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The Role of Proximal and Distal Sequence Variations in the Presentation of an Immunodominant CTL Epitope Encoded by the Ecotropic AK7 MuLV

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An *emv*-14-derived, replication-competent ecotropic murine leukemia virus [MuLV], designated AK7, was previously cloned from the AKXL-5 recombinant inbred mouse strain and partially characterized. While genetically encoding for an envelope-derived immunodominant CTL epitope [KSPWFTTL] located in the transmembrane region of p15TM, this virus, unlike the *emv*-11-derived virus AKR623, fails to be efficiently recognized by AKR/Gross MuLV-specific cytotoxic T lymphocytes [CTL]. AK7 thus provides the opportunity to study the role of retroviral sequence variations that are located outside of the immunodominant epitope as a mechanism of escape from CTL-mediated immune surveillience. In an attempt to identify which region[s] of the AK7 genome could account for its ability to evade efficient recognition by AKR/Gross MuLV-specific CTL, we have constructed recombinant murine retroviruses. The direct influence of a sequence variation twelve amino acids N-terminal to KSPWFTTL was explored with the use of chimeric viruses and determined not to significantly impair the presentation of KSPWFTTL to AKR/Gross MuLV-specific CTL. The long terminal repeat [LTR] derived from the AK7 virus, which possesses only one copy of the 99-base pair transcriptional enhancer in the U3 region, in contrast to AKR623 that possesses two copies of the tandem direct repeat enhancers, was also analyzed for its influence on the presentation of KSPWFTTL. Interestingly, our data indicate that the enhancer region derived from AK7 negatively influences the presentation of KSPWFTTL in the context of a recombinant AKR623 virus.

INTRODUCTION

AKR [H-2^k] mice have a high incidence of spontaneously occuring leukemias believed to be proximally induced by thymotropic mink cell focus-inducing [MCF] retroviruses of the AKR/Gross type. MCF retroviruses are dualtropic, capable of infecting both murine as well as xenogeneic cells, and are formed from the recombination of expressed endogenous ecotropic murine leukemia viruses [emv] with other endogenous xenotropic and polytropic murine leukemia viral sequences [Hartley *et al.,* 1977; Stoye *et al.,* 1991; Elder *et al.,* 1977; Chattopadhyay *et al.,* 1981; Cloyd *et al.,* 1985; Khan, 1984]. The generation of these leukemogenic MCF retroviruses is thus dependent upon the expression of ecotropic MuLV. C57BL/6 [H-2^b] mice have a much lower incidence of spontaneously occuring thymic leukemias and have been shown to generate vigorous H-2K^b-restricted CTL responses against tumors induced by the AKR/Gross MuLV [Green *et al.,* 1979, 1980]. In contrast, AKR mice were unable to mount H-2^k restricted anti-AKR/Gross MuLV CTL responses [Green, 1984]. The C57BL/6-derived T lymphocytes, designated as AKR/Gross MuLV-specific CTL, are directed specifically against an immunodominant T cell epitope [KSPWFTTL] [White *et al.,* 1994a; Sijts *et al.,* 1994; Green and Smith, 1996] located in the p15 transmembrane region [TM] of the envelope protein in almost all emv studied [Coppola *et al.,* 1995] and many MCF MuLV [Sijts *et al.,* 1994; Coppola and Green, 1994].

Three highly related endogenous emv proviruses found in AKR mice, *emv*-11, *emv*-13, and *emv*-14, were previously studied. AKXL recombinant inbred mice, generated from the AKR and C57L progenitor strains, allowed for the independent segregation of these endogenous emv [Steffen *et al.,* 1982]. Interestingly, splenocytes from *emv*-14 positive AKXL-5 mice failed to stimulate an AKR/Gross MuLV-specific CTL response in responder C57BL/6 mice while splenocytes from *emv*-11-positive AKXL mice successfully stimulated the response [Green and Graziano, 1986]. When AKXL mice were used as the responders to generate an AKR/Gross MuLV-specific CTL response, a clear pattern emerged where *emv*-14 negative AKXL-29 mice could mount a full AKR/Gross MuLV-specific CTL response, while *emv*-14-positive AKXL-5 mice were only partially responsive [Green and Rich, 1988]. Furthermore, AKR/Gross MuLV-specific CTL-recognized SC.K^b cells infected with virus isolated from *emv*-11-positive AKXL-21 mice very efficiently. In contrast, SC.K^b cells infected from virus derived from *emv*-14-positive AKXL-5 mice were poorly recognized [White *et al.,* 1990]. Subsequent molecular cloning of the ecotropic AK7 MuLV, derived from *emv*-14-positive AKXL-5 mice, led to the observation that AK7-infected cells were inefficiently recognized by AKR/Gross MuLV-specific CTL, in contrast to cells infected by the *emv*-11-derived molecu-

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lar clone AKR623 which were very efficiently recognized [White *et al.,* 1993]. These findings were curious since both AK7 and AKR623 genetically encoded for the immunodominant KSPWFTTL epitope at TM134–141. Recognition of AK7 MuLV infected cells by AKR/Gross MuLV-specific CTL was dramatically improved, however, when AK7-infected cells were exogenously pulsed with the synthetic peptide KSPWFTTL, suggesting that there was a defect in the presentation of KSPWFTTL that should be endogenously produced from this virus [White *et al.,* 1994b]. Partial sequencing [H. D. White; unpublished observations] of the AK7 retrovirus has elucidated two significant differences, when compared to the efficiently recognized AKR623 virus, that could potentially account for a negative influence on KSPWFTTL presentation. In this report, we investigated the possibility that either a flanking sequence variation located twleve amino acids N-terminal to KSPWFTTL and/or a distal variation located in the U3 region of the long terminal repeat [LTR] encoded by the AK7 MuLV is responsible for the failure of AK7 virally infected cells to be efficiently recognized by AKR/Gross MuLV-specific CTL.

