

# A Mouse Mammary Tumor Virus-Like Long Terminal Repeat Superantigen in Human Breast Cancer

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## ABSTRACT

We previously reported a 660-bp mouse mammary tumor virus (MMTV)-like *env* gene sequence in approximately 38% of human breast cancer DNA, but not in normal breasts or other tumors. This MMTV-like *env* gene sequence was expressed in 66% of the *env* gene-positive human breast cancers. An entire proviral structure was identified in human breast cancer DNA with high homology to MMTV and low homology to known human endogenous retrovirus. MMTV-like long terminal repeat (LTR) sequences were also detected in 41.5% of human breast cancers. They contain hormone-responsive elements, TEF-1 family elements, and the open reading frame for the superantigen (SAG). We have now amplified and sequenced MMTV-like *sag* sequences from 10 human breast cancers, and we found that they are highly homologous to those of MMTV. However, deletions and insertions at the COOH-terminal of *sag* were observed. The immune function of the human MMTV-like LTR SAG was also investigated. The *sag* gene was cloned and expressed in a human B-cell line (Ramos). T-cell proliferation and cytokine releasing assays were performed after cocultivation of T cells with irradiated Ramos SAG-expressing cells. The results indicate that expression of the human SAG stimulates T-cell activation *in vitro*, as the mouse SAG does. Because the T-cell responses *in vitro* are considered similar to those *in vivo*, these results suggest that the human LTR SAG might also play a role in human breast carcinogenesis.

## INTRODUCTION

It has been very well documented that mouse mammary tumor virus (MMTV), a replication-competent type B retrovirus discovered by Bittner more than half a century ago (1), serves as an insertional mutagen to cause mammary carcinomas by activation of cellular proto-oncogenes such as *int*, *wnt*, and *fgf* genes in certain strains of mice (2). Expression of a protein called superantigen (SAG) that is encoded by an open reading frame (ORF) located at the 3' long terminal repeat (LTR) of the provirus in B lymphocytes is required for viral transmission and pathogenesis (3, 4). MMTV is transmitted as an infectious milk-borne particle from mother to offspring. Infected B cells in the gut-associated lymphoid tissue present the SAG at their surfaces to T cells. A significant number of T cells (about 1 in 20) that express the relevant T-cell receptor  $\beta$  chain variable domain (TCR V $\beta$ ) are activated. Both the infected B cells and activated T cells proliferate, followed by cytokine release. This is a critical step to promote amplification and integration of MMTV by augmenting the number of virus-infected lymphocytes. The MMTV LTR contains enhancer and promoter elements, hormone-responsive elements, TEF-1 family elements (5) as well as the ORF of the SAG, which plays an important role in pathogenesis in mouse mammary tumorigenesis (3, 4).

We have investigated whether a MMTV-like agent might play a role in human breast carcinogenesis (6, 7). We have found that a

660-bp sequence homologous to the *env* gene of MMTV is present in 38% of the human breast cancers tested, but not in normal breasts or in other tumors or tissues (8, 9). The prevalence of the sequence is higher in gestational breast cancer (10). Similar findings have since been reported by Etkind *et al.* (11) in breast cancer and lymphomas and by Ford *et al.* (12) in breast cancers from Australia and Vietnam. Expression of this 660-bp sequence has been detected in 66% of *env*-positive tumors using reverse transcription-PCR (13). Finally, a 9.9-kb provirus that is 95% homologous to MMTV but only 57% homologous to human endogenous retrovirus K10, the human endogenous retrovirus most homologous to MMTV, has been amplified with nested extra-long PCR and primer walking from two human breast cancer patients. Fluorescence *in situ* hybridization with a 2.7-kb *env*-LTR probe showed that this MMTV-like sequence is monoallelic inserted in several chromosomes of an *env*-positive primary breast cancer cell line, but not in those of normal breast cells (14). The provirus exhibits typical features of a replication-competent virus. Cloning and sequencing of a complete 1.2-kb 3' MMTV-like LTR displayed an ORF for the SAG and a hormone-responsive element homologous to those of MMTV (15). These results may suggest that this sequence could be functionally involved in human breast cancer development.

To explore whether the human *sag* gene found in human breast cancer has structural and functional features similar to those of mouse *sag*, the *sag* gene sequences from 10 human isolates were sequenced and compared with the mouse gene. Moreover, the human *sag* gene was cloned into an expression vector, and functional assays were performed. The results indicate that the human SAG expressed in human B cells can activate human T-cell functions, thus suggesting that it may play a role in human breast cancer pathogenesis.

## MATERIALS AND METHODS

**Cells.** Ramos, an EBV-negative human B-cell line, was obtained from the American Type Culture Collection (Manassas, VA) and maintained as recommended by the manufacturer. Human T cells were isolated from peripheral blood mononuclear cells with CD3 MicroBeads (MACS, Auburn, CA) following the protocol provided by the manufacturer. The T cells were cultured in RPMI 1640 (Life Technologies, Inc., Rockville, MD) plus 10% FCS (ICN Biochemicals, Costa Mesa, CA).

