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# Mls-1 is encoded by the long terminal repeat open reading frame of the mouse mammary tumor provirus *Mtv-7*

(superantigen/T-cell activation/Vβ deletion)

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**ABSTRACT** **The murine Mls-1 antigen is the prototype of endogenous superantigens, molecules whose activities lead to deletion of T cells expressing certain T-cell receptor Vβ genes from the mature repertoire. However, Mls-1 also stimulates T cells expressing these particular Vβ genes (Vβ6, Vβ7, Vβ8.1, and Vβ9) *in vitro*, making it one of the strongest known T-cell activators. We have recently reported that the *Mls-1* gene is closely linked to the endogenous mammary tumor virus *Mtv-7*. We now demonstrate that Mls-1 is encoded by the open reading frame in the U3 region of the long terminal repeat of *Mtv-7*. However, control of expression of this molecule seems complex, depending on the promoter used for the transfection experiments. The sequence of the *Mtv-7* open reading frame differs from all other known mammary tumor virus open reading frame sequences in the 3′ end, suggesting that the T-cell receptor Vβ specificity is conferred by the C terminus of the molecule. The predicted structure of the protein encoded by the open reading frame is consistent with a type II transmembrane molecule where the C terminus is extracellular.**

The endogenous superantigens comprise a family of molecules that are recognized by the T-cell receptor (TCR) Vβ segment only, regardless of the third hypervariable region and α-chain expression. Since these antigens lead to deletion of T cells expressing particular Vβ genes, they have a profound effect on the mature T-cell repertoire (1–3). The mapping of a Vβ5 tolerizing element close to the endogenous mammary tumor virus (MMTV) *Mtv-9* provided the first hint of the molecular nature of this molecule (4). This observation prompted us to map the various Mls antigens in inbred mice, and we were able to identify Mls-1 with *Mtv-7* and to associate the genes encoding the Mls$^c$ phenotype with *Mtv-6*, *Mtv-13*, and *Mtv-1* (5). Other superantigens have also been associated with the presence of various endogenous and infectious mammary tumor viruses (6–8). Recently it has been shown that the open reading frame (ORF) in the U3 region of the MMTV long terminal repeat (LTR) of two infectious MMTVs and of the proviruses *Mtv-1*, *Mtv-3*, *Mtv-8*, and *Mtv-9* encodes superantigens (9–12).

We have extended these observations to show that the ORF of *Mtv-7* encodes Mls-1. However, from the results we obtained in transfection experiments we surmise that the control of expression of this activity is complex. We have determined the nucleotide sequence of the *Mtv-7* ORF and compared its predicted amino acid sequence to that of other MMTV ORFs. Two polymorphic regions were identified where the *Mtv-7*-derived sequence differs drastically from the others. It is likely that these segments contribute to TCR Vβ specificity and/or constitute superantigen/major histocompatibility complex class II contact sites.§

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## MATERIALS AND METHODS

**Cloning of *Mtv-7*.** Genomic DNA derived from the recombinant inbred mouse strain AKXD-15 was digested with *Eco*RI. A bacteriophage λ Gem-11 (Promega) library containing *Eco*RI fragments larger than 9 kilobases (kb) was prepared and screened for colonies hybridizing to a MMTV LTR probe (13). *Bam*HI-, *Pvu* II-, and *Sst* I-digested phage DNA from positive clones was analyzed by Southern blotting, using a MMTV *env* probe (13). One clone was obtained containing a 12-kb 3′ *Eco*RI junction fragment, consistent with *Mtv-7* (14). A probe specific for the flanking sequence was used in a Southern blot of DNA from recombinant inbred mice. Hybridization of this probe to a band differing in size between *Mtv-7*-positive and *Mtv-7*-negative strains confirmed the identity of the clone (5). The 3′ *Eco*RI fragment was subcloned into the plasmid vector Bluescript KS (Stratagene) (clone II-11, see Fig. 1 for orientation).