MATERIALS AND METHODS

Cell lines

The SC-1 cell line is a continuous fibroblast line from a feral mouse embryo and is cultured in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum, 2 m*M* L-glutamine, 33 μg gentamycin per milliliter, 30 μg of penicillin per milliliter, and 20 μg of streptomycin per milliliter. The MHC class I H-2K^b gene was introduced into SC-1 giving rise to SC.K^b as previously described [White *et al.,* 1990]. B.GV and E♂G2 are H-2^b Gross virus cell surface antigen [GCSA]-positive tumor cell lines derived from a BALB.B congenic and a C57BL/6 mouse, respectively, both induced with the Gross virus passage A MuLV. AKR.H-2^b SL1 is a GCSA-positive tumor cell line derived from a spontaneous leukemia from an AKR.H-2^b congenic mouse. YAC-1 [YAC] is an H-2^a tumor cell line that is used here as a highly natural killer [NK]-sensitive target cell. The B.GV, E♂G2, YAC, and AKR.H-2^b SL1 tumor cell lines were grown in RPMI supplemented with 5% fetal bovine serum, 2 m*M* L-glutamate, 33 μg gentamycin per milliliter, 50 μ*M* 2-mercaptoethanol, 30 μg of penicillin per milliliter, and 20 μg of streptomycin per milliliter. EL4[S] is a C57BL/6-derived T cell lymphoma, and EG7 is a clone of EL4 that has been transfected with an ovalbumin [OVA] cDNA [Moore *et al.,* 1988]. RF33.70 is a C57BL/6-derived anti-OVA-specific T-T cell hybridoma [Rock *et al.,* 1990]. The EG7 and RF33.70 cell lines were grown in either DMEM or RPMI, respectively, both supplemented with 10% fetal bovine serum, 33 μg gentamycin per milliliter, 30 μg of penicillin per milliliter, and 20 μg of streptomycin per milliliter. All cell lines were maintained at 37° in a 5% CO_2 atmosphere.

Viruses

The BM5 ecotropic TM 122E/134K and BM5 ecotropic TM 122K/134K retroviral recombinants were constructed by excising the 846-bp *Bss*HII/*Xba*I fragment from the parental plasmid pBM5 eco and inserting it into the *Bss*HII/*Xba*I site in the polylinker region of the plasmid pNEB193 [New England Biolabs] resulting in the plasmid designated as pNEB-RS1. The 3537-bp *Xba*I/*Bst*XI fragment from pNEB-RS1 was then ligated to the 260-bp *Xba*I/*Bst*XI fragment derived from either the pAKR623 or pAK7 plasmids resulting in the plasmids pNEB-RS1 [623] or pNEB-RS1 [7], respectively. The 846-bp *Bss*HII/*Xba*I fragment from either pNEB-RS1 [623] or pNEB-RS1 [7] was then inserted into the 12-kb *Bss*HII/*Xba*I fragment from pBM5 eco resulting in the plasmids pBM5 eco TM 122E/134K and pBM5 eco TM 122K/134K. The resulting pBM5 eco TM 122E/134K and pBM5 eco TM 122K/134K constructs were then introduced into SC-1 cells by calcium-phosphate-mediated transfection.

The AKR623/7 LTR viral chimera was constructed by excising the 388-bp *Bss*HII fragment from pAK7, which contains only a single copy enhancer, and ligating it to the pAKR623-derived 10540-bp *Bss*HII fragment, from which the tandem direct repeat sequences were already removed. The resulting pAKR623/7 LTR construct was then introduced into SC-1 cells by calcium-phosphate-mediated cotransfection with the plasmid pSV2His. Supernatants from SC-1 cells transfected with pBM5eco TM 122E/134K, pBM5 eco TM 122K/134K, or AKR 623/7 LTR were then collected, briefly spun down, and filtered before using to infect SC.K^b cells that had been cultured in the presence of polybrene for at least 12 hr. Infected SC.K^b cells were then subjected to FACS analysis using the antiviral-specific monoclonal antibodies 24-8, a murine IgG2a mAb specific for emv gp70, and 548 which is a murine IgG2b mAb specific for the p12 [gag] antigen.

Recombinant vaccinia virus encoding for H-2K^b [vaccinia K^b] was kindly provided by Dr. Jack Bennink [NIAID/NIH] and was used to infect SC-1, SC-1 BM5 ecotropic TM 122E/134K, and SC-1 BM5 ecotropic TM 122K/134K cells at a multiplicity of infection [m.o.i.] of 10:1 for 1 hr in BSS/BSA. Vaccinia virus-infected cells were then incubated in DMEM media containing 5% fetal bovine serum for 4 hr before conducting the CTL assay.

RT-PCR and DNA sequencing

The pBM5 eco TM 122E/134K, pBM5 eco TM 122K/134K, and pAKR623/7 LTR generated plasmid constructs were sequenced by thermal cycle dideoxy DNA sequencing [New England Biolabs] using the M13/pUC [−40] oligonucleotide 5′-GTTTTCCCAGTCACGAC-3′ and the M13/pUC [−48] oligonucleotide 5′-AGCGGATAACAATTTCACACAGGA-3′ to confirm TM122E-TM134K, TM122K-TM134K, and the enhancer region of the LTR, respectively. The SC.K^b BM5 eco TM 122E/134K- and SC.K^b BM5 eco TM 122K/134K-infected cell lines were confirmed for the TM122E-

TM134K and TM122K-TM134K regions, respectively, by RT-PCR sequencing using *Taq* polymerase [Perkin–Elmer]. PCR was conducted on extracted RNA [Sigma] reverse transcribed into cDNA [Boehringer-Mannheim] with PCR conditions set at 95° 1 min, 55° 1 min, 72° 2 min for 25 cycles, and directly sequencing the 222-bp PCR product using the oligonucleotides 623/7499 5′-GTACGGGATAGC-ATGGCCAAACTTAGAGAA-3′ and 623/7694 5′-CTACCG-AAATCCTGTCTTTGATAAACTG-3′. The SC.K^b 623/7 LTR cell line was confirmed for the AK7-derived enhancer region by RT-PCR and direct sequencing using the oligonucleotides LTR[+] 5′-GCAAGGCATGGGAAAATACCAGAGCTG-ATG-3′ and LTR[−] 5′-TCCCCGGTCATCTGGGGAACC-TTGAGACAG-3′. The oligonucleotides 623/7499, 623/7694, LTR[+], and LTR[−] were synthesized at the Dartmouth College Molecular Biology Core Facility.

Assay for cell-mediated cytotoxicitiy

AKR/Gross MuLV-specific CTL were generated by immunizing C57BL/6 male mice with 2×10^6 B.GV cells i.p. Approximately 10 days later, the spleen cells were harvested and cultured with irradiated [8000 rads] E♂G2 or AKR.H-2^b SL1 stimulator cells at a responder to stimulator ratio of 40:1 or with stimulating KSPWFTTL peptide at 10 μg/ml at 37° in a 5% CO_2 atmosphere. AntiH-2^b allogeneic CTL were generated by culturing BALB/c splenocytes with irradiated [2000 rads] C57BL/6 splenocytes at a responder to stimulator ratio of 2:1. After 6 days in culture, effector cells were harvested and incubated with ^{51}Cr- labeled target cells at various effector-to-target [E:T] ratios at 37° in a 5% CO_2 atmosphere for 6 hr in the case for fibroblast targets or 4 hr for tumor targets. NK-sensitive YAC target cells were included in all CTL assays and always observed to lyse at values less than 2%. Percentage of specific lysis was calculated according to the following formula: $\{[A - B] \div C\} \times 100$ where $A = {}^{51}$Cr released from target cells incubated with CTL, $B = {}^{51}$Cr released by target cells incubated alone, and C = total freeze–thaw releasable ^{51}Cr [approximately 80%]. Lytic unit values were defined as the number of effectors required for 30% lysis and were determined by plotting percentage of lysis values versus the log of the effector cell number and expressed as the number of lytic units per 10^6 effector cells.