**PCR.** DNA was isolated from human tumors as described previously (8). The GeneAmp Kit (Perkin-Elmer Cetus, Norwalk, CT) was used to amplify the *sag* gene. The reaction contained 500 ng of genomic DNA and 100 ng of each primer in a total volume of 50  $\mu$ l. To obtain an intact *sag* sequence, primers were designed using the Genetics Computer Group Wisconsin Package program. Their sequences are described in Table 1. Amplification conditions were as follows: denaturing at 95°C for 1.5 min, annealing at 62°C for 2 min, and extension at 72°C for 2 min for 35 cycles; and a final extension at 72°C for 7 min. Analysis of the PCR products was done by 1% agarose gel electrophoresis. To confirm the PCR results, Southern hybridization was performed using a labeled internal oligonucleotide. The oligonucleotide was terminal labeled with [<sup>32</sup>P]ATP (New England Nuclear, Boston, MA) by the T4 polynucleotide kinase, and purified with a TE MIDI SELECT G-25 column (5 Prime  $\rightarrow$  3 Prime, Inc., Boulder, CO) following the protocol supplied by the manufacturer. The hybridization solution contained 5 $\times$  saline/sodium phosphate/EDTA, 2.5 $\times$  Denhardt's solution, 0.1% SDS, and 100  $\mu$ g/ml calf thymus DNA. Prehy-

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Table 1 Primer and probe sequences and location in the MMTV<sup>a</sup> 3' LTR

Primers	Sequence (5' to 3')	Location
LTRorf-5	ATGCCGCGCCTGCAGCAGCAGAA	8698-8722
LTRorf-3	TTATTTATTATACCTTATGTCAAA	9637-9661
LTR5	GGTGCAACACGGGACTTAT	9308-9327

<sup>a</sup>MMTV, mouse mammary tumor virus; LTR, long terminal repeat.

bridization took place at 42°C for 4 h, followed by hybridization at 42°C overnight in the same solution with the addition of 1 × 10<sup>6</sup> cpm/ml radiolabeled probe. The filter was washed with 2× SSC and 0.1% SDS twice for 15 min and exposed to Kodak film overnight at -70°C.

**Cloning and Sequencing.** The PCR products were ligated into pCRII vector from Original TA Cloning Kit (Invitrogen, San Diego, CA) and transformed into INV<sup>F</sup> One Shot competent cells (Invitrogen), and the white colonies were selected. Isolation and purification of plasmid were done using the Plasmid Maxi Kit (Qiagen, Chatsworth, CA). EcoRI digestion and 1% agarose gel electrophoresis as well as Southern blot hybridization were performed to detect the cloned insert. The cloned DNA was sequenced by automated DNA sequencing. The resulting sequences were compared with

known sequences in the GenBank using the Genetics Computer Group Wisconsin Package program.

**Western Blot Analysis.** Ramos cells were washed with 1× PBS and lysed with 0.5 ml of lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, and freshly added protein inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) per T75 flask (Fisher HealthCare, Houston, TX). The lysate was transferred to a microcentrifuge tube, incubated on ice for 20 min, and then centrifuged at 13,000 × g for 5 min at 4°C. The protein concentration in the supernatant was determined by the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules CA). Lysates containing 80 μg of protein were purified with the His bind purification kit (Novagen, Inc.), electrophoresed through a 10% SDS-polyacrylamide gel, and finally transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). Expression of LTR SAg was detected using a monoclonal anti-herpes simplex virus (HSV) antibody (Ab) as a primary Ab at a 1:6,400 dilution and horseradish peroxidase-labeled antimouse IgG as a secondary Ab at a 1:2,000 dilution. Subsequently, a rapid immunodetection was done using the enhanced chemiluminescence Western blotting protocol (Amersham Life Science). Actin expression was detected using an anti-total actin Ab (Santa Cruz Biotechnology Biochemistry) as a primary Ab at a 1:4,000 dilution and an alkaline phosphatase-

Fig. 1. Comparison of the COOH-terminal long terminal repeat superantigen sequences from human breast cancer with those from mouse mammary tumor virus (BR<sub>6</sub> strain, GenBank accession number M15122). Red letters indicate the region in which specific deletions and insertions occurred in human isolates.

