

# Identification of a Proviral Structure in Human Breast Cancer<sup>1</sup>

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## ABSTRACT

Involvement of a virus similar to mouse mammary tumor virus (MMTV) in human breast cancer has long been postulated but never demonstrated. We have detected by PCR a 660-bp sequence similar to the *env* gene of MMTV but not to the known endogenous viruses, in 38% of human breast cancers examined (Wang *et al.*, *Cancer Res.*, 55: 5173–5179, 1995). This sequence was expressed in 66% of the *env*-positive tumors as detected by reverse transcription-PCR (Wang *et al.*, *Clin. Cancer Res.*, 4: 2565–2568, 1998).

In this article we report the amplification of a whole proviral structure from each of two human breast carcinomas that were *env* positive. Using nested extra-long PCR and primers from specific MMTV sequences, overlapping *env*-long terminal repeat (LTR), LTR-*gag*, *gag-pol*, and *pol-env* segments were successfully amplified. The 9.9-kb provirus is 95% homologous to MMTV but only 57% to human endogenous retrovirus K10 in 3.5 kb of the *gag* and *pol* genes. The provirus displays typical features of a replication competent virus, plus the open reading frame for the superantigen and the glucocorticoid responsive element. Fluorescence *in situ* hybridization with a 2.7-kb *env*-LTR sequence of an *env*-positive breast cancer cell line revealed that the sequence is inserted in several chromosomes but not in chromosomes from normal breast cells.

The origin of the MMTV-like sequences is uncertain. Because they are undetectable in normal tissues, because the similarity between the two isolates is high (96%), and because they maintain open reading frames, they appear to be exogenous.

## INTRODUCTION

The participation of a virus similar to MMTV<sup>3</sup> in human breast cancer has long been postulated but never demonstrated (1, 2). Despite a large body of positive evidence, the presence of endogenous retroviral sequences with 58% homology to MMTV (3, 4) and the failure to observe virus particles in tumors have confounded the evidence.

In an effort to exclude confusion with known endogenous retroviruses, we selected a 660-bp region of the MMTV *env* gene that has low homology to HERV-K10, the prototype human retrovirus most similar to MMTV (4), designed primers to this *env* gene region, and used the PCR to detect the 660-bp sequence. We found this specific *env* gene sequence in 38% of unselected breast cancers of American women. The sequence was rarely present (2%) in normal breasts but was not found in other tumors or tissues (5). The sequence was detected by Southern blot hybridization under stringent conditions. Similar results have been reported recently by Etkind *et al.* (6). We have also shown that this sequence is expressed in 66% of the

sequence-positive tumors and in some breast cancer cell lines (7). These observations also suggested that the sequence was present in a very low copy number. When the phenotypic characterization of tumors containing the sequence was studied, a correlation was found between *env*-positive tumors and expression of laminin receptor (8), a characteristic parameter of breast cancer aggressiveness (9). In addition to the *env* sequences, we have reported the detection of MMTV-like LTR sequences in human breast cancers that were *env* sequence positive. A 1.6-kb *env*-LTR sequence was also found that provided evidence that there was continuity between the two genes.<sup>4</sup>

We set out to establish if other retroviral genes homologous to MMTV are present in *env* gene-positive human breast cancers and, if so, whether they are distinct from the known endogenous proviruses, which are defective. We have now amplified, cloned, and sequenced a series of overlapping fragments that correspond to a 9.9-kb proviral sequence from each of two human breast cancers. Characterization of this entire sequence indicated that it is highly homologous to MMTV but not to HERV-K10 and that it has potentiality for replication.

## MATERIALS AND METHODS

Normal breast MCF-10F cell line (a gift from Dr. Raphael Mira y Lopez, Mount Sinai School of Medicine) and breast cancer EK-2 cells were maintained as previously described (7). MTSV1–7 cell line (a gift from Dr. Joyce Taylor-Papadimitriou, Imperial Cancer Research Fund London, United Kingdom) was maintained as reported (10). Tumors were designated MSSM 1 and 2. Both tumors were poorly differentiated infiltrating ductal carcinomas with positive lymph nodes.