Antibodies

Two monoclonal antibodies [mAb] specific for gp70 were used: 35/299, a rat IgG2a mAb specific for ecotropic gp70 [Pinter *et al,* 1982]; and 24-8, a murine IgG2a mAb with a broad specificity for the gp70-p15E-associated complex [Portis *et al.,* 1982]. Two mAbs specific for p15TM were also used: 9E8, an ascites-derived murine IgG2a mAb specific for the p15E^a determinant [Lostrom *et al.,* 1979]; and 19VIII-E8, an ascites-derived murine IgG2b mAb specific for the p15E^b determinant [Lostrom *et al.,* 1979]. The H-2K^b-specific mAb HB41 [28-13-3s], a murine IgM antibody [Ozato *et al.,* 1981], and HB158 [Pharmingen], an affinity purified mouse IgG2a mAb were also used.

Flow cytometric analysis

Cell surface antigen expression was measured by indirect immunofluorescence. Then 1×10^6 cells washed with staining medium consisting of RPMI 1640 containing 0.2 % [wt/vol] bovine serum albumin [Sigma Chemical] were incubated with the appropriately diluted mAb for 1 hr at 4°. The samples were washed twice in staining medium and then treated with the appropriately diluted FITC-conjugated secondary antibody [goat anti-mouse or goat anti-rat immunoglobulin]. Cells were incubated at 4° for 1 hr with the secondary antibody and then washed once with staining medium before fixing the cells in 1% parafomaldehyde. Background fluorescence values were determined with the following irrelevant monoclonal antibodies used as isotype controls: OKT8 [CRL 8014, murine IgG2a] for 24-8, 9E8 and HB158; OKM1 [CRL 8026, murine IgG2b] for 19VIII-E8; 35-12 [murine IgM] for HB41; and purified rat IgG2a for 35/299. Flow cytometry analysis was performed on a FACScan [Becton–Dickinson immunocytometry systems] set on a linear amplifier.

IL-2 biosassy

The IL-2 content in stimulated T cell hybridoma culture supernatants was measured in a quantitative bioassay with CTLL-2 indicator cells [Gillis *et al.,* 1978]. Hybridoma cultures were prepared with 10^5 RF33.70 cells in the presence or absence of various antigen presenting cells [APC] in 200 μl media in flat bottom microtiter wells in duplicate. After a 20-hr incubation at 37°, serial dilutions of the stimulated versus control hybridoma supernatants were tested for their ability to support the proliferation of the highly IL-2-sensitive CTLL-2 cell line.

RESULTS

TM122K does not appear to negatively influence the presentation of the TM134-141 CTL epitope [KSPWFTTL] in SC.K^b cells

To identify which region[s] of the AK7 MuLV genome could account for its ability to evade efficient recognition by AKR/Gross MuLV-specific CTL, known sequences of the AK7 MuLV were compared to the very efficiently recognized AKR623 MuLV since these are two very similar viruses, having originated from a common primordial emv [Steffen *et al.,* 1982]. Sequencing in the vicinity of KSPWFTTL in the p15TM region of the envelope gene revealed that AK7 encodes for a predicted lysine at position TM122 instead of the glutamic acid residue found in the AKR623, SL3-3, and MCF247 viruses that are representative of viruses efficiently recognized by AKR/Gross MuLV-specific CTL [Coppola and Green, 1994; White *et al.,* 1993] [Fig. 1A].

