a DMBA-LV-induced primary tumor with recombinant plasmid p64.5 (48) cloned from a BALB/c mammary tumor induced by MMTV (C3H) (23). Recombinant plasmid pB6-3'VC contains a 6.6-kb *EcoRI* insert consisting of approximately 4.1 kb of *pol env* and LTR sequences and 2.5 kb of 3' adjacent cellular DNA. The corresponding insert in p64.5 from the wild-type MMTV (C3H) provirus was a *HindIII* fragment and was therefore 0.2 kb shorter at the 5' end than was the insert in pB6-3'VC. p64.5 contained 0.7 kb of cellular flanking sequences.

The plasmids (1.0 µg/µl each) to be compared were digested with *EcoRI* or *HindIII* in 100 µl of 70% formamide–0.3 M NaCl–10 mM Tris hydrochloride (pH 8.5)–1 mM EDTA. The DNAs were denatured at 70°C for 5 min, renatured at 30°C for 2 h, and directly spread for electron microscopy. φX174 DNAs were used as single- and double-stranded internal length standards (47).

Nucleotide sequence determination. Plasmids pB6-3'VC and p2-5'VC were digested with *BglII* and *HindIII*, and the 1.3-kb fragments containing the LTR sequences were inserted into the M13 vectors as described previously (33, 40). Recombinant DNAs were isolated, single-stranded DNA was prepared by the method of Dale et al. (14), and, after binding of the appropriate primer, the DNA was cleaved by digestion with the appropriate restriction endonuclease and a series of deletion mutants were generated by digestion with T4 polymerase as described by Dale et al. (14). Poly(A) tails were added, and, following ligation, the molecules were used to transform the JM109 strain of *E. coli*. From the series of overlapping deletion mutants obtained representative mutants of the appropriate size classes were sequenced by the

FIG. 1. Comparison of the restriction endonuclease maps of unintegrated linear DMBA-LV DNA (line 2) and of a reconstructed molecular clone of the DMBA-LV genome (p3-35) (line 3) with those of four mammary tumor-inducing type B isolates of MMTV [line 4, (C3H)f (37); line 5, RIII (22); line 6, GR (7); and line 7, C3H (13, 42)] and of the endogenous provirus from AJ mice (19) (line 8). The viral genome locations for the different type B DNA probes used are indicated in the lower part of the figure; the probes were pMMTVrep (line 1), pgagpol (line 2), penv (line 3), pgag (line 4), and pLTR (line 5). Cellular sequences are indicated by wavy lines (lines 1 and 6). The restriction endonucleases used were *Bam*HI (B), *Bgl*II (Bg), *Bst*EII (Bs), *Cla*I (C), *Eco*RI (E), *Hind*III (Hi), *Hpa*I (H), *Kpn*I (K), *Pst*I (Ps), *Pvu*II (P), *Sac*I (S), *Xba*I (X), and *Xho*I (Xh). In the top portion of the figure, the straight line in line 1 represents the approximate sizes of the *gag-pol-env*-encoded sequences, and the rectangular boxes represent the LTR regions of the genome.

method of Sanger et al. (41). Two additional LTRs, one 5' and one 3', were also sequenced. They were derived from virus-cell junction fragments cloned into plasmid pGem4 (Promega) at the *Bam*HI site for the 3' LTR and at the *Hin*dIII-*Bgl*II site for the 5' LTR. Overlapping sequential deletion mutants were prepared by using the Erase a Base system (25) and sequenced as described above (41). The number of independent overlapping deletion mutants sequenced was such as to give rise to four complete sequence determinations for each of the LTRs.

RESULTS

Restriction endonuclease mapping. The restriction endonuclease map for the unintegrated linear DMBA-LV DNA is

shown in Fig. 1. This map was generated by digestion of unintegrated linear DMBA-LV DNA isolated from 485-10 cells with the enzymes listed, followed by hybridization with DNA probes specific for various regions of the genome of MMTV (C3H). The use of these probes was justified because in earlier studies (4) we showed by liquid hybridization that the DMBA-LV type B retrovirus shares 85 to 90% nucleotide sequence homology with MMTV (C3H). The sizes of the cleavage fragments derived from unintegrated linear DMBA-LV DNA were confirmed by double digestions of a reconstructed molecular clone of the DMBA-LV genome and the use of region-specific genome probes.