	1		50
MMTVBR6-C-Sag	GTTGACTCAG	GAAGAGAAAA	ACGACATGAA ACAACAGGTA CATGATTATA
BB-C-Sag	GTTGTCTCAA	GAGGAAAAAG	ACGACATGAA ACAACAGGTA CATGATTATA
A488-C-Sag	GTTGTCTCAA	GAAGAAAAAG	ACGACATGAA ACAACAGGTA CATGATTATA
A658-C-Sag	GTTGTCTCAA	GAGGAAAAAG	ACGACATGAA ACAACAGGTA CATGATTATA
R23-C-Sag	GTTAACTCAA	AAAGAAAAAG	ACGACATGAA ACAACAGGTA CATGATTATA
R46-C-Sag	GTTAACTCAA	AAAGAAAAAG	ACGACATGAA ACAACAGGTA CATGATTATA
I84A-C-Sag	GTTAACTCAA	AAAGAAAAAG	ACGACATGAA ACAACAGGTA CATGATTATA
A612-C-Sag	GTTGTCTCAA	GAAGAAAAAG	ACGACATGAA ACAACAGGTA CATGATTATA
R29-C-Sag	GTTAACTCAA	AAAGAAAAAG	ACGACATGAA ACAACAGGTA CATGATTATA
R16-C-Sag	GTTAACTCAA	AAAGAAAAAG	ACGACATGAA ACAACAGGTA CATGATTATA
A735-C-Sag	GTTGTCTCAA	NAAGAAAAAG	ACGACATGAA ACAACAGGTA CATGATTATA
	51		100
MMTVBR6-C-Sag	TTTATTTGGG	AACAGGAATG	AGCAGCATTT GGGGAAAGAT TTTTC...AT
BB-C-Sag	TTTATCTAGG	AACAGGAATG	...CACTTTT GGGGAAAGAT TTTCC...AT
A488-C-Sag	TTTATCTAGG	AACAGGAATG	...CACTTTT GGGGAAAGAT TTTCC...AT
A658-C-Sag	TTTATCTAGG	AACAGGAATG	...CACTTTT GGGGAAAGAT TTTCC...AT
R23-C-Sag	TTTATCTAAG	AACAAGAATA	AGC . GTTT . GGAAAAAGAT TTTTCATTAT
R46-C-Sag	TTTATCTAAG	AACAAGAATA	AGC . GTTT . GGAAAAAGAT TTTTCATTAT
I84A-C-Sag	TTTATCTAAG	AACAAGAATA	AAC . GTTT . GGAAAAAGAT TTTTCATTAT
A612-C-Sag	TTTATCTAGG	AACAGGAATG	GAC . GTTT . GGGGAAAGAT TTTTCATTAT
R29-C-Sag	TTTATCTAAG	AACAAGAATA	AAC . GTTT . GGGGAAAGAT TTTTCATTAT
R16-C-Sag	TTTATCTAAG	AACAAGAATA	AAC . GTTT . GGAAAAAGAT TTTTCATTAT
A735-C-Sag	TTTATCTAGG	AACAGGAATG	AAC . GTTTG GGGGAAAGAT TTTTCATTAT
	101		150
MMTVBR6-C-Sag	ACCAAGGAGA	GGACAGT . GG	CTGCACTAAT AGAGCAC . TA TTCTG . CAAA
BB-C-Sag	ACCAAGGAGG	GGACAGT . GG	CTGGACTAAT ANAACAT . TA TTCTG . CAAA
A488-C-Sag	ACCAAGGAGG	GGACAGT . GG	CTGGACTAAT AGAACAT . TA TTCTGCCAAA
A658-C-Sag	ACCAAGGAGG	GGACAGTGGG	CTGGACTAAT AGAACAT . TA TTCTG . CAAA
R23-C-Sag	ACCAAGGAGG	GGGCAGTGGC	TAGACAATT . . AGAACACA TTTCTGCGGA
R46-C-Sag	ACCAAGGAGG	GGGCAGTGGC	TAGACAATT . . AGAACACA TTTCTGCGGA
I84A-C-Sag	ACCAAGGAGG	GGGCAGTGGC	TAGACAATT . . AGAACACA TTTCTGCGGA
A612-C-Sag	ACCAAGGAGG	GGGCAGTGGC	TAGACAATT . . AGAACACA TTTCTGCGGA
R29-C-Sag	ACCAAGGAGG	GGGCAGTGGC	TAGACAATT . . AGAACACA TTTCTGCGGA
R16-C-Sag	ACCAAGGAGG	GGGCAGTGGC	TAGACAATT . . AGAACACA TTTCTGCGGA
A735-C-Sag	ACCAAGGAGG	GGGCAGTGGC	TAGAACAATT AGAACACA TTTCTGCGGA
	151		177
MMTVBR6-C-Sag	GACTTATGGC	ATGAGTTATT	ATGATTA
BB-C-Sag	AACTTATGGC	ATGAGTTATT	ATGAATA
A488-C-Sag	AACTTATGGC	ATGAGTTATT	ATGAATA
A658-C-Sag	AACTTATGGC	ATGAGTTATT	ATGAATA
R23-C-Sag	TACCTTTGAC	ATAAGGTATA	ATAAATA
R46-C-Sag	TACCTTTGAC	ATAAGGTATA	ATAAATA
I84A-C-Sag	TACCTTTGAC	ATAAGGTATA	ATAAATA
A612-C-Sag	TACTTTTGGC	ATGAGCTATA	ATGGATA
R29-C-Sag	TACCTTTGAC	ATAAGGTATA	ATAAATA
R16-C-Sag	TACCTTTGAC	ATAAGGTATA	ATAAATA
A735-C-Sag	TACTTTTGGC	ATGAGCTATA	ATGGATA

MMTVBR6-C-Sag	LTQEEKNDMK	QQVHDYIYLG	TGMSSIWGKI	FH .TKERTV
BB-C-Sag	LTQEEKDDMK	QQVHDYIYLG	TGM.HFWGKI	FH .TKEGTV
A488-C-Sag	LTQEEKDDMK	QQVHDYIYLG	TGM.HFWGKI	FH .TKEGTV
A658-C-Sag	LTQEEKDDMK	QQVHDYIYLG	TGM.HFWGKI	FH .TKEGTV
R23-C-Sag	LTQKEKDDMK	QQVHDYIYLR	TRI.SVWKKI	FH.YTKEGAV
R46-C-Sag	LTQKEKDDMK	QQVHDYIYLR	TRI.SVWKKI	FH.YTKEGAV
I84A-C-Sag	LTQKEKDDMK	QQVHDYIYLR	TRI.NVWKKI	FH.YTKEGAV
A612-C-Sag	LSQEEKDDMK	QQVHDYIYLG	TGM.DVWGKI	FH.YTKEGAV
R29-C-Sag	LTQEEKDDK	QQVHDYIYLR	TGM.NVWKKI	FH.YTKEGAV
R16-C-Sag	LTQEEKDDIK	QQVHDYIYLG	TGM.NVWGKI	FH.XTXXGAV
A735-C-Sag	LSQXKDDMK	QQVHDYIYLG	TGM.NVWGKD	FSLYQGGGSG

Fig. 2. Comparison of the COOH-terminal long terminal repeat superantigen peptides from human breast cancer with those from mouse mammary tumor virus BR<sub>6</sub>. \*, stop codon.

MMTVBR6-C-Sag	AALIEHYSK	TYGMSYYD*	41	59
BB-C-Sag	AGLIXHYSK	TYGMSYYE*		
A488-C-Sag	AGLIEHYSK	NLWHELL*		
A658-C-Sag	GWTNRTLFCK	NLWHELL*		
R23-C-Sag	ARQLEHISAD	TFDIRYNK*		
R46-C-Sag	ARQLEHISAD	TFDIRYNK*		
I84A-C-Sag	ARQLEHISAD	TFDIRYNK*		
A612-C-Sag	ARQLEHISAD	TFGMSYNG*		
R29-C-Sag	ARQLEHISAD	TFXMRYNK*		
R16-C-Sag	AXQXXHIXX	TXGMSYNG*		
A735-C-Sag	ANNXKQHFCR	YFWHELL*		

conjugated donkey anti-goat IgG (ICN Biomedicals, Aurora, OH) as a secondary Ab at a 1:2,000 dilution. The signal was developed with nitroblue tetrazolium (Sigma, St. Louis, MO) and 5-bromo-4-chloroindolyl phosphate (Sigma), as shown in Fig. 6.