Breast carcinomas and tissue from reduction mammoplasties of normal breasts were harvested fresh, and aliquots discarded by pathologists were transported on ice and frozen at  $-70^{\circ}\text{C}$  until used. The protocol for these studies was approved by the Institutional Review Board. DNA was extracted from tumors and tested for *env* sequences as previously described (5). To determine whether the DNA could be amplified in large sequences, a 2,150-bp segment of the  $\beta$ -actin gene was first amplified with XL-PCR, using rTth DNA polymerase (Perkin-Elmer), under the conditions recommended by the manufacturer. Primers used were as follows: actin 1, 5' TCC GAC CAG TGT TTG CCT TTT ATG 3' (positions 921 to 944); actin 2, 5' ACT GCT GTC ACC TTC ACC CTTC 3' (positions 3150 to 3171). The 100- $\mu\text{l}$  reaction mixture containing 2 units of enzyme, 10  $\mu\text{l}$  10 $\times$  buffer provided by the manufacturer, 1  $\mu\text{g}$  DNA, 20 ng of each primer, 200  $\mu\text{M}$  of each nucleotide, and 1 mM Mg acetate was incubated at 93 $^{\circ}\text{C}$  for 1 min for 1 cycle, then for 16 cycles at 93 $^{\circ}\text{C}$  for 1 min and 68 $^{\circ}\text{C}$  for 10 min, followed by 93 $^{\circ}\text{C}$  for 1 min and 68 $^{\circ}\text{C}$  for 10 min, increasing 15 s each cycle for 12 cycles, and then at 72 $^{\circ}\text{C}$  for 10 min ending at 4 $^{\circ}\text{C}$ .

### Amplification Cloning and Sequencing of 2.7-kb *env*-LTR Sequence.

Two rounds of XL-PCR were performed to amplify a 2.7-kb *env*-LTR sequence. Primers 5L and LTR3 were used for both rounds. The product of the reaction was detected in 1% agarose gel electrophoresis and by Southern blot hybridization with an internal sequence (LTR5) that was end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP (NEN, Boston, MA) using the T4 polynucleotide kinase (New England Biolabs). The radiolabeled oligonucleotide was purified using a TE MIDI select G-25 column (5 Prime  $\rightarrow$  3 Prime, Boulder, CO) following the protocol provided by the manufacturer. Hybridization was carried out as

Received 3/28/00; accepted 12/12/00.

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<sup>1</sup> Supported in part by the T. J. Martell Foundation for Leukemia, Cancer, and AIDS Research; The Kash Family Foundation; The Chemotherapy Foundation; The Jane Grinberg Research Fund; The Ellen Block Memorial Fund; and The Myra Shaw Anti-Cancer League.

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<sup>3</sup> The abbreviations used are: MMTV, mouse mammary tumor virus; HERV-K10, human endogenous retrovirus K10; LTR, long terminal repeat; MSSM, Mount Sinai School of Medicine; FISH, fluorescence *in situ* hybridization; XL, extra long; ORF, open reading frame; HMTV, human mammary tumor virus.

<sup>4</sup> Y. Wang, I. Pelisson, S. M. Melana, J. F. Holland, and B. G-T. Pogo. Detection of MMTV-like, LTR, and LTR-*env* gene sequences in human breast cancer, submitted for publication.

Table 1 Primer and probe sequences and location in the MMTV genes

Primers	Sequence (5'-3')	Location
LTR5	GGTGGCAACCAGGGACTTAT	735-754 or 9308-9327
LTR3	CGAACAGACACAAACACACG	1345-1364 or 9918-9937
GAG2	ACGGACTCACCACAGGGA	1565-1582
GAG3	GACAGCTTGTCTACCTCTGT	2053-2072
GAG4	GATGATGAGCTAATCCTTCC	2299-2318
GAG5	TGGGGATTATGTTTTATG	2643-2660
GAG7	AGTATTCTACCTTTGTAAAGC	3143-3163
POL4	TTCAAAAACTTGTAAAGGG	3393-3412
POL7	AAAGGAGAAGAATAGCACA	3756-3775
POL8	CCTTGTGGGGAAGAGACATT	4135-4154
POL5	TGTAGCAGTCCCTAAAGGAT	4501-4520
POL6	AAATTAACACGGGAGAGTT	5021-5040
POL3	GGGCTTTACTAAATAATTC	5345-5364
POL2	GTGGATTACCTTTGTAAT	5811-5830
POL10	GTCCCTGATATGACCAATG	5851-5870
POL1	GCTTCGCCCTTCCGGGCG	6214-6231
POL9	TTGAGGAATGCCCATGTATGC	6269-6289
POL3'NR	CCGATCCAAAACCTATGGTC	6576-6595
ENV5R	GACCCAGATTGGTGTTCGGCA	6685-6706
ENV1	AAATCAAAACCATGTATTTGG	7061-7080
5L	CCAGATCGCCTTAAGAAGG	7376-7395
EPR	AAACTCACCCCTGGTCAG	7453-7471
3L	TACAGGTAGCAGCACGTATG	7951-7970

Reverse primers were made based on the specific sequences and indicated by R.

described previously (5, 7). The same strategy was used to amplify and sequence the 2.7-kb *env*-LTR fragment from tumors MSSM 1 and MSSM 2.

