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Amplification of Mouse Mammary Tumor Virus Genomes in Non-Mammary Tumor Cells

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Extra proviral copies of mouse mammary tumor virus (MMTV) are known to be present in the genomes of certain T-cell lymphomas of mice. Analysis of additional non-mammary tumor cell types known to express MMTV transcripts and antigens revealed the presence of extra acquired MMTV proviruses in a pituitary tumor cell line, a macrophage line, and Leydig testicular tumor cells. The nature of the amplified MMTV proviruses in these various tumor cell types differed with regard to copy number and presence of alterations in the long terminal repeat region.

Virus-induced mouse mammary tumors generally contain one or more additional integrated mouse mammary tumor virus (MMTV) genomes over and above the endogenous proviral copies present in the particular mouse strain genome (2). The pattern of the extra integrated proviruses in mammary tumors reflects the clonal origin of the tumors, and in most cases, at least one of the acquired viral genomes is integrated near a cellular gene, whose activation by the virus is believed to initiate the transformation process (4, 19). This mechanism for the initiation of oncogenic transformation of mouse mammary gland cells by MMTV is supported by numerous studies, and a number of target cellular genes have been identified (4, 19). Although as its name implies, MMTV was originally identified as a result of its association with mouse mammary tumors, many studies have also reported the expression of MMTV antigens and transcripts in tissues and tumors other than mammary glands or mammary tumors (6, 16, 18, 20, 24). Among the non-mammary cell types expressing MMTV, the most extensively studied have been certain mouse T-cell lymphomas which have been found to contain many newly acquired (amplified) MMTV copies located in novel genomic locations (6, 16). In contrast to most mammary tumors, the T-cell lymphomas were observed to contain many more copies of newly integrated MMTV proviruses. Sequence analysis of the amplified MMTV proviruses of a GR T-cell lymphoma provided the intriguing observation that the acquired MMTV genomes contained long deletions in their long terminal repeat (LTR) regions (17). Additional studies of the amplified MMTV genomes of T-cell lymphomas of mouse strains GR (15, 17), C57BL/6 (11), and DBA/2 (12) have all confirmed that the newly acquired proviruses all contain deletions in their LTR regions. Although not identical, the deletions in all the isolates appear to be similar in length and location within the LTR. All the extra proviral copies appear to have been derived from the same transcript, containing a deletion of about 300 to 500 base pairs and encompassing the 3′ end of the MMTV LTR open reading frame (11, 12, 15, 17). An infectious, thymotropic type B retrovirus has also been found to contain a similar deletion as well as a rearrangement in its LTR (1). One additional related report describes an exogenous renal tropic MMTV variant which contains a 90-base-pair substitution with a sequence of unknown origin in the 3′ end of the LTR open reading frame (25).

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To further investigate the phenomenon of MMTV amplification, we analyzed additional non-mammary tumor cells known to express MMTV antigens for the presence of extra proviral copies. Cells of the radiation-induced LAF_1 mouse pituitary tumor line AtT-20 have been reported to contain large numbers of A-type particles, the precursor cores of MMTV (24). Analysis of AtT-20 cell DNA by digestion with restriction endonuclease *Eco*RI, followed by agarose gel electrophoresis, blotting, and hybridization with an MMTV LTR probe (1.4-kilobase [kb] *Pst*I fragment of the 5′ LTR [13]) revealed numerous extra proviruses to be present in the AtT-20 cell genome (Fig. 1, lane B) compared with control LAF_1 mouse liver DNA (Fig. 1, lane A). The DBA/2 mouse monocyte-macrophage cell line P388 (10) expresses MMTV transcripts (7) and was also found to contain many extra MMTV proviral copies in its genome (Fig. 1, lane D) compared with control DNA of its parental strain DBA/2 (Fig. 1, lane C). T-cell lymphoma S49 (9), a BALB/c line known to contain numerous acquired MMTV genomes (6), is shown in lanes F and G of Fig. 1. Lane G had a shorter exposure than that for lane F, permitting the resolution of some individual bands. All samples consisted of 10 μg of *Eco*RI-digested genomic DNA hybridized and exposed under identical conditions. The genomes of AtT-20 (Fig. 1, lane B) and P388 (Fig. 1, lane D) cells contain so many additional proviruses that the *Eco*RI-generated virus-cell junction fragments form a continuum, and except for a few of the smaller bands, lower exposures of the autoradiograms did not resolve individual bands. The five endogenous proviral *Eco*RI fragments of BALB/c DNA, ranging in size from 6.7 to 16.7 kb, are shown in lane E of Fig. 1.