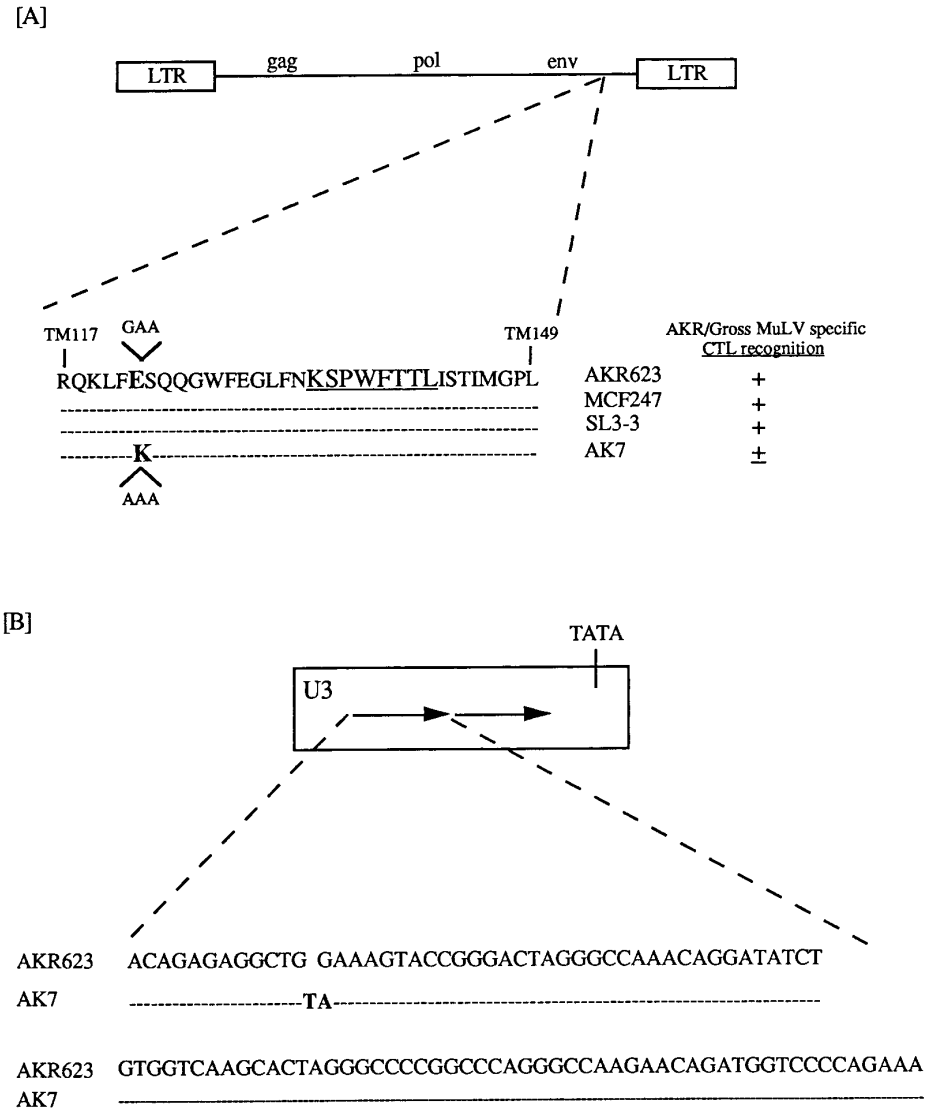


FIG. 1. Comparative sequences of the AKR623 [Herr, 1984], SL3-3 [Lenz], MCF247 [Kelly *et al.,* 1983], and AK7 [this study] retroviruses. A represents amino acid sequences corresponding to TM117–149 found within the p15TM region of the *envelope* gene. The immunodominant KSPWFTTL peptide is underlined. The nucleotides GAA found in AKR623, SL3-3, and MCF247 encode for the negatively charged glutamate while the AK7-derived AAA sequence encode for a positively charged lysine residue at amino acid position 122. The data documenting the relative susceptibilities of cells infected by these retroviruses to AKR/Gross MuLV-specific CTL was derived from published studies [Coppola and Green, 1994; White *et al.,* 1993]. The determination for SL3-3 was done with NB-tropic SL3-3 [Coppola *et al.,* 1996; Thomas *et al.,* 1993]. B illustrates the nucleotide sequence of a single copy of the transcriptional enhancer found in the U3 region of the LTR. The single copy of the AK7 transcriptional enhancer is identical to either of the two AKR623 tandem direct repeats with the exception of a T insertion followed immediately by a G → A substituion. (GenBank Accession No. AF019257).

To explore the possibility that CTL epitope processing and/or presentation might be affected by this nonconservative change, we examined this sequence variation for its specific influence on the presentation of KSPWFTTL. Two viral chimeras were constructed utilizing the BM5 ecotropic virus as the viral backbone [Fig. 2]. The highly infectious BM5 ecotropic virus is normally not recognized by H-2K^b-restricted AKR/Gross MuLV-specific CTL since it encodes for RSPWFTTL instead of KSPWFTTL at TM134-141 [Coppola *et al.,* 1995]. Two recombinant retroviruses, BM5 ecotropic TM 122E/134K and BM5 ecotropic TM 122K/134K, were generated so that they both encoded for the KSPWFTTL epitope, but with either a glutamate or a lysine, respectively, at position TM122. Positioning the KSPWFTTL epitope along with the corresponding TM122-encoded amino acid in a common viral backbone in this fashion thus allowed us to directly study the influence of this flanking sequence variation on the presentation of KSPWFTTL. SC-1 fibroblast cells expressing either of the two chimeric retroviruses were then assayed at the target level against H-2K^b-restricted AKR/Gross MuLV-specific CTL, derived from C57BL/6 mice by *in vivo* priming and secondary *in vitro* stimulation with MHC compatible AKR/Gross MuLV-induced tumor cells, as demonstrated previously in our lab [Green, 1982]. To introduce the MHC re-

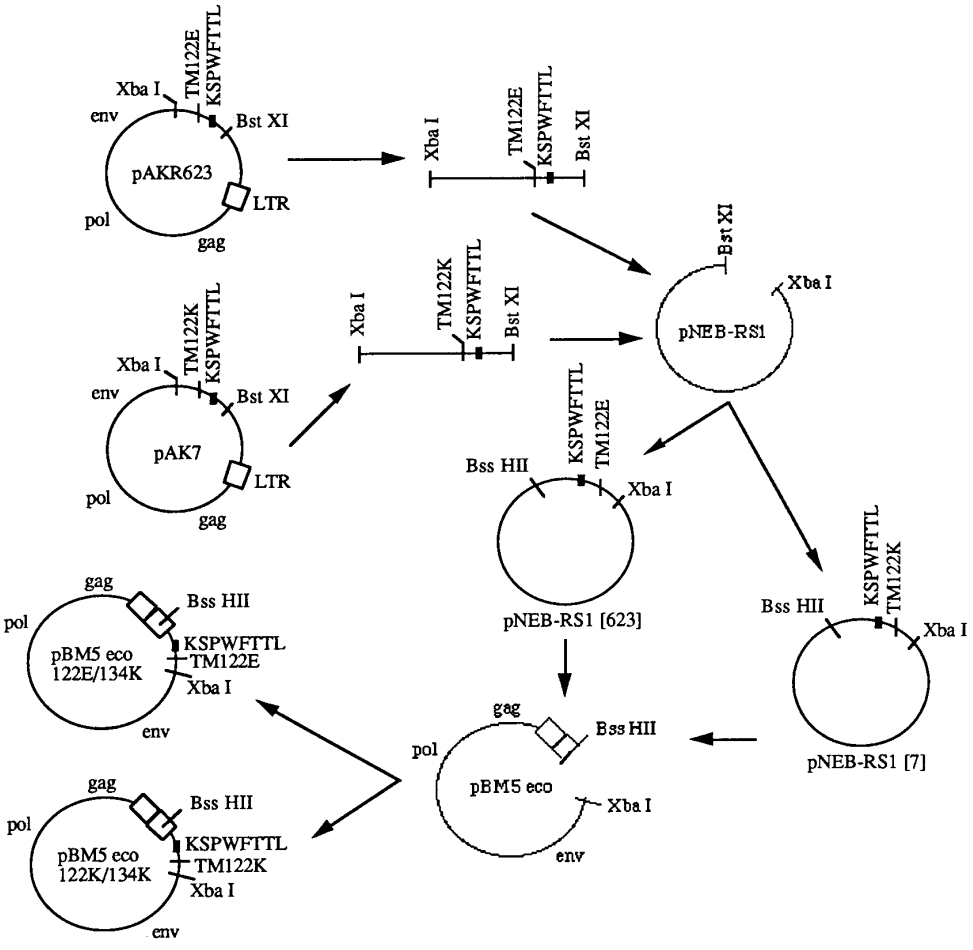


FIG. 2. Construction strategy of the BM5 ecotropic TM 122E/134K and BM5 ecotropic TM 122K/134K viral chimeras used in this study as detailed under Materials and Methods. The steps leading to the formation of pNEB-RS1 have not been depicted for clarity.

striction element into these cells, recombinant vaccinia virus encoding for H-2K^b was used to infect the SC-1 cells containing either chimeric retrovirus.