**Amplification and Cloning of 1.3-kb LTR-*gag* Sequence.** Primers LTR5 and GAG4 were used to amplify a 1.58-kb LTR-*gag* sequence. Nested PCR with primers LTR5 and GAG3 was then performed, using AmpliTaq polymerase following conditions recommended by the supplier (Perkin-Elmer) to amplify 1.3 kb. Perfect-match PCR Enhancer (Stratagene) was used to increase product specificity. Thermocycling conditions were as follows: 1 cycle of 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 70°C for 2 min and by 1 cycle of 72°C for 10 min. The 1.3-kb PCR product was electrophoresed in 0.8% agarose gel followed by Southern blot hybridization with labeled LTR3. The 1.3-kb sequence was cloned and sequenced. An additional 265-bp sequence in the *gag* gene was amplified with primers GAG3R and GAG4 and directly sequenced from the PCR product. The same strategy was used to amplify 1.3-kb LTR-*gag* fragment from tumors MSSM 1 and MSSM 2.

**Amplification and Cloning of a 0.8-kb *pol-env* Sequence.** Conditions for PCR were the same as for amplification of LTR-*gag*. The first-round PCR was performed with primers GAG4R and EPR and nested PCR with primers POL3'R and 5LR. The 0.8-kb PCR product was electrophoresed in 2% agarose gel, and Southern hybridization with labeled ENV1 was performed. The same

strategy was used to amplify the 0.8-kb *pol-env* junction from tumor MSSM 1 and MSSM 2.

**Amplification and Cloning of a 2.2-kb 5' *gag-pol* Sequence from Tumor MSSM 1.** The same XL-PCR conditions as above were used. Primers GAG4R and EPR were used for the first-round of PCR, followed by nested PCR with primers GAG4R and POL5R. A 2.2-kb fragment was electrophoresed, and Southern hybridization with labeled POL4 was performed.

**Amplification and Cloning of a 3.09-kb 3' *gag-pol* Sequence from Tumor MSSM 1.** The XL-PCR conditions described above were used to amplify a 3.09-kb viral sequence. The first-round PCR was performed with primers GAG4R and EPR followed by nested PCR with primers GAG7 and POL1. Perfect-match PCR Enhancer (Stratagene) was added to the PCR reaction. Thermocycling conditions were as follows: 93°C for 1 min for 1 cycle and then 25 cycles of 93°C for 1 min, 55°C for 1 min, 68°C for 10 min, and 1 cycle at 72°C for 10 min. The 3.09-kb PCR product was electrophoresed in 1% agarose gel, followed by Southern hybridization with labeled POL5. An additional 380-bp sequence between *pol* and *env* genes was amplified with primers POL1R and POL3'R, hybridized with labeled POL9, and sequenced directly from the PCR product.

**Amplification of Sequences from Tumor MSSM 2.** A different strategy was used to amplify and clone some of the sequences from the second tumor. The same primers and PCR conditions were used to clone the 2.7-kb *env*-LTR, 1.3-kb LTR-*gag*, and 0.8-kb *pol-env* fragments. However, the *gag-pol* region was amplified with primers GAG2 and POL8R for the first-round PCR and with primers GAG3R and POL8R for the next-round PCR, and Southern blot hybridization was performed with labeled probe POL4. The *pol-env* region was amplified with primers POL8 and 3L for the first-round PCR and then with primers POL8 and ENV5R for the second-round PCR. The same PCR conditions were used as described for amplification of *pol-env* sequences. The amplified PCR products were separated by 2% agarose gel electrophoresis, and Southern hybridization with labeled POL5 was carried out as above.

**Cloning and Sequencing of the Amplified Sequences.** The PCR products were ligated into the pCR II vector from Original TA Cloning Kit (Invitrogen, San Diego, CA) and grown in INVαF' One shot competent cells (Invitrogen). White colonies were selected, and isolation and purification of the plasmids were accomplished using Plasmid Maxi Kit (Qiagen, Chatsworth, CA). *Eco*RI digestion and 1% agarose gel electrophoresis and Southern blot hybridization with labeled probes were performed to test for the presence of cloned inserts, and finally, the purified DNA was sequenced by automated DNA sequencing. The sequences were then compared with known sequences of MMTV, HERV-K10, and others through the GenBank program.

**FISH.** The 2.7-kb *env*-LTR fragment was used as a probe after nick-translation with dioxigenin (Boehringer Mannheim). The conditions described by Najfeld *et al.* (11) were used for hybridization and detection.