**Expression Vector Constructs.** The *Bam*HI–*Eco*RI fragment of the 3′ *Mtv-7* junction fragment was subcloned into the pBABE hygromycin-resistance vector (15) to prepare pMo-BE. This fragment contains the complete *Mtv-7 env* gene and LTR as well as 8 kb of flanking host sequences. To prepare pMo-ES, II-11 was digested with *Hin*dIII and religated, removing most of the flanking sequence. The *Eco*RI–*Sal* I fragment from this construct was subcloned into the pBABE puromycin-resistance vector (15). pMo-BS was made by subcloning the *Bam*HI–*Sca* I fragment containing the entire *Mtv-7 env* gene and parts of the LTR into the *Bam*HI–*Sna*BI sites of the pBABE hygromycin-resistance vector. *Sca* I cuts in the middle of the ORF; thus this vector allows expression of the *env* gene alone. For the pMo-BC construct, the *Hpa* II fragment containing the *Mtv-7 env* and U3 region of the LTR was subcloned into the *Cla* I site of Bluescript KS (Stratagene). A *Bgl* II–*Eco*RI fragment containing only the ORF was subcloned into the pBABE hygromycin-resistance vector. pMo-ES was digested with *Bgl* II and religated to make pMo-Bgl. This procedure removed the *env* gene; thus pMo-Bgl contains only the ORF. The ORF was cloned with PCR using the following set of primers (see Fig. 4*A* for primer binding sites): 5′-GGGAATTCATGCCGCGCCTGCAG-3′ and 5′-GGGTCGACAGATCTCCGCAAGTAGACCTG-3′. The amplified fragment was digested with *Eco*RI–*Sal* I and subcloned into the pBABE puromycin-resistance vector to prepare pMo-ORF. The PCR amplified ORF fragment was subcloned into the *Eco*RI–*Bgl* II sites of the pSP72 polylinker (Promega). The *Sal* I–*Bgl* II fragment of this construct was

Abbreviations: MMTV, mouse mammary tumor virus; Mo-MLV, Moloney murine leukemia virus; TCR, T-cell receptor; LTR, long terminal repeat; ORF, open reading frame; IL, interleukin; FcγR, γ chain of the Fc receptor.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M90535).

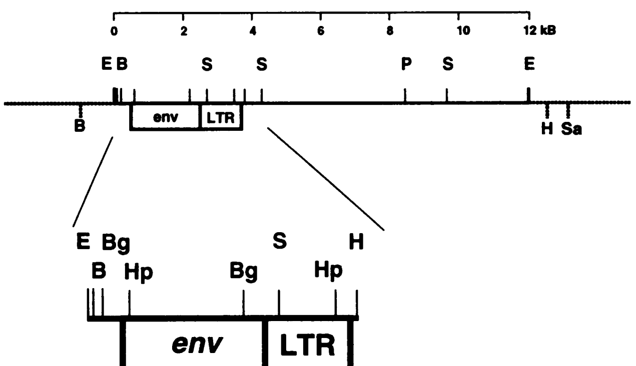



FIG. 1. Restriction map of the 12-kb *Eco*RI 3′ *Mtv-7* junction fragment. The upper map shows the whole fragment. Dotted lines indicate Bluescript sequences, and three restriction sites from the polylinker are shown for orientation. The lower map shows the *Eco*RI–*Hin*dIII fragment containing the 3′ *Mtv-7* sequences. B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa* II; S, *Sca* I; Sa, *Sal* I; P, *Pvu* II.

subcloned into the *Sal* I–*Bam*HI sites of pHβAPr-1-neo (16) to make pHβA-ORF (see Figs. 1 and 3).

**Southern Blot Analysis.** Digested genomic DNA was Southern blotted to Zetabind (AMF-Cuno), as described (17), and hybridized with a $^{32}$P-labeled MMTV LTR probe (13).

**Sequencing.** The plasmid II-11 and subclones thereof were used for DNA sequencing. Both strands were sequenced using Sequenase Version 2.0 (USB), with two plasmid-specific primers ("KS" and "SK") and six MMTV oligonucleotide primers, based on the MMTV (C3H) sequence and chosen for their conservation among other published MMTV sequences. MMTV plus strand primers were U3-3 (nucleotides 343–357), U3-1 (nucleotides 438–452), and U3-5 (nucleotides 647–661). Minus strand primers were U3-2 (nucleotides 1121–1106), U3-6 (nucleotides 828–814), and U3-4 (nucleotides 397–383) (see Fig. 4*A* for nucleotide sequences). Either double-stranded DNA or single-stranded DNA rescued with VCSM13 helper phage (Stratagene) was used as template.