**Construction of pLTR SAg COOH-Terminal Fusion Protein.** The TriEx-1.1 expression vector (Novagen, Inc.) was used to construct a His-tagged LTR SAg protein plasmid named pLTRSag. A 5' primer, LTRsag-5, was designed to remove the LTR SAg initiating ATG codon and to create a 5' *NcoI* site. The 3' primer, LTRsag-3, took away the stop codon of the LTR SAg and made a 3' *EcoRI* site. To amplify the insert, the primers LTRsag-5 (5'-CCGGAATTCGGTTTATTATACCTTTGTCA-3') and LTRsag-3 (5'-AAATAGGTCTCACATGCCGCGCCTGCAGCA-3') were used under the following conditions: initial denaturation at 94°C for 2 min for 1 cycle; 30 cycles of denaturation (94°C, 45 s), annealing (55°C, 30 s), and extension (72°C, 60 s); followed by a final extension at 72°C for 10 min.

The amplified cDNA and TriEx-1.1 vector were digested with *NcoI* and *EcoRI*, gel purified, and ligated with T4 DNA ligase at 16°C for 15 min to get pLTR SAg. One  $\mu$ l of the ligation reaction was transfected into NovaBlue Singles competent cells.

Sequencing analysis of pLTR SAg clone revealed that the inserted sequence has a 100% similarity with the original PCR product. Furthermore, 1  $\mu$ l of a 1:50 dilution of a miniprep (~1 ng of plasmid) was transformed into Tunner (DE3) *placI* competent cells. The *pLacI* plasmid carried by this host is compatible with pTriEx recombinants and serves as the source for additional *lac* repressor, which is necessary for isopropyl-1-thio- $\beta$ -D-galactopyranoside-inducible expression of target genes in *Escherichia coli* using pTriEx vector.

**Expression of LTR SAg Fusion Protein.** Tunner cells were transformed with pLTRSag and grown in LB media containing 50  $\mu$ g/ml ampicillin at 37°C with shaking at 250 rpm, to reach an  $A_{600\text{ nm}}$  of approximately 0.5–1.0. Isopropyl-1-thio- $\beta$ -D-galactopyranoside was added to a final concentration of 1 mM for full induction. Another aliquot served as uninduced control. The liquid culture was incubated at 37°C with shaking for 4 h. Cells from the liquid culture were harvested by centrifugation at 10,000  $\times$  g for 10 min, and the pellet was completely resuspended at room temperature in BugBuster (Novagen, Inc.) reagent by gentle vortexing. The cell suspension was incubated on a shaking platform at a slow setting for 15 min at room temperature. The insoluble cell debris was removed by centrifugation at 16,000  $\times$  g for 20 min at 4°C. The soluble extract was loaded directly onto SDS-PAGE. The pellet was resuspended in BugBuster reagent, and lysozyme was added to a final concentration of 200  $\mu$ g/ml. The inclusion bodies were collected by centrifuging the suspension at 16,000  $\times$  g for 15 min at 4°C. The insoluble pellet was washed several times by resuspending it in 20 mM Tris-HCl (pH 7.5), and the supernatant was removed by centrifugation at 10,000  $\times$  g for 5 min. The final pellet was resuspended in 1% SDS with heating

and vigorous mixing. To verify the expression of the LTR SAg fusion protein, 10% SDS-PAGE and Western blot analysis with anti-HSV monoclonal Ab were performed as shown in Fig. 4.

**Cotransfection.** Although the TriEx-1.1 expression vector is designed to allow target gene expression in vertebrate cells, *E. coli*, and baculovirus insect cells, it lacks a selection marker in the mammalian system. For that reason, we cotransfected pLTRSag and pEGFP-1 (plasmid for enhanced green fluorescent protein; BD Biosciences Clontech, Palo Alto, CA), which carries the neomycin-selective marker and allows G418 selection into Ramos cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the protocol provided by the manufacturer. A TriEx-1.1 vector without the insert was also cotransfected with pEGFP under the same conditions, as a mock control. The positive transfectants were sorted by flow cytometric analysis via standard methods using a FACS 440 (Becton Dickinson, Mountain View, CA). Two pLTRSag-positive Ramos cell lines named pLTRSAG-1, and pLTRSAG-2 were established after selection with G418 (Life Technologies, Inc.).

**Immunofluorescence Assay.** Ramos cells (transfectants, mock transfectants, and parental cells) were plated on a multiple sites slide (Carlson Scientific, Inc., Peotone, IL). The slide was air dried and fixed with 50% methanol for 10 min at 4°C, followed by two washes with 1 $\times$  PBS. Incubation for 1 h at room temperature with the primary Ab (anti-HSV Ab) at a 1:4000 dilution was performed, followed by three washes with 1 $\times$  PBS and incubation with the secondary Ab (goat antimouse IgG1-FITC; Southern Biotechnologies, Birmingham, AL) at a 1:2000 dilution for 1 h at room temperature. Afterward, the slide was washed four times with a large volume of 1 $\times$  PBS, air dried, and sealed with coverslips. Cells were examined under a fluorescence microscope.

**T-Cell Proliferation Assay.** Human T cells from normal donors were purified with a CD3<sup>+</sup> T-cell enrichment column. An aliquot containing 1  $\times$  10<sup>5</sup> T cells and 1  $\times$  10<sup>4</sup> irradiated (3000 rads) pLTRSag-transfected Ramos cells was cocultured in 1 well of a 96-well plate for 72 h. After 60 h of culture, the cells were pulsed with 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine for 12 h (New England Nuclear). All cultures were harvested and counted by liquid scintillation spectroscopy.