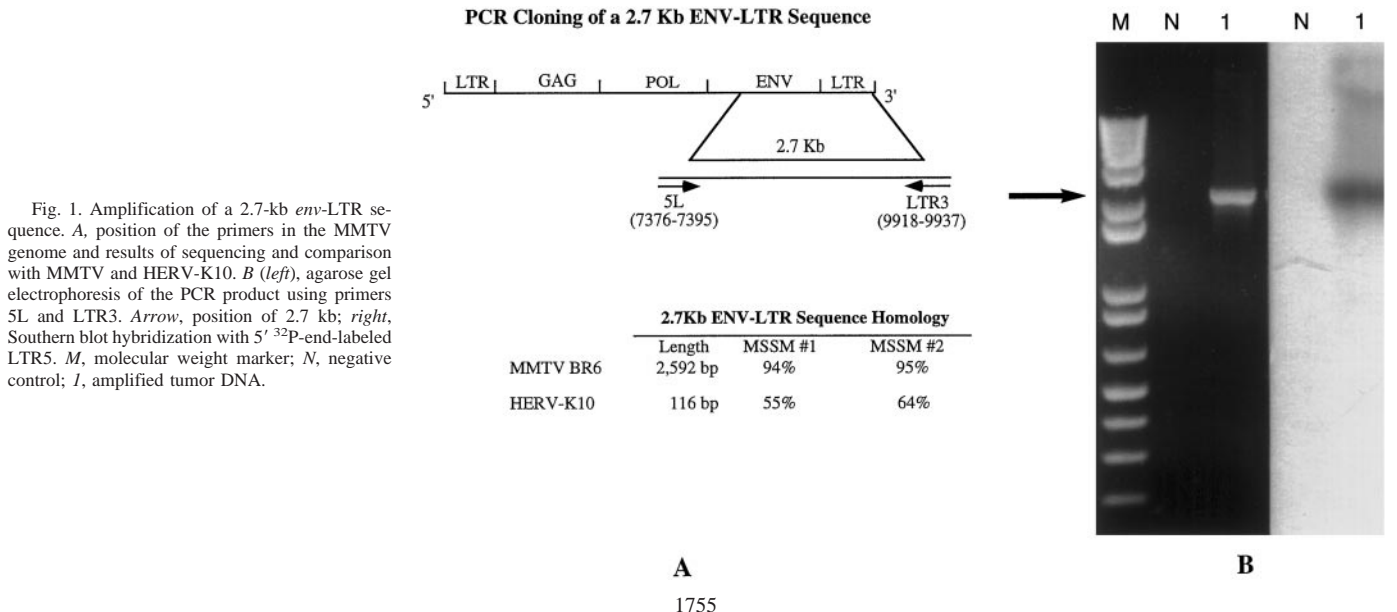
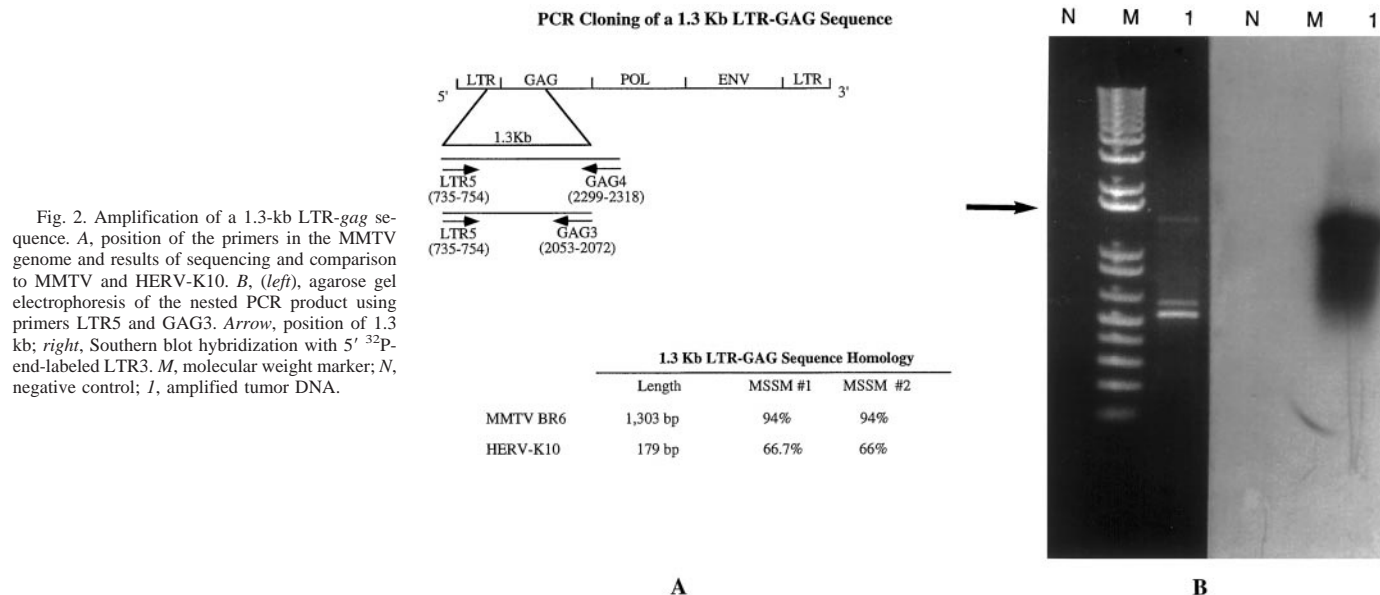