**Cell Lines and Medium.** LBB.A, an Mls-1-expressing B-cell hybridoma derived from a fusion of BALB/c and RF/J parents, its Mls-1-negative variant LBB.11, and the Vβ6 T-cell hybridoma RG17 were a kind gift of A. Glasebrook (18). Cells were maintained in complete medium, consisting of RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum, 10 mM Hepes (pH 7.2), 50 μM mercaptoethanol, 2 mM glutamine, 100 units of penicillin per ml, and 100 μg of streptomycin per ml (JRH).

**Complement Lysis.** Ly17.1 is an allelic form of the FcγR that maps to the distal part of chromosome 1 near *Mtv-7* (19). LBB.A and LBB.11 cells were incubated on ice for 20 min with an anti-Ly17.1-specific monoclonal antibody (gift of S. Kimura) or an anti-E$^k$ monoclonal antibody (14.4.4s, ATCC), followed by complement treatment for 30 min at 37°C. Viability of the cells was assessed by trypan blue exclusion. Specific lysis was determined as 100 × (1 − cell count after monoclonal antibody plus complement/cell count after complement only).

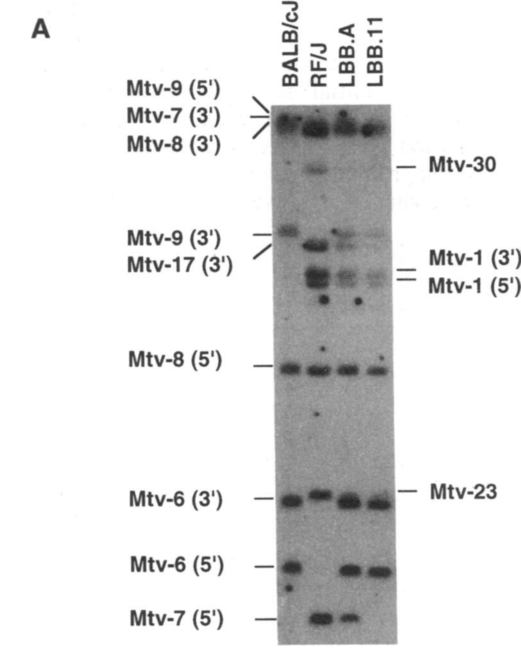
**Transfection.** pBABE-derived plasmids were linearized with *Not* I, and the pHβA-ORF plasmid was linearized using *Nde* I (both obtained from New England Biolabs). LBB.11 cells (5 × 10$^6$ per 0.5 ml of cold PBS) were electroporated at 1250 μF, 200 V (Andersen Systems, Brookline, MA), using 30 μg of DNA. Cells were cultured in complete medium at 5 × 10$^5$ per ml. After 2 days antibiotics were added at the following concentrations: hygromycin B (Boehringer Mannheim), 0.75 mg/ml; puromycin (Sigma), 2.5 μg/ml; G418 (GIBCO/BRL), 0.5 mg of active material per ml.

**Functional Assay.** Stable transfectants (5–10 × 10$^6$) were incubated overnight in 1 ml of medium containing 100 units of murine recombinant interleukin 4 (IL-4) (DNAX) to increase Mls expression (4). Untreated transfectants varied in their stimulatory capacity (results not shown). Cells were washed and mixed (1 × 10$^5$) with 2 × 10$^5$ Vβ6-positive RG17 T-cell hybrids (20) in 0.4 ml of complete medium and incubated for 24 hr. All groups were set up in duplicates. Plates were frozen, and 100 μl of thawed supernatant was tested in quadruplicate for IL-2 concentration, using the IL-2-dependent cell line HT-2. Proliferation of the HT-2 cells was measured by incorporation of [$^3$H]thymidine (ICN). Expression of Mls-1 was quantitated by comparing the [$^3$H]thymidine incorporation obtained after stimulation with transfected cells to that obtained with the untransfected cell line LBB.11 (stimulation index). Stimulation indexes >5 were considered positive for Mls-1 expression. The positive control cell line LBB.A yielded stimulation indexes between 70 and 200.