Cellular DNAs containing amplified MMTV genomes were next screened for the presence of deletions in the acquired proviruses by digestion with endonucleases *Msp*I and *Pst*I. *Msp*I digestion of MMTV proviruses generated a characteristic internal fragment 3 kb in length, containing the *env* gene and most of the 3′ LTR (Fig. 2). Figure 2 shows two duplicate blots of the same samples, except that the left panel shows hybridization with the MMTV LTR probe and the right panel shows hybridization with a MMTV *env* probe (1.8-kb *Pst*I fragment of the *env* gene [13]). If a deletion was present within the LTR or *env* regions, then the 3′ *Msp*I internal fragment would migrate at a lower-molecular-weight range, as can be seen for the AtT-20 DNA (Fig. 2, lane B) and the S49 DNA (Fig. 2, lane F). The extra number of proviruses present in the three tumor cell DNAs was re-

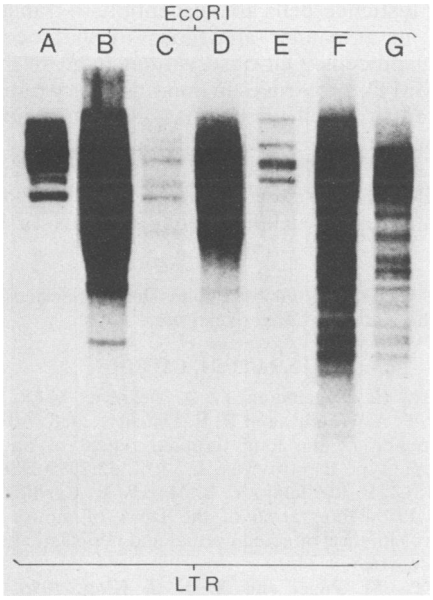

FIG. 1. Restriction endonuclease *Eco*RI analysis of genomic DNAs isolated from different cells. Lanes: A, LAF_1 mouse liver; B, AtT-20 cells (pituitary tumor LAF_1 mouse cells); C, DBA/2 mouse liver; D, P388 cells (macrophage cell line of DBA/2 mouse strain); E, BALB/c mouse liver; F, S49.1 cells (T-cell lymphoma of BALB/c mouse strain); G, same as lane F but with a shorter exposure. Hybridization was done with an MMTV LTR probe.

flected by the increased intensity of the internal 3′ *Msp*I fragments (Fig. 2, lanes B, D, and F). The amplified proviruses present in P388 cell DNA did not appear to contain deletions by this analysis (Fig. 2, lane D). To determine whether the deletions were present in the LTR or *env* portions of the 3′ *Msp*I fragments, a similar analysis was performed with *Pst*I-digested DNAs. *Pst*I digestion of MMTV proviruses generated a 1.4-kb internal fragment encompassing the 5′ LTR and a 1.8-kb internal fragment