A comparison of the DMBA-LV DNA restriction map with the published data for the four mammary tumor-inducing isolates and the molecular clone of the AJ provirus is shown in Fig. 1. Unique cleavage sites for the enzymes (*Eco*RI, *Hind*III, and *Kpn*I) were present in the same locations in the DMBA-LV genome as in the genomes of the other isolates. Cleavage with *Bam*HI distinguished DMBA-LV DNA from all of the other isolates. As in the endogenous AJ MMTV provirus, the *Bam*HI site located toward the 3' end in the *env* gene is missing in DMBA-LV, and, like MMTV (RIII) DNA, DMBA-LV DNA also has *Bam*HI sites at the beginning of the *pol* gene. The 4.1-kb (identified in some publications as 3.7-kb [13, 22]) internal *Pst*I fragment, which has been considered as a marker for mammary tumor-inducing potential, is also generated from the DMBA-LV genome.

Heteroduplex analysis. To compare the genome of the newly isolated provirus with other MMTV genomes, we prepared heteroduplexes between the 5' *Eco*RI proviral fragment of DMBA-LV present in p2-5'VC and the complete provirus of MMTV (GR) (7) and between the 3' *Eco*RI proviral fragment of DMBA-LV present in pB6-3'VC and the corresponding type B retroviral sequences cloned from an MMTV (C3H)-induced mammary tumor (48). The heteroduplexes were then examined in the electron microscope to determine if regions of nonidentity were present. Nonhomologies smaller than 50 bp are not detected by this technique. Thirty heteroduplexed molecules of each combination were analyzed (an example of one of each is shown in Fig. 2). No sequence divergence was detected in the *gag*, *pol*, or *env* genes. However, a striking alteration was seen in the 5' and 3' LTRs (arrows). A substitution loop was observed about 520 nucleotides downstream from the 5' end of the LTR and 320 nucleotides upstream from the 3' end of the LTR. One of the strands measured 160 bases, and the other strand measured 440 bases.

Heteroduplexes were also formed between the 5' and 3' *Eco*RI fragments from the type B proviral copy present in kidney adenocarcinomas (MMTV-K)(23) and DMBA-LV. A substitution loop was found in approximately the same position as in heteroduplex molecules formed between the *Eco*RI fragments of MMTV (C3H) and DMBA-LV (data not shown).

Nucleotide sequencing of DMBA-LV LTRs. Direct information on the alterations in the region of the substitution loop was obtained by nucleotide sequencing of the LTRs from four different DMBA-LV proviral copies present in a DMBA-LV-induced primary tumor. The complete nucleotide sequence for three of the four DMBA-LV LTRs was obtained, and the sequence was identical for all copies. For the fourth copy, only the substituted region was sequenced, and the sequence obtained was identical to that found for the other three proviral copies. The sequence for the altered region in the 3' LTR of p6B-3'VC is shown in Fig. 3A. The

region of interest extends from the cap site (position 0) to nucleotide -656 upstream of the cap site, approximately in the middle of the LTR. A stretch of 440 nucleotides of the wild-type sequence has been deleted starting at position -656 and extending to position -216 (upstream from the cap site) and has been replaced by 122 nucleotides derived from sequences flanking the deleted region (Fig. 3). This deletion-substitution region leaves the glucocorticoid regulatory response element intact. Examination of the nucleotide sequence of the substituted region revealed a tandem repeat structure (outlined in Fig. 3B) which consisted of a tandem repeat derived from the sequences flanking the deleted

region at both its 5' and 3' ends (Fig. 3A). This substitution generated a 5'-B A' B' A' B' A-3' structure with each repeat consisting of three components, each with a length of 62 bp. The A segment consisted of 43 bp, and the B segment consisted of 15 bp, with the two being separated by the tetranucleotide ACAG. No such repeat structure is present in the MMTV (C3H) LTR. Of most interest are the sequences which constituted the A' repeat segment. It consisted of 43 nucleotides and included the distal glucocorticoid response element (CAAAGTGTCTT) (28). As a result of the rearrangement, the DMBA-LV LTR contains three of these elements spaced by 50 nucleotides.