Fig. 1. Amplification of a 2.7-kb *env*-LTR sequence. A, position of the primers in the MMTV genome and results of sequencing and comparison with MMTV and HERV-K10. B (left), agarose gel electrophoresis of the PCR product using primers 5L and LTR3. Arrow, position of 2.7 kb; right, Southern blot hybridization with 5' <sup>32</sup>P-end-labeled LTR5. M, molecular weight marker; N, negative control; I, amplified tumor DNA.



**RESULTS**

The primers and probes used for amplification and sequencing of the proviral sequences are shown in Table 1 together with their location in the MMTV genome (12). All PCRs were performed with tumor and normal breast DNAs. Only the amplified fragments for tumor DNA are shown, because normal DNA failed to be amplified with these primers.

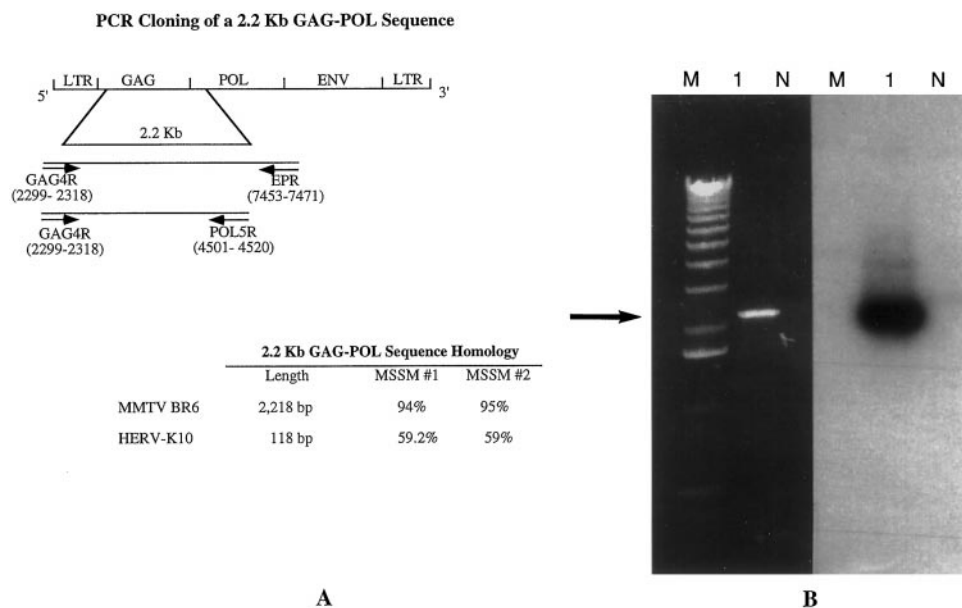
**Amplification of a 2.7-kb *env*-LTR Sequence.** In Fig. 1A, the position of the primers is indicated, and in Fig. 1B, the product of two rounds of XL-PCR using primers 5L and LTR3 is shown as well as Southern blot hybridization with probe LTR5. The results of sequencing and comparison to MMTV strain BR6 and to endogenous HERV-K10 are summarized in Fig. 1A. The 2.7-kb sequence was 94 and 95% homologous to MMTV in 2.59 kb of the two breast cancers but only 55 and 64% homologous to HERV-K10 in 116 bp (GenBank accession number AF243039).

**Amplification of a 1.3-kb LTR-*gag* Sequence.** In Fig. 2A, the position of the primers is indicated, and in Fig. 2B, the product of

nested PCR with primers LTR5 and GAG4, followed by PCR with primers LTR5 and GAG3, is shown as well as Southern blot hybridization with probe LTR3. The 1.3-kb sequence from the two tumors showed 94% homology to MMTV in 1.3-kb and 66 to 67% homology to HERV-K10 in 179 bp (Fig. 2A; GenBank Accession number AF248271).