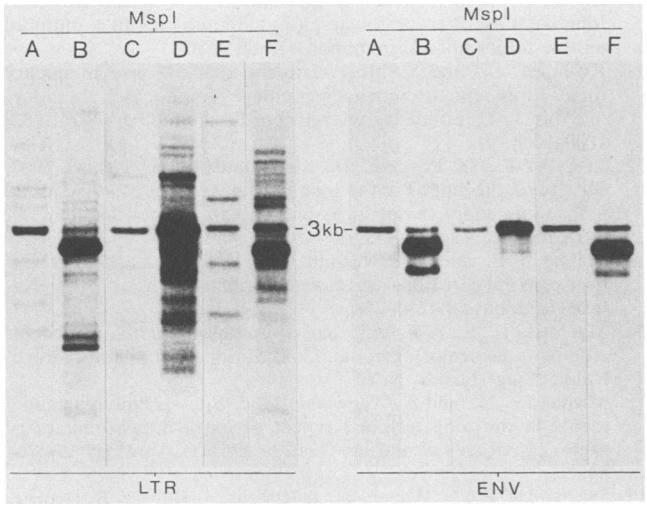

FIG. 2. Restriction endonuclease *Msp*I analysis of genomic DNAs isolated from different cells. Lanes: A, LAF_1 mouse liver; B, AtT-20 cells; C, DBA/2 mouse liver; D, P388 cells; E, BALB/c mouse liver; F, S49.1 cells. Hybridization was done with an MMTV LTR probe and an *env* probe.

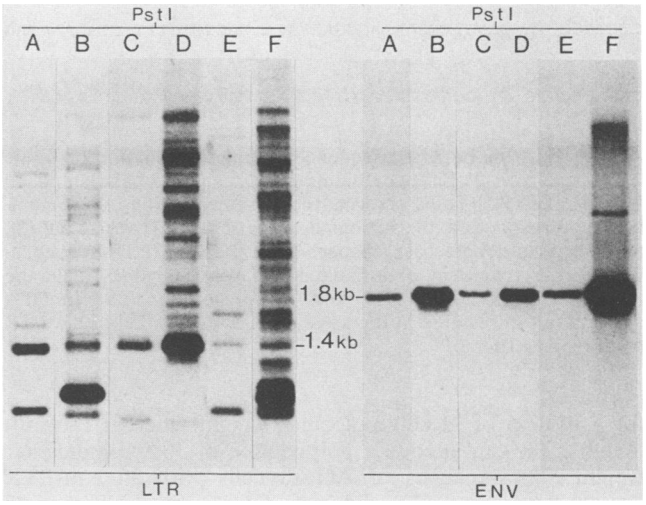

FIG. 3. Restriction endonuclease *Pst*I analysis of genomic DNAs isolated from different cells. Lanes: A, LAF_1 mouse liver; B, AtT-20 cells; C, DBA/2 mouse liver; D, P388 cells; E, BALB/c mouse liver; F, S49.1 cells. Hybridization was done with an MMTV LTR probe and an *env* probe.

containing the *env* gene. Both the AtT-20 and S49 DNAs showed the presence of amplified LTR-specific fragments shorter than 1.4 kb (Fig. 3, left panel, lanes B and F), whereas no such band was visible in P388 PstI-digested DNA (Fig. 3, left panel, lane D). This analysis indicates that the amplified proviruses in P388 cells carry full-length LTRs, although it does not rule out the possibility of substitutions or rearrangements. Hybridization of the *Pst*I digests with the envelope probe (Fig. 3, right panel) showed that all samples contained only the full-length 1.8-kb internal *Pst*I *env* fragment. The *Pst*I analysis suggests that the deletions were present in the LTRs of the amplified proviruses of the AtT-20 and S49 DNAs.

To define the alterations in the LTRs of the amplified MMTV proviruses in AtT-20 pituitary tumor cells, the 1.1-kb *Pst*I LTR fragment (Fig. 3, left panel, lane B) was eluted from a preparative gel and subcloned into the plasmid pGEM 3Z (Promega Biotec) by standard techniques (14). The sequence of the *Pst*I LTR fragment was obtained by the Sanger dideoxy technique (23) in combination with the exonuclease III deletion subcloning technique (8). The entire *Pst*I fragment proved to be 1,069 base pairs long, and a comparison with the full-length LTR of the C3H exogenous MMTV (5) indicated that the amplified MMTV LTR in AtT-20 cells contained a deletion of 391 base pairs located between bases 623 and 1015 (Fig. 4). The size and location of the deletion within the LTR were very similar to the deletions present in the amplified proviruses of the T-cell lymphomas. A 4-base sequence, TTCT, is inserted between the boundaries of the deleted sequence (Fig. 4). The sequence TTCT is found in the deleted region at positions 924 to 927 in the full-length LTR; whether this is the sequence that is retained in the deleted LTR is not known. Aside from the deletion, the rest of the LTR sequence of the AtT-20 provirus is highly conserved relative to the C3H virus, showing only a 3.5% divergence. One substitution that does however have a profound effect is the replacement of an A by a T at position 579, resulting in the creation of a stop codon in the LTR open reading frame (Fig. 4). The resulting open reading frame in the LTR of the amplified provirus of AtT-20 cells could code