Figure 3 shows that vaccinia virus K^b-infected target cells containing the BM5 ecotropic TM 122K/134K viral chimera, which harbors the TM122K variation derived from the AK7 MuLV, were recognized with a similar degree of efficiency as the BM5 ecotropic TM 122E/134K viral chimera which contains the normal glutamate at TM122 derived from the AKR623 virus. This experiment was performed three times with similar results. To further validate this finding and demonstrate that these results were not due to an artifact, such as a crossreactive epitope introduced by the vaccinia virus infection, or were influenced by a limitation in the K^b expression levels that could be achieved by the recombinant vaccinia virus infection, the previously characterized SC.K^b cell line was alternatively used at the target level since it endogenously produces the appropriate restriction element. As Fig. 4 demonstrates, SC.K^b target cells, whether infected by the BM5 ecotropic TM 122K/134K or the BM5 ecotropic TM 122E/134K viral chimera, were also recognized with similar efficiencies by the AKR/Gross MuLV-specific CTL. This experiment was performed three times with similar results. Although the data displayed in Figs. 3 and 4 suggest that the BM5 ecotropic TM 122K/134K viral chimera may be slightly more efficiently recognized than the BM5 ecotropic TM 122E/134K viral chimera, repeated experiments have indicated that this small difference was not a consistent and significant result. RT-PCR was performed from the RNA extracted from these two infected SC.K^b cell lines, and direct sequencing of the PCR products confirmed the identity of both chimeric retroviruses. These results collectively suggested that the flanking amino acid sequence variation from glutamate to lysine found at position TM122 in the AK7 MuLV does not negatively impact the final presentation of the immunodominant KSPWFTTL epitope to H-2K^b-restricted antiviral CTL.

The AK7-derived transcriptional enhancer partially influences the ability of AKR623/7 LTR-infected cells to be recognized

Another region of the AK7 MuLV genome that could have potentially accounted for the inefficient recognition of AK7 MuLV-infected cells by AKR/Gross MuLV-specific CTL was its long terminal repeat [LTR]. It was previously

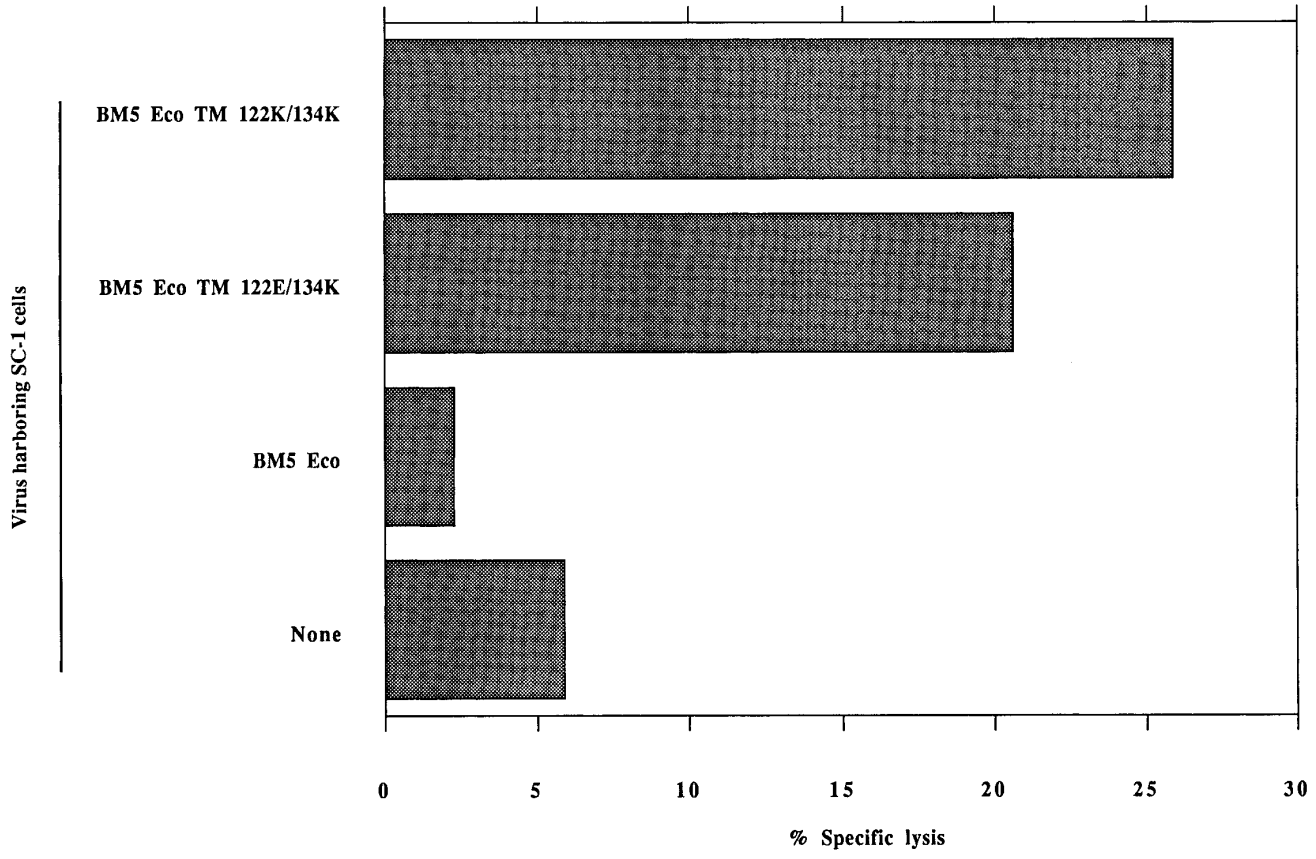


FIG. 3. TM122K does not negatively influence TM134–141 processing and presentation in vaccinia K^b infected SC-1 cells. SC-1 cells harboring either the BM5 eco TM 122E/134K or the BM5 eco TM 122K/134K chimeric virus were infected with the recombinant vaccinia K^b virus at a multiplicity of infection of 10:1 5 hr before the B.GV-primed and E♂G2 tumor-stimulated AKR/Gross MuLV-specific CTL were added at an effector to target ratio of 100:1 in a standard chromium release assay as described under Materials and Methods. Target cell spontaneous release values ranged from 6 to 9%.

demonstrated by RFLP analysis that the AK7 retrovirus contains only a single copy of the 99-bp transcriptional enhancer found in the U3 region of its LTR [White *et al.,* 1993], while in contrast, the AKR623 retrovirus contains two copies of the enhancer, otherwise known as the tandem direct repeat sequences. Other than this difference, sequencing of this region has revealed that AK7 has a highly homologous enhancer sequence when compared to AKR623 [this study]. In fact, the AK7 enhancer sequence only differs from the AKR623 sequence by two nucleotides as depicted in Fig. 1B. This difference in sequence allowed for the easy and definitive identification of the two different LTRs even if a spontaneous tandem duplication of the AK7 LTR direct repeat had occured.