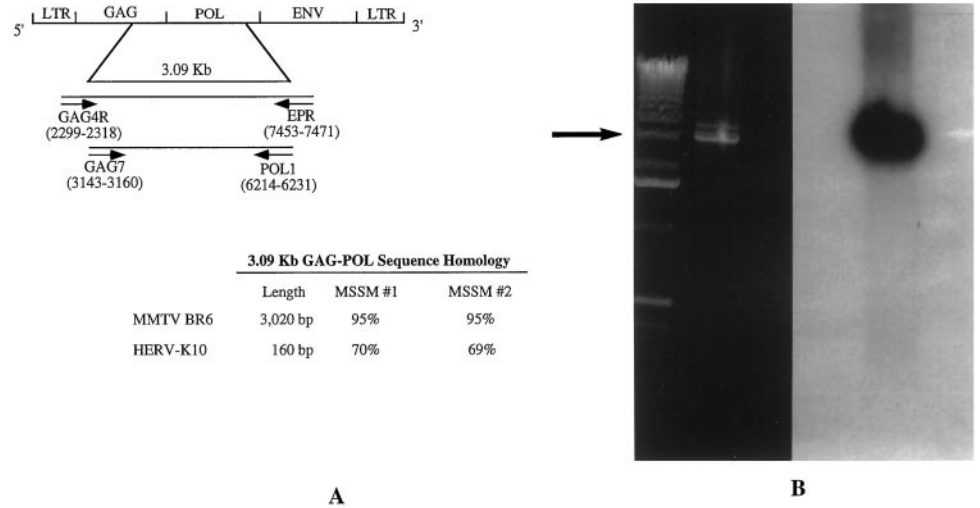
**Amplification of a 2.2-kb 5' *gag-pol* Sequence.** In Fig. 3A, the position of the primers is indicated, and in Fig. 3B, the product of nested XL-PCR with primers GAG4R and EPR, followed by PCR with primers GAG4R and POL5R, is shown as well as Southern blot hybridization with probe POL4. The 2.2-kb sequence from the two tumors was 94 to 95% homologous to MMTV over its entire length but only 59% homologous to HERV-K10 in 118 bp (Fig. 3A). The 2.2-kb sequence contains the ORFs for the dUTPase and the protease (*prt*) genes (GenBank accession number AF248272).

**Amplification of a 3.09-kb *gag-pol* Sequence.** In Fig. 4A, the position of the primers is indicated, and in Fig. 4B, the product of nested XL-PCR with primers GAG4R and EPR, followed by PCR

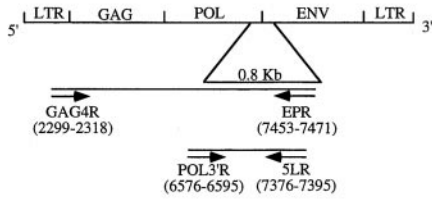


PCR Cloning of a 3.09 Kb GAG-POL Sequence

Fig. 4. Amplification of a 3.09-kb 3' *gag-pol* sequence. A, position of the primers in the MMTV genome and results of sequencing and comparison to MMTV and HERV-K10. B, (left), agarose gel electrophoresis of the PCR product using primers GAG7 and POL1. Arrow, position of 3.09 kb; right, Southern blot hybridization with 5' <sup>32</sup>P-end labeled POL5. M, molecular weight marker; N, negative control; I, amplified tumor DNA.



PCR Cloning of a 0.8 Kb POL-ENV Sequence



	Length	MSSM #1	MSSM #2
MMTV BR6	745 bp	94%	95%
HERV-K10	25 bp	60%	60%

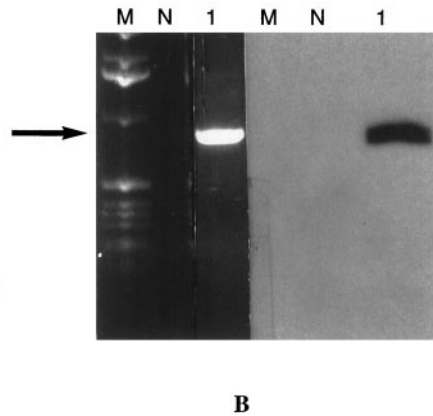


Fig. 5. Amplification of a 0.8-kb *pol-env* sequence. A, position of the primers in the MMTV genome and results of sequencing and comparison to MMTV and HERV-K10. B, (left), agarose gel electrophoresis of the PCR product using primers POL3'R and 5LR. Arrow, position of 800 bp; right, Southern blot hybridization with 5' end labeled POLA. M, molecular weight marker; N, negative control; I, amplified tumor DNA.

with primers GAG7 and POL1, is shown as well as Southern blot hybridization with POL5. The 3.09-kb sequence from the two tumors was 95% homologous to MMTV in 3.02 kb and 69 to 70% homologous to HERV-K10 in 160 bp (Fig. 4A; GenBank accession number AF248269).

**Amplification of a 0.8-kb *pol-env* Sequence.** In Fig. 5A, the position of the primers is indicated, and in Fig. 5B, the product of the XL-PCR with GAG4R and EPR, followed by PCR with primers POL3'R and 5LR, is shown as well as Southern blot hybridization with probe ENV1. The 0.8-kb sequence from the two tumors was 94

and 95% homologous to MMTV in 745 bp and 60% to HERV-K10 in 25 bp (Fig. 5A; GenBank Accession number AF248270).