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          560
C*T*CAGGCCTA GAAGTA*CAAA AGGGAAAAT*A GAGTGT*G*TTT GT*AAAAATAG

          610
GAGACAGGTG GTGGCAACCA GGGTTCTAAC TGTTCTTAA*C AC*AAGGATGT
                       623      1015
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FIG. 4. A 100-base-pair sequence from the central portion of the 1,069-base-pair, 5′ LTR-containing *Pst*I fragment of the amplified MMTV of AtT-20 cells, showing the major alterations. Base numbering corresponds to the analogous bases of the C3H virus LTR (5), and asterisks denote base changes from the C3H LTR sequence. The 391-base-pair deletion is flanked by bases 623 and 1015, and the inserted sequence, TTCT, is boxed. The substitution of a T for an A at position 579 resulted in the creation of a stop codon in the LTR open reading frame.

for a protein of 21,800 molecular weight whereas the full-length LTR can encode a polypeptide of 36,800 molecular weight (22). Analysis of AtT-20 cells with an anti-LTR protein antiserum (22) did not detect any LTR-specific products, either with or without phorbol ester treatment of the cells (results not shown). The theoretical size of the LTR translation product in AtT-20 cells is very similar to that observed in EL-4 T-cell lymphoma cells (21).

A type of tumor cell which has been often reported to express MMTV antigens, transcripts, and A particles, is the Leydig testicular tumor cell (18, 20). Restriction endonuclease *Hin*dIII and *Bgl*I analysis of I-10 cells (26), Leydig testicular tumor cells of a BALB/c mouse, showed one extra MMTV proviral band to be present in the genome of these cells (Fig. 5, lanes B). The extra band appears to represent the 3′ half of a provirus, since it also hybridized with *env* probe (Fig. 5, lanes B). This band is not as apparent in *Eco*RI digests because it migrated very closely with one of the endogenous proviral bands (results not shown). Analysis with *Msp*I and *Pst*I did not indicate the presence of deletions in the extra proviral fragment (results not shown). As opposed to the tumor cells described above (AtT-20, P388, and S49), which contain numerous extra acquired proviral bands, the Leydig tumor cell genome appears to be analogous to that of a mammary tumor, carrying only one extra partial provirus. In view of the finding that the MMTV LTR contains regions responsive to androgens (3), it is feasible

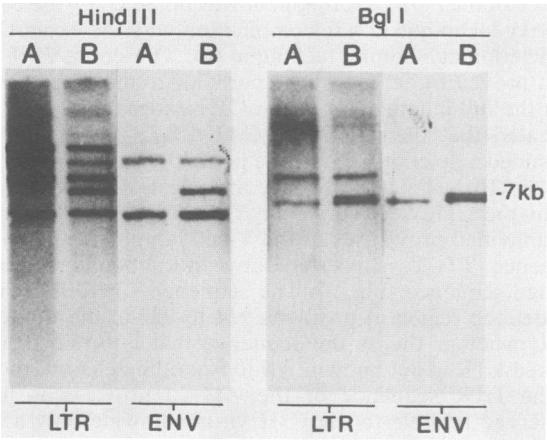

FIG. 5. Analysis of BALB/c mouse liver DNA (lanes A) and Leydig testicular tumor cell line I-10 DNA (lanes B) with restriction endonucleases *Hin*dIII and *Bgl*I. Hybridizations were done using the MMTV LTR or *env* probes as indicated on the bottom of the panels.

that Leydig testicular cells are susceptible to transformation by MMTV in a manner analogous to that occurring in mammary gland cells. Our observations indicate that amplification of MMTV proviruses in non-mammary tumor cells is not restricted to T-cell lymphomas, that alterations in the LTR are not always present, and that copy number can vary between 1 extra viral genome to 20 or more. Whether the acquired MMTV proviruses in these non-mammary tumor cells activate certain cellular genes remains to be determined.

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