In order to explore what influence the AK7 retroviral LTR had on the presentation of KSPWFTTL, a viral chimera was constructed in the context of the AKR623 retrovirus. The AKR623/7 LTR recombinant retrovirus has its LTR derived from AKR623, with the exception that the tandem direct repeat sequences were excised and replaced with the single copy enhancer sequence derived from the AK7 viral LTR. As demonstrated in Figs. 5A and 5B, SC.K^b cells infected with the AKR623/7 LTR retroviral chimera were recognized at levels intermediate to that of AKR623 or AK7 infected cells, based either on examination of specific lysis values or on lytic unit calculations [legend to Fig. 5]. This experiment was conducted a total of four times with Fig. 5A representing one such experiment and Fig. 5B depicting the average of the three other experiments. Although there was some variation in the degree of recognition by antiviral CTL of cells infected by the AKR623/7 LTR chimeric virus, relative to AKR623- or AK7-infected SC.K^b cells, SC.K^b cells infected by the AKR623/7 LTR chimera were never as poorly recognized as AK7-infected cells, indicating the existence of other AK7 sequences that interfered with the functional presentation of peptide 12. RT-PCR was performed from the RNA extracted from the AKR623/7 LTR-infected SC.K^b cell line and direct sequencing of the PCR product verified the presence of only the AK7-derived enhancer. These data thus collectively suggest that the transcriptional enhancer derived from AK7 played an important, but not the only, negative role in the presentation of KSPWFTTL.

Retroviral LTR enhancer regions are generally considered to function as a positive regulator of viral transcription. As Fig. 1B depicts, the AK7 retrovirus contains only a single copy of the 99-bp transcriptional enhancer found in the U3 region of its LTR while the AKR623 virus pos-