**ELISA.** T-cell enrichment column-isolated human CD3<sup>+</sup> T cells were cocultured with irradiated (3000 rads) pLTRSag-transfected Ramos cells at a ratio of 10:1 cells per well in a 96-well plate. Supernatants were harvested after 72 h, and cytokines were measured by ELISA (R&D Systems Europe, Oxford, United Kingdom). Controls included untreated T cells, empty vector-transfected Ramos cells (mock-transfected cells), and untransfected Ramos cells.

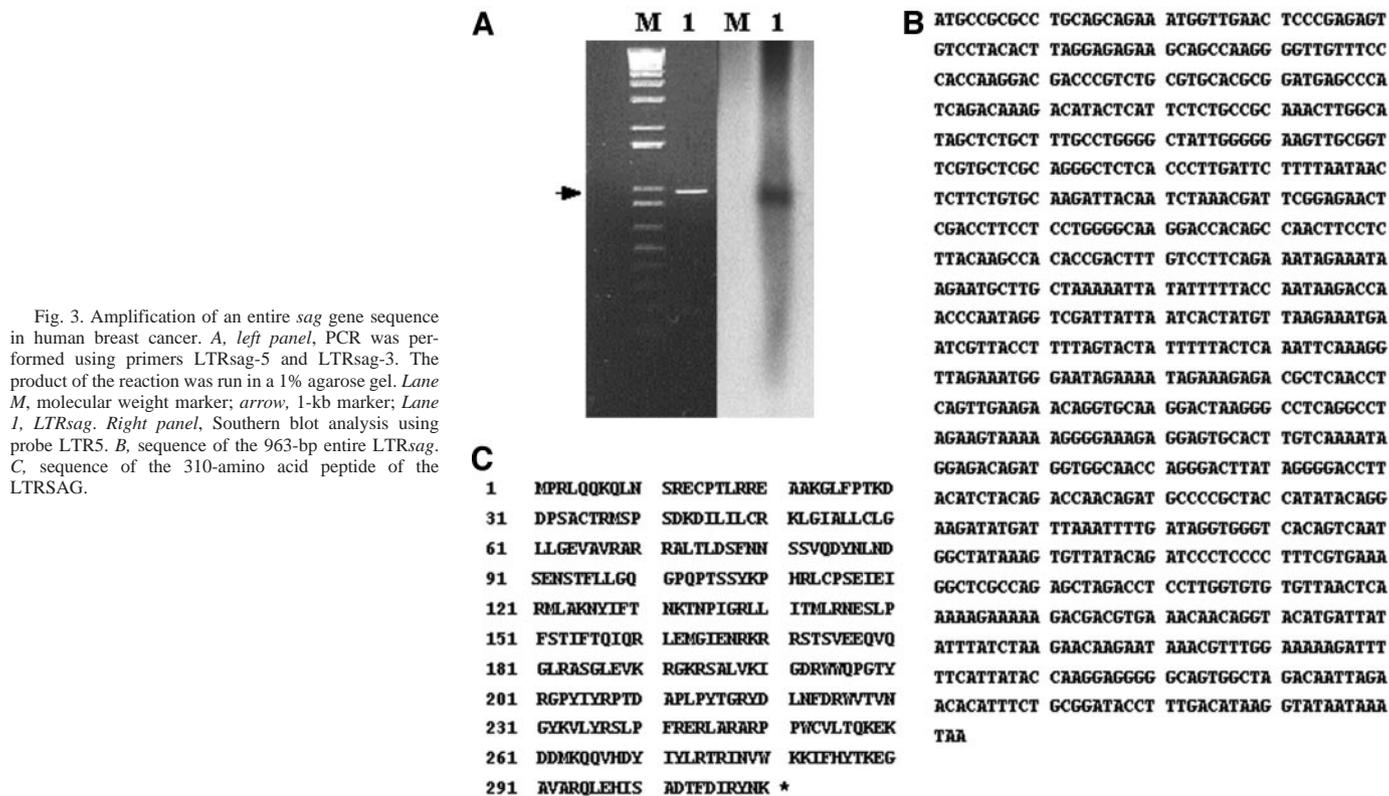


Fig. 3. Amplification of an entire *sag* gene sequence in human breast cancer. **A**, left panel, PCR was performed using primers LTRsag-5 and LTRsag-3. The product of the reaction was run in a 1% agarose gel. Lane M, molecular weight marker; arrow, 1-kb marker; Lane 1, LTRsag. Right panel, Southern blot analysis using probe LTR5. **B**, sequence of the 963-bp entire LTRsag. **C**, sequence of the 310-amino acid peptide of the LTRsag.

## RESULTS

**Sequence Differences between the COOH-Terminal of LTR *sag* of MMTV and the Human LTRs.** Although the human MMTV-like LTR sequences have a high homology to MMTV (15), sequencing of 10 COOH-terminal LTR 177-bp *sag* human isolates showed that there are certain specific deletions and insertions at the COOH-terminal of the *sag* sequences that clearly differentiate them from the MMTV (Fig. 1). The predicted peptide sequences also revealed a variable region in the COOH-terminal of the SAg in the human-derived tumors as shown in Fig. 2. Differences between the human isolates can also be observed. Comparison with other strains of MMTV revealed that there are other specific deletions and insertions (data not shown).

**Amplification of an Entire *sag* Sequence from Human Breast Cancer.** To obtain an intact *sag* sequence, PCR was performed in a MMTV-like LTR-positive breast cancer sample (I84) with a pair of primers, LTRorf-5 and LTRorf-3 (Table 1), which were designed based on the MMTV sequence to amplify the ORF from the start codon to the stop codon. A 963-bp PCR product was obtained, which hybridized with the internal probe LTR5 (Fig. 3). Analysis of the sequence showed that it has a complete ORF without any early stop codon and a 96% similarity to MMTV *sag*, but no similarity to the human endogenous retrovirus K10 LTR because the LTR of human endogenous retrovirus K10 is much shorter than the analyzed sequence and only carries a truncated *sag* sequence. Sequencing of the inserted sequence showed a 100% similarity with the original PCR product. A predicted amino acid sequence revealed a high homology to MMTV SAg protein, with the exception of some COOH-terminal variations as shown in Fig. 2.