FIG. 2. Heteroduplex mapping of the complete genome of type B thymotropic retrovirus DMBA-LV. (A) Electron micrograph and tracing of a heteroduplex molecule between the circularly permuted complete provirus of MMTV (GR) opened at the *Eco*RI site (7) and plasmid p2-5'VC linearized by *Eco*RI. The sequence divergence was located 520 ± 30 bp from the 5' end of the 5' LTR marked by the junction of the double-stranded and single-stranded portions of the heteroduplex molecule. The bar represents 1.0 kb. The lengths of the single-stranded (a to d) and double-stranded (A and B) regions of the 30 molecules were measured. The mean values $\pm$ standard deviations in kilobases were as follows: a, 0.19 ± 0.02 ; A, 4.92 ± 0.25 ; b, 0.46 ± 0.03 ; B, 0.52 ± 0.03 ; c, 4.52 ± 0.36 ; and d, 3.00 ± 0.20 . (B) Electron micrograph and tracing of a heteroduplex molecule between inserts in p64.5 from MMTV (C3H) (23) from a mammary tumor and pB6-3'VC carrying an *Eco*RI 3' fragment of the DMBA-LV genome. The sequence divergence was located 330 ± 20 bp upstream of the 3' end of the double-stranded region of the molecule. The bar represents 1.0 kb. The lengths of the single-strand (a to e) and double-strand (A and B) regions of the 30 heteroduplex molecules were measured. The mean values $\pm$ standard deviations in kilobases were as follows: a, 0.27 ± 0.04 ; A, 2.97 ± 0.11 ; b, 0.44 ± 0.06 ; B, 0.33 ± 0.02 ; c, 0.16 ± 0.02 ; d, 0.61 ± 0.09 ; and e, 2.84 ± 0.16 .

In the normal wild-type LTR the *orf* region has a coding capacity sufficient to encode a 36-kilodalton protein (319 amino acids). For DMBA-LV LTR the divergence would start at amino acid 179 and extend to amino acid 185, where the protein would be terminated. Complete sequencing of the whole DMBA-LV LTR from three proviral copies revealed that in the regions outside the deletion-substitution loop there were, in all three LTRs, a total of 17 identical point mutations when compared with the MMTV (C3H) LTR sequence. Of the 17, 15 occurred in the C-terminal end of *orf*. Of these 15 changes, 6 would result in no amino acid change, 5 would be conservative, and 4 would result in the introduction of nonconserved amino acids.

DISCUSSION

DMBA-LV is a type B retrovirus with a tropism for T cells (3). The virus can be readily distinguished from the three endogenous type B proviral copies present in CFW/D mice (15). The virus is highly leukemogenic and induces T-cell lymphomas with a very short latent period (1, 3, 6). The induction of mammary tumors with this virus (via standard protocols of intraperitoneal injection and forced breeding) has never been observed (2). The leukemogenic properties of the virus can be completely neutralized by a monoclonal antibody directed toward MMTV envelope glycoprotein (2). All thymic lymphomas contain multiple DMBA-LV proviral

FIG. 3. (A) Nucleotide sequence of the 3' portion of the region of the DMBA-LV LTR in which the deletion-substitution loop was detected. The sequence shown starts at -70 from the cap site (position 0) and continues upstream through the deletion-substitution region. The upper line shows the nucleotide sequence of the DMBA-LV LTR determined as described in Materials and Methods, and the lower line shows the DNA sequence of the LTR of MMTV (C3H) as reported previously (31). The flanking sequences which make up the substituted region are indicated by circles for the upstream sequences and squares for the downstream sequences. The position of the tetranucleotide ACAG is indicated by underlining. The termination codon (T) in frame with the LTR open reading frame is indicated. (B) Diagram showing the repeat structures (A'B'A'B') in the substituted region of the LTR of DMBA-LV relative to the flanking regions (A and B) from which they were derived. The numbers in the third line represent the numbers of nucleotides in each of the two segments which constitute the direct tandem repeats. The solid bars in the second line indicate the positions of the distal glucocorticoid regulatory element-binding sites. The numbers in the fourth line represent the numbers of nucleotides separating the glucocorticoid regulatory element-binding sites. The asterisks represent the ACAG repeat sequence. The solid bars in the fourth line indicate the positions of the additional copies of the distal glucocorticoid regulatory element-binding sites.

copies (15). In this work we described the molecular cloning and sequence characterization of DNA fragments from four different DMBA-LV provirus-cell junction sites present in a single primary DMBA-LV-induced thymic lymphoma. From these an intact copy of the DMBA-LV genome was reconstructed. The cloned genome had complete restriction endonuclease identity with unintegrated linear DMBA-LV DNA

present in the same tumor cell line, which was the source of all of the infectious tumor-inducing DMBA-LV. Further, the nucleotide sequences of the LTRs from three different proviral copies were identical (including 17 point mutations), and the nucleotide sequence of the deletion-substitution region of the fourth was identical to that of the other three copies. Thus, all LTRs must have been derived from the same parental virus. We therefore concluded that the thymic lymphomas were induced by an infectious type B retrovirus with a unique alteration in the U_3 region of the LTR.