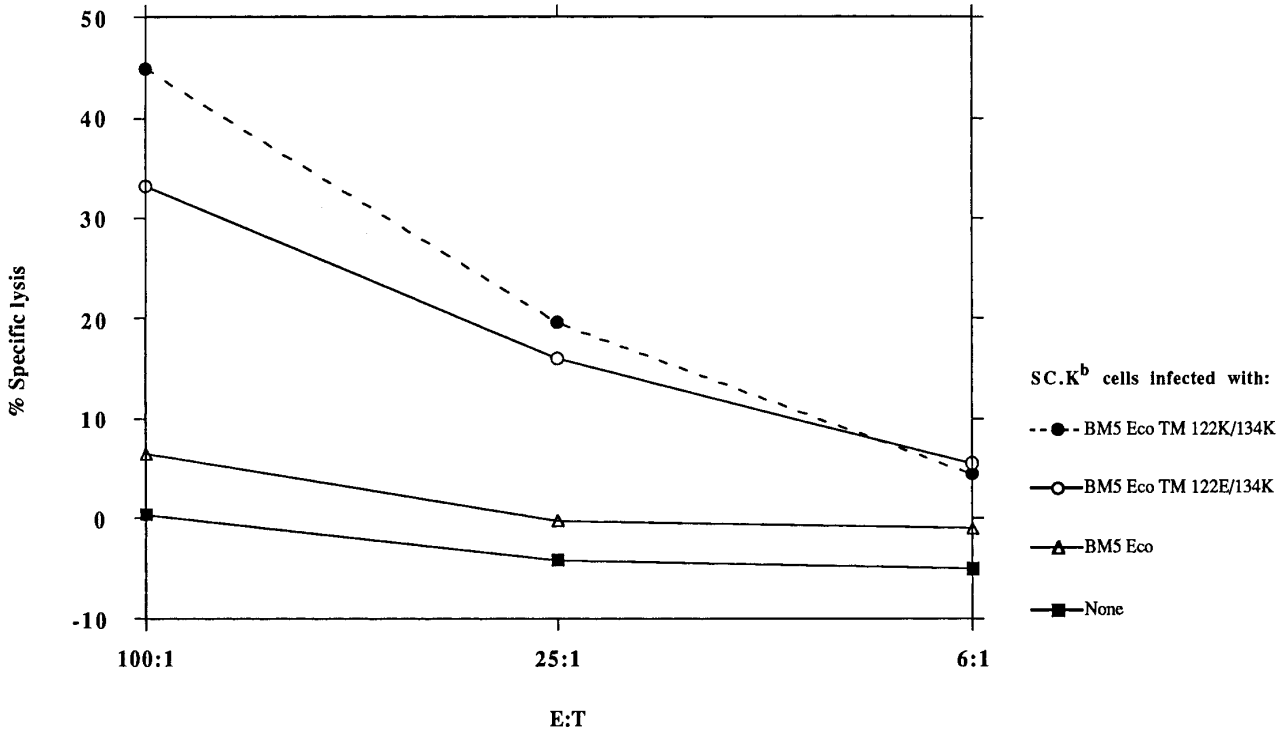


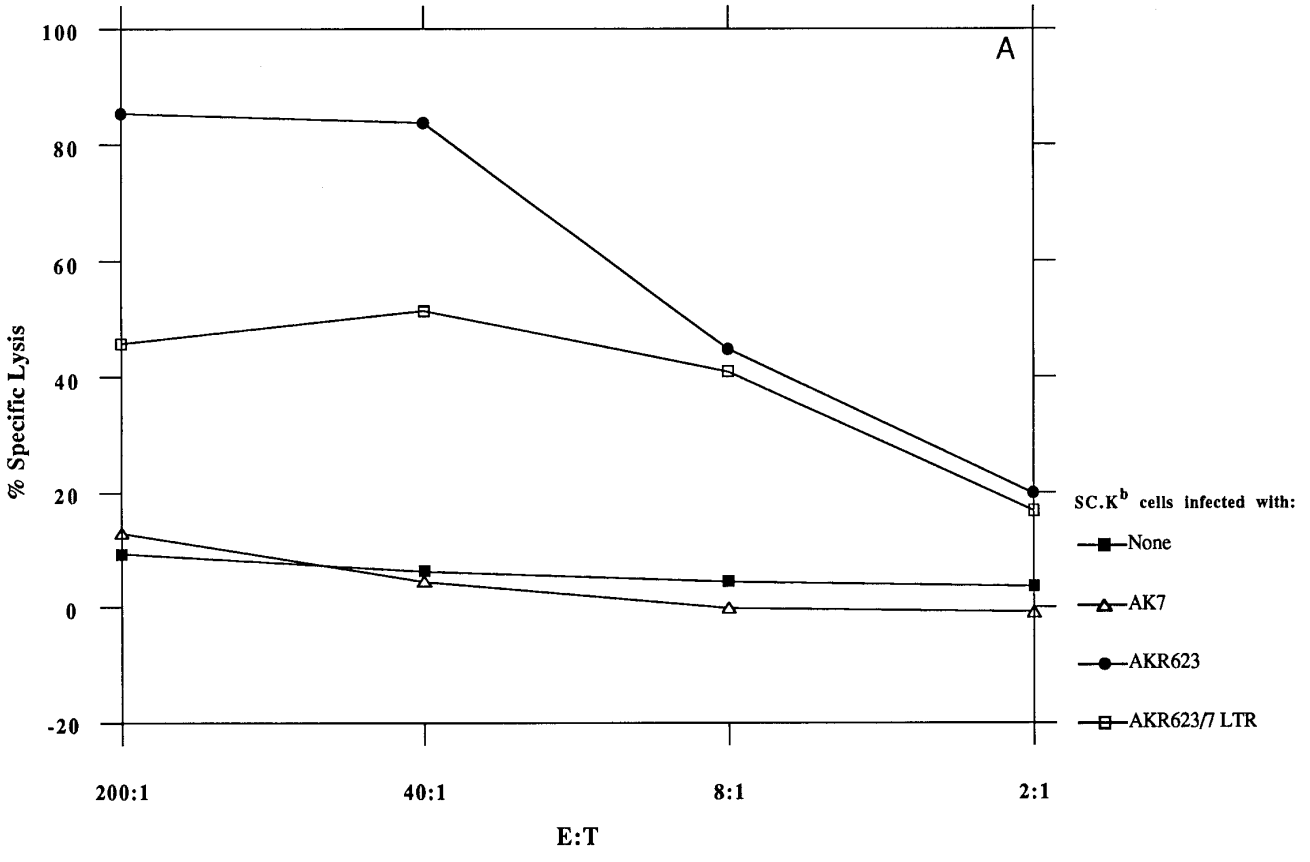
FIG. 4. TM122K does not negatively influence TM134–141 CTL epitope processing and presentation in SC.K^b cells. SC.K^b cells infected with either the BM5 Eco TM 122E/134K or the BM5 Eco TM 122K/134K chimeric retrovirus were used as target cells in a standard chromium release assay with B.GV-primed and AKR.H-2^b.SL1 tumor-stimulated AKR/Gross MuLV-specific CTL. Target cell spontaneous release values ranged from 15 to 23%.

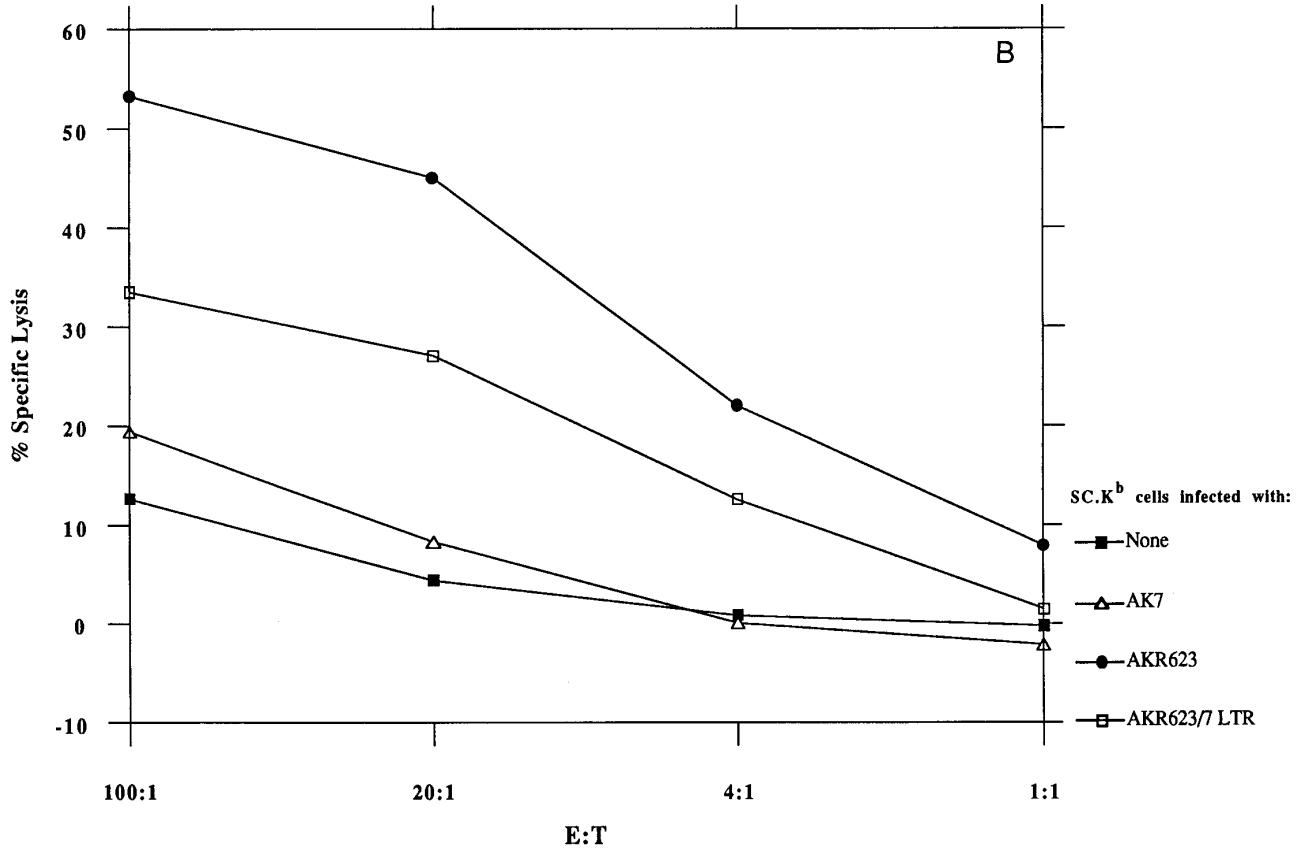
sesses two copies of the enhancer. To address whether viral *env* gene expression was equivalent at the protein level for AKR623 versus AK7-infected SC.K^b cells, flow cytometry was utilized to quantitate gp70 and p15TM. As demonstrated in Fig. 6, the data for mAb 35/299, which detects gp70; as well as for 9E8, which is specific for p15TM; show that both gp70 and p15TM are expressed at either roughly equivalent or greater levels in AK7-infected SC.K^b cells in comparison to AKR623-infected SC.K^b cells. The mAb 24-8, which recognizes the viral gp70-p15E-associated complex, and the mAb 19VIII-E8, specific for p15TM, were also used to confirm that SC.K^b cells infected with AK7 expressed at least as much cell surface gp70 and p15TM as AKR623 infected SC.K^b cells [data not shown]. Thus, the inefficient recognition of AK7-infected SC.K^b cells by AKR/Gross MuLV-specific CTL that was consistently observed previously [White *et al.,* 1993 and 1994b] and here in Figs. 5A and 5B was not due to lower levels of expression of either the envelope protein or more specifically, p15TM, within which peptide 12 is located. Figure 6 also demonstrates that the cell surface expression levels for H-2K^b, the restriction element for peptide 12 presentation, were essentially the same in either AKR623 or AK7 infected SC.K^b cells. Similar results were also obtained with a second H-2K^b specific mAb [HB158, data not shown].