## RESULTS AND DISCUSSION

**Identification of a Competent Recipient Cell Line for Transfection Studies.** The expression of endogenous superantigens

A

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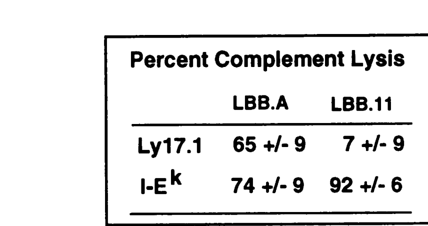

Percent Complement Lysis

| | LBB.A | LBB.11 |
|---|---|---|
| Ly17.1 | 65 +/- 9 | 7 +/- 9 |
| I-E$^k$ | 74 +/- 9 | 92 +/- 6 |

FIG. 2. The Mls-1-negative variant of LBB.A, LBB.11, has lost a fragment of chromosome 1, bearing *Mtv-7* and the Ly17.1 allele of the γ chain of the Fc receptor (FcγR). (*A*) Southern blot analysis. Genomic DNA from BALB/c and RF/J spleen cells and LBB.A and LBB.11 B-cell hybridomas was digested with *Pvu* II and hybridized to a MMTV-specific $^{32}$P-labeled probe. The junction fragments of the various *Mtv* proviruses are marked. (The RF/J genome contains at least one other *Mtv* provirus not readily distinguished in this experiment.) LBB.A has only one copy of *Mtv-7*, whereas LBB.11 is *Mtv-7*-negative. (*B*) Expression of the Ly17.1 allele on LBB.A and LBB.11 cells.

is complex and poorly understood, potentially complicating efforts to identify DNA sequences encoding them. Although primary B cells express Mls activity (20, 21), expression is abrogated in most B-cell lines. The mechanism for this suppression is unknown, but we were concerned that it might also operate on transfected genes. Only one *in vitro* maintained B-cell hybridoma, LBB.A, is known to express Mls-1 (18). We determined by Southern blotting that an Mls-1 loss variant of LBB.A, LBB.11, has lost *Mtv-7* (Fig. 2). Furthermore, we established by tissue typing that this cell line does not express the Ly17.1 allele of the Fc$\gamma$R, which is closely linked to Mls-1 on chromosome 1 (19) (Fig. 2). Thus, this cell line has suffered a deletion of part of chromosome 1 encoding Mls-1, yet still expresses a V$\beta$3 stimulatory Mls$^c$ phenotype (results not shown), presumably encoded by the two MMTV proviruses *Mtv-1* and *Mtv-6* (5). This cell line was used as recipient for our transfection studies.

**Cloning of *Mtv-7* and Expression Studies.** To test directly whether Mls-1 is encoded by *Mtv-7*, we cloned this provirus from the recombinant inbred mouse strain AKXD-15. A 12-kb *Eco*RI fragment, containing the *env* gene, the 3′ LTR, and about 8 kb of flanking host sequences, was isolated and subcloned into the pBABE hygromycin-resistance vector (15), which contains the Moloney murine leukemia virus (Mo-MLV) LTR as promoter/enhancer (pMo-BE). This construct was made to ensure a high level of expression of the *env* gene product, our initial candidate for Mls-1, since *env* encodes a known cell surface molecule (22). LBB.11 cells stably transfected with this construct expressed the Mls-1 phenotype when preincubated with IL-4 (Fig. 3). Without this treatment somewhat erratic results were obtained (results not shown). This lymphokine has been shown to potentiate the response to other endogenous superantigens (4).

To further define the sequences encoding Mls-1, we tested various deletion constructs. First, removal of the 8-kb flanking sequence had no effect on activity, confirming the role of MMTV sequences (pMo-ES). Surprisingly, however, neither the *env* (pMo-BS) nor the LTR ORF alone (pMo-BC) conferred significant Mls-1 activity in conjunction with a Mo-MLV LTR (Fig. 3), regardless of IL-4 preincubation. Since these results seemed to contrast findings of other groups (9–12), we prepared two additional constructs containing the *Mtv-7* ORF—one by using other restriction sites for isolating this fragment (pMo-Bgl) and the other by cloning the ORF without additional sequences using the PCR. Since the same negative expression results were obtained with these constructs, we suspected that the apparent discrepancy in expression might reside with the specific promoter. Thus, we tested the ORF in conjunction with the human $\beta$-actin promoter, known to direct efficient expression in a wide variety of cells (16), and found high levels of activity (pH$\beta$A-ORF) (Fig. 3). The dependence on IL-4 seemed to be less pronounced in these transfectants (results not shown).