**Expression of MMTV-like LTR SAg COOH-Terminal-Tagged Fusion Protein in *E. coli*.** To construct a LTRsag expression plasmid, the initiating ATG codon in the 5' end of the *sag* was removed, a *Nco*I site was created by PCR with primer LTRsag-5 (5'-AATAG-GTCTCACATGCCGCGCCTGCAGCA-3'), and the stop codon of

the 3' end of the *sag* was replaced by an *Eco*RI site, which was generated with primer LTRsag-3 (5'-CCGGAATTCGGTTTATTAT-ACCTTATGTCA-3'). The entire *sag* sequence with two modified ends was then ligated into a TriEX-1.1 expression vector that encodes optional HSV tag and His tag sequences at the distal end of the multiple cloning site to enable the construction of COOH-terminal-tagged fusion proteins.

LTR SAg fusion protein was purified with the His purification column and identified by Western blot with monoclonal HSV Ab. The fusion protein migrated as a  $M_r$  37,000 peptide, the expected molecular weight as shown in Fig. 4.

**Cotransfection and Expression of pLTRsag in a Human B-Cell Line.** The human B-cell line Ramos, was cotransfected with pLTRsag and pEGFP. The ratio of untransfected cells:cotransfected

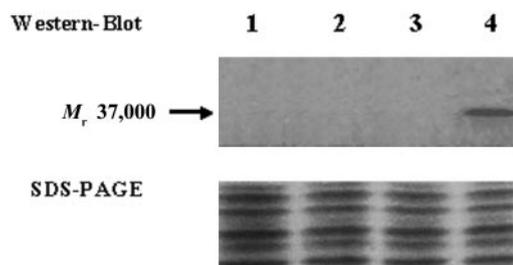
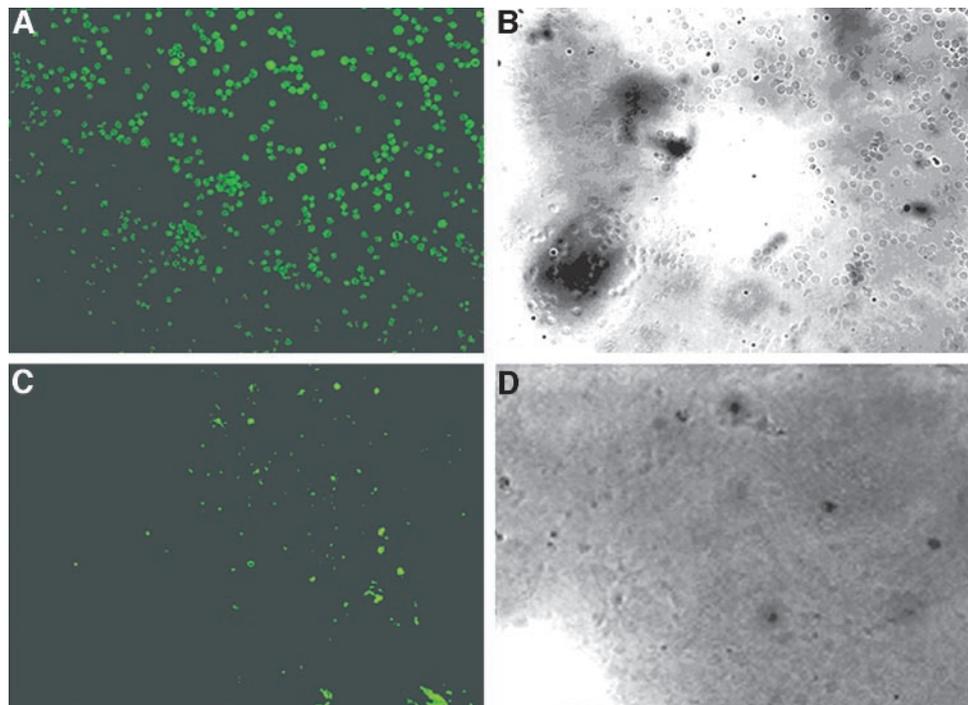


Fig. 4. Expression of mouse mammary tumor virus-like long-terminal repeat superantigen in *E. coli*. Bottom panel, SDS-PAGE. Expression was induced with isopropyl-1-thio- $\beta$ -D-galactopyranoside, and the protein lysate was prepared as described in "Materials and Methods." Approximately 80  $\mu$ g of protein lysate were loaded per lane and resolved on 10% SDS-polyacrylamide gel. To normalize for loading, the gel was visualized by staining with Coomassie Blue. Top panel, Western blot analysis. The proteins were electrophoretically transferred from the SDS-polyacrylamide gel onto polyvinylidene difluoride membrane, and immunodetection was performed using the enhanced chemiluminescence Western blotting protocol. An anti-herpes simplex virus monoclonal antibody was used as a primary antibody. Lane 1, control supernatant; Lane 2, control pellet; Lane 3, induced supernatant; Lane 4, induced pellet.

Fig. 5. Transfection and expression of pLTRSAG in a human B-cell line. Ramos cells were transfected by using Lipofectamine 2000 (Invitrogen), and the fusion protein was detected by immunofluorescence assay. *A*, pLTRSAG-1-transfected Ramos cells observed under a fluorescence microscope. *B*, pLTRSAG-1-transfected Ramos cells observed under a light microscope. *C*, untransfected Ramos cells observed under a fluorescence microscope. *D*, untransfected Ramos cells observed under a light microscope.



cells was found to be only  $4:10^5$  after these cells were sorted by fluorescence-activated cell sorting. The cotransfected cells were then amplified and selected by G418 treatment. Two transfected cell lines were established, named pLTRSAG-1, and pLTRSAG-2. The transfected cells were analyzed by immunofluorescence assay. The results are shown in Fig. 5. As seen in Fig. 5A, most of the cells expressed the fusion protein. The expression of pLTRSAG was also investigated by Western blot as shown in Fig. 6. The TriEx-1.1 vector (without insert) and pEGFP-1 were transfected into Ramos cells. The fusion protein was only expressed in the pLTR SAG-1 and -2 cells. The function of the MMTV-like SAG in both cell lines was then explored by the T-cell proliferation assays and detection of cytokine release.