The whole proviral structure is shown in Fig. 6. Total homology from the two carcinomas was 95% to MMTV in 9.9 kb and 57% to HERV-K10 in 3.5 kb. Homology to the endogenous retrovirus was seen primarily in the *pol* gene, which is known to be conserved among different retroviruses and partially in the *gag* gene. Similarity between the two tumors was 96%, indicating that they were closely related but not identical. The differences were randomly distributed across the provirus genome.

Fig. 6. Structure of proviral sequences in human breast cancer showing genes, ORFs, and sequence homology to MMTV and HERV-K10.

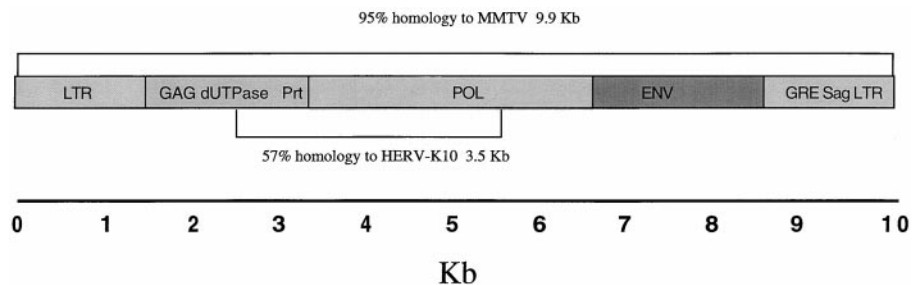
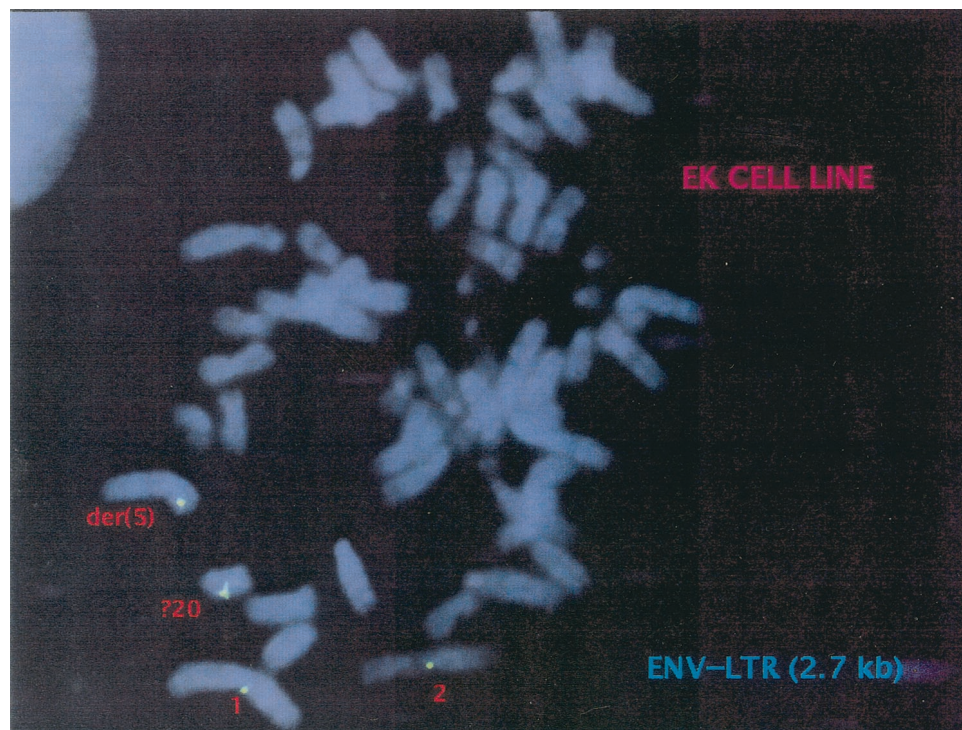


Fig. 7. Metaphase chromosomes from EK-2 cell line showing the 2.7-kb *env*-LTR signals on four chromosomes.



To learn more about the genomic localization of the proviral sequences, FISH analysis was performed using the 2.7-kb *env* LTR sequence as probe in EK-2 cells, a primary cell line derived from the pleural fluid of a patient with *env*-positive breast cancer. These cells have been karyotyped and shown to be human. They contain *env*, LTR, *gag* and *pol* sequences, as well as *env*-LTR and *gag-pol* junctional sequences, as detected by amplification with PCR.<sup>5</sup> An average of 3.5 signals per cell were detected in 80% of the 100 interphase EK-2 cells counted, whereas only 2 cells of the 100 normal breast MTSV1-7 cells counted and 2 of the 100 MCF-10 normal breast cells counted showed signals. Between 1 and 14 signals were observed in different chromosomes in nine metaphases in EK-2 cells, but the labeled chromosomes could not be identified with certainty. Fig. 7 shows one metaphase with hybridization signals produced by the 2.7-kb *env*-LTR sequence on four chromosomes.