These experiments demonstrate that the ORF alone can confer Mls-1 activity, in agreement with findings that ORFs of other exogenous and endogenous MMTVs encode superantigen activity (9–12). However, these results also show an underlying complexity in the expression that we do not yet fully understand, given the effect of different promoters. An additional activity (called *naf*) assigned to the ORF has been reported to decrease expression for certain specific enhancer/promoters, such as the LTR of Rous sarcoma virus (23).

| 1<br>Promoter | 2<br>Genes expressed | 3<br>Cloned fragment | 4<br>Vector construct | 5<br>Stimulation index (Cell lines tested) |
|---|---|---|---|---|
| MoMLV LTR | *env* and *orf* | B LTR E | pMo-BE | 50 -110 (8) |
| | | E H<br>*env* *orf* | pMo-ES | 70 (1) |
| | *env* | B S<br>*env* | pMo-BS | 1.0 (3) |
| | *orf* | Bg Hp | pMo-BC | 1.0 - 2.0 (24) |
| | | Bg H | pMo-Bg | 1.0 - 2.0 (6) |
| | | (PCR)<br>*orf* | pMo-ORF | 1.0 (10) |
| Human β-Actin | *orf* | *orf* | pHβA-ORF | 90 - 150 (2) |

FIG. 3. Mls-1-specific stimulatory capacity of LBB.11 transfectants. Antigen-specific production of IL-2 by the RG17 T-cell hybridoma mixed with cell lines containing the indicated expression constructs was measured. All results shown are with transfectants that were preincubated with IL-4; the absence of IL-4 led to erratic results in some transfectants. Column 1 indicates the promoter used for the expression construct; column 2 shows the genes that can be expressed with this construct; column 3 shows the cloned fragments with the essential restriction sites; column 4 indicates the names of the constructs; column 5 shows the stimulation index. The number of transfectants tested is indicated in parentheses.