**Human T-Cell Proliferation Induced by MMTV-Like LTR SAG.** Three proliferation assay experiments, each done in triplicate, were performed. The results, as analyzed by StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA), showed a significant difference in proliferation between the T cells cocultured with pLTRSAG-transfected Ramos cells, mock-transfected Ramos cells, and untransfected Ramos cells ( $P < 0.001$ ) as seen in Fig. 7.

**Secretion of Cytokines from the Activated Human T Cells Stimulated by MMTV-Like LTR SAG.** The release of interleukin-2, interleukin-10, tumor necrosis factor  $\beta$ , and IFN- $\gamma$  from untreated T cells, activated T cells cocultured with pLTRSAG-1 or pLTRSAG-2,

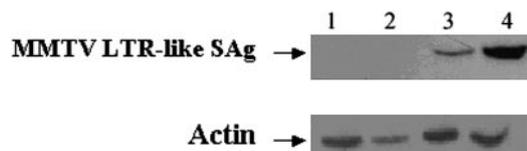


Fig. 6. Expression of mouse mammary tumor virus long terminal repeat (LTR)-like superantigen (SAG) in transfected human B cells. Western blot analysis was performed as described in "Materials and Methods." Protein lysates were prepared from  $10^7$  cells, and the fusion protein was purified by using a His bind purification kit (Novagen, Inc.). *Top panel*, expression of mouse mammary tumor virus LTR-like SAG was detected only from pLTRSAG-transfected human B-cell lines (pLTRSAG-1 and pLTRSAG-2). *Bottom panel*, expression of actin showed that a comparable amount of protein was loaded for all of the samples. *Lane 1*, control parental Ramos cell line; *Lane 2*, mock-transfected Ramos cells; *Lane 3*, pLTRSAG-2-transfected Ramos cells; *Lane 4*, pLTRSAG-1-transfected Ramos cells.

### T-cell proliferation

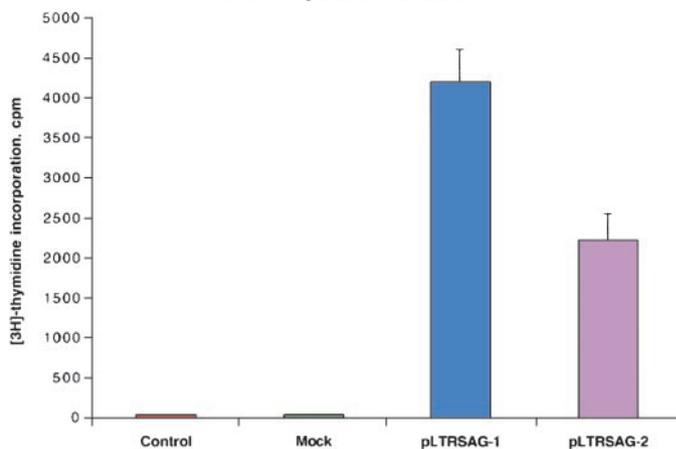


Fig. 7. Human T-cell proliferation induced by mouse mammary tumor virus-like long terminal repeat superantigen. Human T cells were isolated from CD3<sup>+</sup> T-cell enrichment column. T cells ( $10^5$ ), together with  $10^3$  irradiated (3000 rads) pLTRSAG-transfected Ramos cells (human B-cell line), were cocultured in each well of a 96-well plate for 72 h. After 60 h of culture, the cells were incubated with  $1 \mu\text{Ci/well}$  [ $^3\text{H}$ ]thymidine for 12 h (New England Nuclear). All cultures were harvested, and radioactivity was determined by liquid scintillation spectroscopy.

mock-transfected and untransfected Ramos cells was investigated by ELISA. Triplicates from three individual experiments were performed. The results were also analyzed by StatView 4.5. A strong stimulation by MMTV-like LTR SAG on cytokine secretion of human T-cells is revealed when compared with untreated T cells or T cells cocultured with untransfected or mock-transfected Ramos cells ( $P < 0.001$ ) as shown in Fig. 8.

### DISCUSSION

We have reported previously that a 630-bp MMTV-like LTR sequence was present in 41.5% of human breast cancer DNA and that an entire 1.2-kb LTR was cloned and sequenced from breast cancer (15).