A comparison of the restriction endonuclease map of DMBA-LV with those of exogenous and endogenous MMTV isolates indicated that DMBA-LV does have unique cleavage sites, and these unique sites are present in the coding regions corresponding to all three genes in the viral genome. Two-dimensional peptide mapping studies of *gag* p28 also showed that DMBA-LV has tryptic and chymotryptic peptides which clearly distinguish it from milk-borne and endogenous MMTV isolates (W. G. Robey, G. A. Dekaban, and L. O. Arthur, unpublished results).

It is also clear from both the heteroduplex mapping studies and the nucleotide sequence data that the LTR of DMBA-LV is significantly different from those present in both the mammary tumor-inducing MMTV isolate (C3H) and the type B isolate associated with kidney adenocarcinomas (23). In comparison with the MMTV (C3H) LTR, a total of 440 nucleotides have been deleted and replaced by 122 nucleotides, all derived from the sequences flanking the detected region. This alteration did not interrupt the glucocorticoid regulatory element, and the nature of the origin of the substituted region resulted in the insertion of two additional copies of the distal glucocorticoid regulatory element (8).

The 440 nucleotides deleted constitute the 3'-terminal half of the *orf*, resulting in the production of a prematurely terminated protein of 179 amino acids and a new C terminus of 5 amino acids. Further, of the 15 point mutations in this prematurely terminated protein, 4 would lead to the introduction of nonconserved amino acids. The biological significance of the *orf* coding sequence in the LTR region is still unclear. Perhaps if the *orf* protein is necessary for biological function, as suggested by Smith et al. (43), then in T cells the N-terminal portion is sufficient.

It was of interest to compare structurally the alteration in the DMBA-LV LTR with those reported for other type B retroviral isolates which have been shown to be associated with nonmammary tumors. A summary of the data from the most extensively studied tumor systems is shown in Fig. 4. The LTRs present in the type B proviral copies in the nonmammary tumor systems studied all had alterations in the 3' end of the U_3 region. As noted previously (34), the deletions were not random but occurred around similar sites. In certain cases the deleted sequences were replaced by sequences flanking the 5' end of the deletion (tumor line 34) or sequences flanking both the 5' and 3' ends of the deletion (tumor line 42 and DMBA-LV). In all cases the inserted sequences had a tandem repeat structure. The alteration present in the LTR of the MMTV associated with kidney adenocarcinomas (48) was unique in that only 131 nucleotides were deleted and replaced by 91 bp of unknown origin.

Studies from a number of laboratories using a variety of murine type C retroviruses (10, 11, 27, 46) have shown that nucleotide sequences in the U_3 region of the LTR are major determinants of disease specificity and may be responsible for the T-cell tropism of Moloney leukemia virus (9, 17, 49). Whether the MMTV LTR contains sequences which are responsible for the strict target cell specificity of mammary

FIG. 4. Diagrammatic comparison of LTR alterations in type B retroviruses associated with nonmammary tumors. The cap site is designated 0. The positions of the glucocorticoid regulatory element (GRE) and the end of *orf* (END ORF) are indicated. The numbers under the broken lines indicate the numbers of nucleotides deleted. The letters at the beginning of the broken lines represent the tandem repeat segments. The row of x's in MMTV-K (23) represents an insertion of 90 nucleotides of unknown origin. The data for MMTV GR tumors are from Michalides et al. (34, 36), those for the C57BL lymphoma cell lines are from Dudley et al. (20, 21), and those for the DBA/2 lymphoid cell line are from Lee et al. (30).

tumor-inducing isolates is not known. The data accumulating from studies with non-mammary-tumor-inducing isolates suggest, as pointed out by Wellinger et al. (48), that such sequences may be present in the 3' end of the U₃ region.

Ideally, it should be possible to show that upon transfection, the molecularly cloned, reconstructed DMBA-LV genome would yield infectious T-cell thymoma-inducing virus. However, such experiments with molecularly cloned type B retroviruses are proving much more difficult than similar experiments with type C retroviruses. To date there have been reports from three different laboratories that molecular clones of three MMTV isolates (an endogenous, nonexpressed copy [19]; a permuted form of an endogenous, tumorigenic copy [7]; and a hybrid genome [38]) have been successfully transfected. Integrated proviral copies were detected, and viral RNA and proteins were expressed intracellularly. However, particle production was very low (39). In no case has it been possible to initiate disease in animals from supernatants from cells transfected with cloned MMTV DNA (H. Diggelmann and J. K. Ball, unpublished data). The situation is further complicated by the lack of suitable in vitro assays for MMTV-producing cells (18, 39). Thus, such experiments with the reconstructed molecular clone of DMBA-LV may also yield negative results. In anticipation of these difficulties we have adopted a long-term approach in which we shall assess the tumorigenic properties of the molecular clone of DMBA-LV by using the clone to prepare strains of transgenic mice.

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