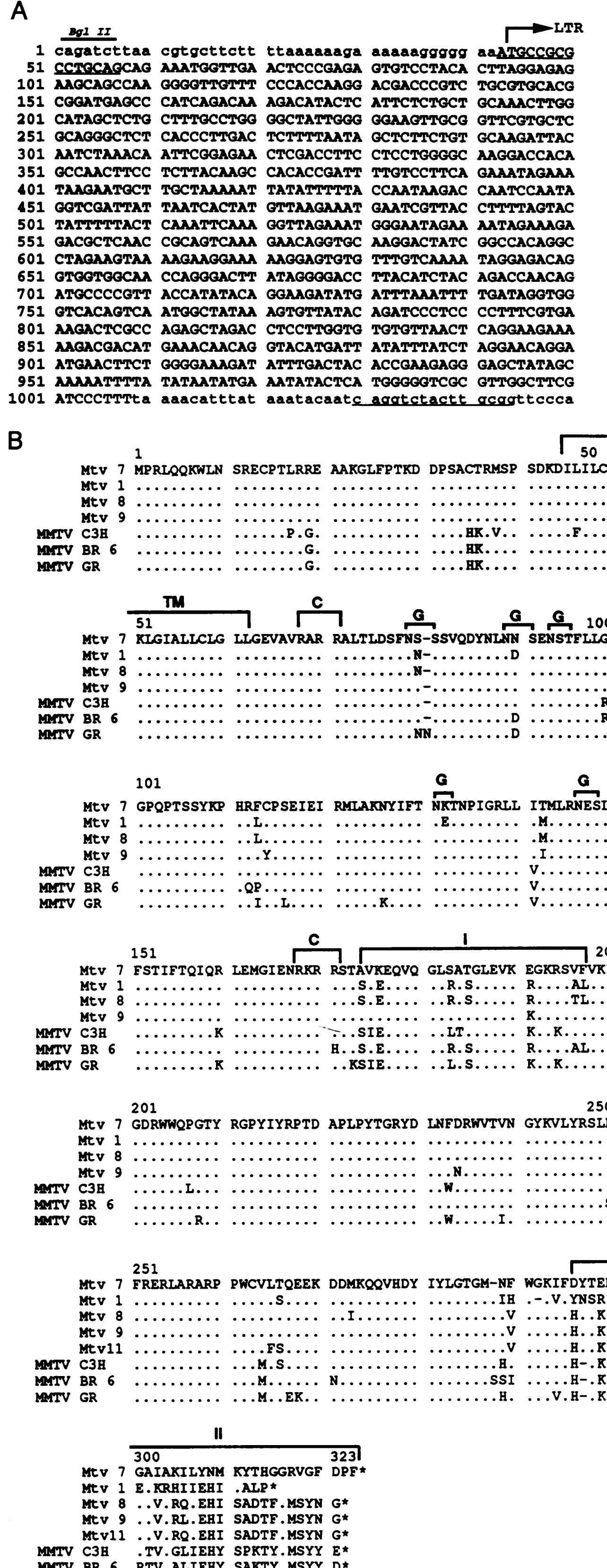


FIG. 4. Nucleotide sequence and predicted amino acid sequence of the *Mtv-7* ORF. (*A*) The nucleotide sequence of the ORF is shown in uppercase letters (43–1008). Nucleotides 1–92 are from the *env* gene, and nucleotides 1009–1050 are from the 3′ untranslated region in U3. The *Bgl* II site (nucleotides 2–7) in *env* is marked. The beginning of the LTR is marked with an arrow. The primer binding sites for the PCR cloning of the ORF are underlined. (*B*) Comparison of predicted amino acid sequences encoded by various MMTV LTR ORF genes (13, 26–29). TM, putative transmembrane segment; C, putative cleavage site; G, N-linked glycosylation sites; I, polymorphic region I; II, polymorphic region II.

Conceivably, such an effect operating on the Mo-MLV LTR might be responsible for the phenomena we have observed here. It is possible that the ORF protein, translated from the spliced 3′ LTR message, down-regulates the MMTV promoter, thus acting as a self-regulator of expression. This would explain why it has been notoriously difficult to detect expression of this protein, even in mammary tumor cells (24, 25). Alternatively, expression of the ORF at high levels might be toxic to cells, leading to unpredictable selection of variant clones with low expression.

**Nucleotide Sequence and Predicted Amino Acid Sequence of *Mtv-7* ORF.** To ascertain whether and how distinct sequences in the ORF might correlate with distinct superantigen activities (i.e., V$\beta$ recognition), we have determined the nucleotide sequence of the *Mtv-7* LTR ORF (Fig. 4*A*) and compared its predicted amino acid sequence to that of other MMTV ORFs (13, 26–29) (Fig. 4*B*). The overall structure is highly conserved among endogenous and exogenous viruses, but the *Mtv-7* sequence diverges significantly in two regions from that of the other known ORF sequences: region I (from amino acid 174 to amino acid 197) differs at dispersed amino acids, most of which constitute nonconservative changes. Region II (from amino acid 295 to amino acid 322) is located at the C terminus, where the last 14 amino acids are unique for *Mtv-7*. It is, therefore, plausible that one or both of these regions contribute specificity to the binding site of the TCR V$\beta$ chain. Additionally, these regions could influence the superantigen/major histocompatibility complex class II interaction, presumably an important factor determining the magnitude of the elicited T-cell response because of the observed hierarchy in the capacity of the various class II molecules to support superantigen activity. Specific recombinants to test the role of these regions must be made.

**Conclusions.** The possibility that the V$\beta$ specificity resides in the C terminus would seem to require that this region be exposed on the cell surface. Such a structure is consistent with the presence of a stretch of mostly hydrophobic amino acids at amino acids 45–63, which might indicate a type II transmembrane protein. Furthermore, the five potential N-linked glycosylation sites on the C-terminal side of the putative transmembrane segment show that this portion must lie outside the membrane, since translation and expression studies have shown that the protein encoded by the ORF is modified by about this amount of N-linked glycosylation (24, 25). Of interest are two arginine-rich segments (RXRR) that resemble cleavage sites in retrovirally encoded *env* proteins (22). It is possible, therefore, that the MMTV ORF-encoded protein consists of a transmembrane and an extracellular peptide. Experiments are necessary to confirm these predictions by analyzing the protein encoded by the ORF. Antisera have been raised against recombinant *Mtv-7* ORF protein and peptides, which should provide the tools for these studies (D. Mottershead, N. Mohan, M. Subramanyam, and B.T.H., unpublished data).

Because the ORF of various MMTVs has been shown to encode superantigen activities (refs. 9–12, and present results), we propose that this gene be renamed "*sag*." This proposal is similar to the suggestion of Choi *et al.* (9), but is in agreement with conventions for naming retroviral genes (30, 31).

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