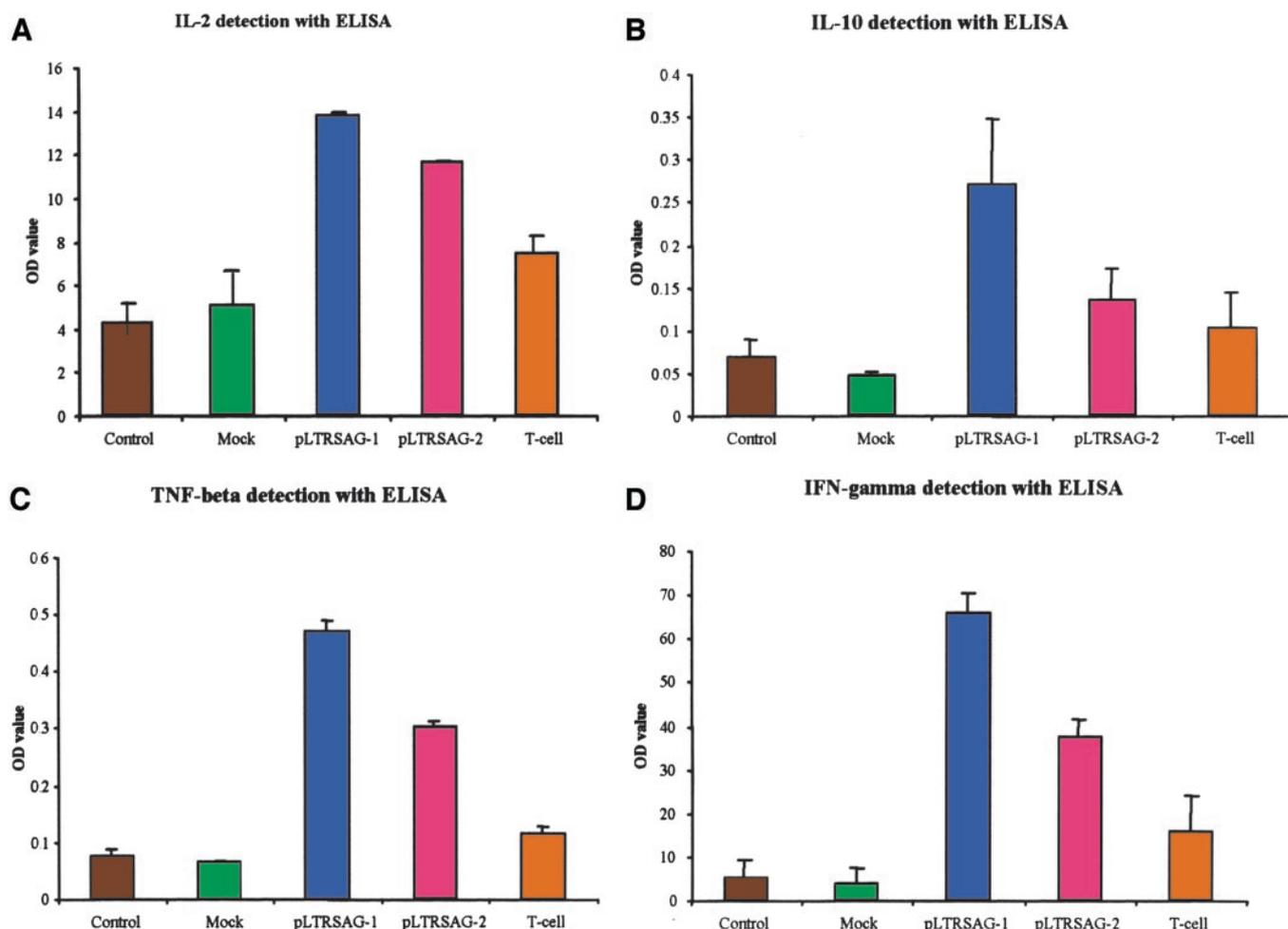


Fig. 8. Secretion of cytokines from the activated human T cells stimulated by mouse mammary tumor virus (MMTV)-like long terminal repeat (LTR) superantigen (SAG). T-cell enrichment column-isolated human CD3<sup>+</sup> T cells were cocultured with irradiated (3000 rads) pLTRSAG-transfected human B cells at a ratio 10:1 cells/well in a 96-well plate. Supernatants were harvested after 72 h, and cytokines were measured by ELISA (R&D Systems Europe). A, interleukin-2 secretion by MMTV-like LTR SAG-stimulated human T cells. B, interleukin-10 secretion by MMTV-like LTR SAG-stimulated human T cells. C, tumor necrosis factor  $\beta$  secretion by MMTV-like LTR SAG-stimulated human T cells. D, IFN- $\gamma$  secretion by MMTV-like LTR SAG-stimulated human T cells.

In this study, results of the cloning and sequencing indicated that 10 human partial 3' LTR isolates were highly homologous to the MMTV LTR *sag* gene, but not to the endogenous virus LTR. Unique differences in the MMTV-like LTR sequences were also found in the human isolates. Although these differences did not result in an early stop codon on the human MMTV-like *sag*, the possible effect on its function was unknown. To perform functional assays for this protein, amplification of an intact *sag* sequence by PCR and construction of a *sag* fusion protein expression plasmid (pLTRSAG) were necessary. Cotransfections with pLTRSAG and pEGFP into the human B-cell line Ramos revealed that the protein is expressed. The data from T-cell proliferation and cytokine release assays clearly indicate that this human LTR SAG can strongly stimulate human T-cell response, just as the MMTV LTR *sag* does in the mouse. Although these are *in vitro* assays, similar responses are thought to occur *in vivo*. The sequence differences do not appear to affect the role of the SAG. This is probably due to the fact that insertions and deletions are on the COOH-terminal sequences of the LTR SAG, a domain that is also variable in the mouse LTR. Because the MMTV LTR SAG plays an essential role in MMTV pathogenesis in mouse (3, 4, 16), it is reasonable to speculate that the human MMTV-like LTR SAG could also have a similar function in human breast.

The origin of the MMTV-like sequences is uncertain at the moment. An association between the frequency of breast cancer and the

presence of *Mus domesticus* has been established, thus implicating the mouse virus (17). Whether MMTV infects human cells is controversial. Infection of human epithelial cells by some variants of MMTV has been shown in the past (18), but recently, Zhang *et al.* (19) were not able to show infectivity in human cells. By contrast, Xu *et al.* (20) showed that MMTV can infect human epithelial biliary cells and induce manifestations of primary biliary cirrhosis. Additional studies on receptor expression in different tissues and MMTV variants are necessary to resolve this issue.

This is the first demonstration that *sag* sequences isolated from human breast cancer are able to perform similar functions to those in the mouse. The results suggest that an analogous mechanism to the mouse SAG might be operating in humans. It is important to emphasize that this likeness does not mean "causation." Epidemiological and virological requirements have to be fulfilled to prove that a virus causes malignancy (7). Our laboratory is actively working on these lines.

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