## DISCUSSION

The results reported here indicate that the whole proviral structure, homologous to MMTV, is present in the genome of two breast cancers that were positive for the *env* gene sequence. Although primary tumors are extremely heterogeneous, the preponderance of evidence from positive results in cell lines and negative results in other tissues (5) supports the concept that these sequences are related to the mammary cancer cells.

The strategy followed for detecting the proviral sequences was the use of primers with low homology to known genes and viral endogenous sequences, followed by amplification of sequences of contiguous genes using the putative primers from the known sequences (chromosome walking).

Analysis of the proviral sequences demonstrates that they have potential for expression and replication. The LTRs contain all of the enhancer and promoter elements characteristic of a replication com-

petent MMTV-like virus as well as the glucocorticoid responsive element and the superantigen sequences. The other genes did not show evidences of deletions or early stop codons as do replication-incompetent endogenous retroviruses (13). The *gag* gene contains the dUT-Pase and *prt* ORFs (Fig. 6).

The putative HMTV overall homology to MMTV is 95% in both isolates. However, when 14 cloned MMTV-like *env* human sequences were compared with MMTV strains, the homology varied from 84 to 99%, suggesting that they are homologous to but not identical with MMTV (14). Furthermore, sequencing of 10 LTR human isolates revealed that there are certain specific deletions and insertions at the COOH-terminal of the superantigen sequences that clearly differentiate them from the MMTV strains.<sup>5</sup>

Comparison of the two isolates with the HERV-K10, the endogenous retrovirus closely related to MMTV (4, 15), revealed 57% homology in 3.5 kb of the *pol* and *prt* genes. Endogenous retroviruses contain deletions and early stop codons in different genes that make them replication incompetent. This is not the case of the two HMTV isolates.

The HERV-K family, which accounts for 1% of the human genome, is expressed to different degrees among individuals and cell types (16). HERV-K10 is expressed in some teratocarcinoma cell lines (13). One of these defective proviral elements, the HML6 BC1, was found to be expressed in only 1 breast carcinoma of 60 examined (17). Yin *et al.* (17) concluded that the role of their element in breast cancer remains unclear.

The origin of the proviral sequences that we have detected only in breast carcinomas is not yet established. Their absence from normal breast samples (2%) and from other tissues of the same patients whose tumors contain the sequences (5) suggests an exogenous origin. The possibility that they are endogenous and detectable only in tumors because of aneuploidy is improbable, because many aneuploid tumors analyzed were negative and many euploid tumors were positive (8). The likelihood of a hybrid virus, resulting from recombination between endogenous and exogenous viruses has been proposed (2), but

<sup>5</sup> Y. Wang, I. Pelisson, S. M. Melana, J. F. Holland, and B. G-T. Pogo, unpublished observations.

analysis of the sequences described here does not support this postulate. The sequences represent a complete competent virus, closely related to MMTV.

To ascertain if the sequences reported here play a role in breast cancer pathogenesis, it is necessary to localize the sites of insertion and to show evidence for viral expression and infectivity. MMTV promotes cell growth through insertional mutagenesis, with activation or inactivation of nearby genes that are related to growth control. Thus, it is important to establish where these sequences are localized in the human genome and whether they promote expression of nearby genes. Among the multiple random insertion sites, only one may be of importance.

With regard to viral expression, it has been shown by immunofluorescence that envelope glycoprotein (gp52) expression occurred in 39% of the breast cancers studied (18). Viral particles have been reported in 37% of breast cancer biopsies (19), in milk (20), and in breast cancer cell lines in culture (15). Antibodies against gp52 have been detected in the sera of 25% of American women with breast cancer (21), indicating immunoreactivity. By analogy to other virus-induced tumors, we can speculate that viral infection-promoting cell growth could have taken place early, but by the time breast cancer develops, only certain viral genes necessary to maintain the malignant state are expressed. We have observed viral particles in *env*-gene-positive specimens and are seeking specific gene activation patterns in tumors.

The presence of an *env* gene sequence highly homologous to MMTV in 38% of American women's breast cancers (5), the expression of these sequences in breast cancers (7), the results from FISH analysis indicating presence of the *env*-LTR sequence only in breast cancer cells, and the identification of an entire proviral sequence in two breast carcinomas taken together support the interpretation that a substantial proportion of breast cancer is associated with retroviral sequences that may represent an HMTV.

Recently, provocative epidemiological inference for the participation of MMTV in human breast cancer has been published (22).

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