DISCUSSION

In summary, previous experiments have demonstrated that when SC.K^b fibroblast target cells, infected with either AKR623 [*emv*-11 derived] or AK7 [*emv*-14 derived] and expressing similar steady state levels of retroviral antigens were compared, AKR/Gross MuLV-specific CTL recognized AKR623-infected cells much more efficiently than AK7-infected cells [White *et al.,* 1993]. Recognition of AK7 virally infected SC.K^b cells by AKR/Gross MuLV-specific CTL was dramatically improved when these cells were exogenously pulsed with the synthetic KSPWFTTL peptide representing the immunodominant CTL epitope [White *et al.,* 1994b]. These findings were intriguing since AK7 genetically encoded for KSPWFTTL [Fig. 1], suggesting that a defect in KSPWFTTL processing and presentation was occuring. Allogeneic CTL directed against H-2^b had also been used to demonstrate that the failure of *emv*-14-infected SC.K^b cells to be efficiently recognized by AKR/Gross MuLV-specific CTL was not due to the downregulation of MHC class I [White *et al.,* 1990].

One alternative mechanism that we address in the present study is the influence of sequence variations encoded by AK7 that may affect KSPWFTTL processing and/or presentation. CTL recognize antigens as short peptides bound to the MHC class I molecule, thus imposing the requirement that viral proteins be proteolytically processed before they can be presented to T cells. As a result, not only are the recently defined MHC class I allele-specific motifs important [Rammensee *et al.,* 1993 and 1995], but the cleavage preferences of the proteasomal complex could potentially affect the availability of peptides either quantitatively and/or qualitatively [Eggers *et al.,* 1995; Niedermann *et al.,* 1995]. The specific mecha-


A
100
80
60
40
20
0
-20
% Specific Lysis
200:1
40:1
8:1
2:1
E:T
SC.K^b cells infected with:
None
AK7
AKR623
AKR623/7 LTR


B
60
50
40
30
20
10
0
-10
% Specific Lysis
100:1
20:1
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1:1
E:T
SC.K^b cells infected with:
None
AK7
AKR623
AKR623/7 LTR

nism of the proteolytic machinery that cleaves endogenously synthesized viral proteins into smaller peptides is incompletely understood, and flanking sequences may hinder the creation of immunogenic peptides, such as by making the epitopes more sensitive to proteolysis, resulting in smaller nonantigenic peptides [Niedermann *et al.,* 1995; Ossendorp *et al.,* 1996]. In addition to the possibility of affecting proteolysis of the viral proteins, flanking sequence variations with respect to the immunodominant KSPWFTTL could also influence the effectiveness of KSPWFTTL containing peptides to be translocated, via the TAP transporters, into the lumen of the ER for the subsequent binding to class I [Neisig *et al.,* 1995]. Moreover, when KSPWFTTL containing peptides are translocated into the ER, the possibility exists that flanking sequences may affect the ability to trim the peptides to the appropriate size for MHC class I binding. The precise role of flanking sequences in MHC class I antigen presentation, however, is not fully understood and remains controversial. While evidence has been reported demonstrating that flanking sequences can influence the presentation of MHC class I-restricted peptides, other cases exist where no influence is observed as well [Egger *et al.,* 1995; Niedermann *et al.,* 1995; Ossendorp *et al.,* 1996; Neisig *et al.,* 1995; Eisenlohr *et al.,* 1992; Koup, 1994; Del Val *et al.,* 1991; Hahn *et al.,* 1991 and 1992; Fu *et al.,* 1993; Mylin *et al.,* 1995; Bergmann *et al.,* 1994 and 1996; Shastri *et al.,* 1995; Couillin *et al.,* 1994; Chimini *et al.,* 1989; Yellen-Shaw *et al.,* 1997a and 1997b].

We approached these possibilities by constructing retroviral chimeras. To directly assess the influence of the only proximally located flanking sequence variation encoded by AK7, we positioned a small region containing the KSPWFTTL epitope along with the corresponding lysine or glutamate flanking sequence variation [Fig. 1], into a common viral backbone [BM5 ecotropic]. In the data presented in this report, we find that the existence of a positively charged lysine at position TM122, as opposed to a negatively charged glutamate, 12 amino acids N-terminal to KSPWFTTL in AK7 does not negatively impact the presentation of the immunodominat KSPWFTTL. This direct comparison was performed both in vaccinia K^b-infected SC-1 cells and in SC.K^b cells, the latter in which there should have been no limitation of expression of the K^b restricting element or complicating factors due to vaccinia virus infection. Our findings thus suggest that the processing and presentation machinery involved in the display of KSPWFTTL to H-2K^b-restricted CTL is not influenced by the specific TM122K variation.

The other prominent disparity between the AK7 and AKR623 viruses resides within the U3 region of the LTR. The U3 region of the retroviral LTR contains the transcriptional promoter and enhancer elements that are required for viral gene expression. The transcriptional enhancer region found in the murine retroviral LTR is usually observed as a direct repeat of 50 to 100 nucleotides and contains binding sites for various cellular transcription factors [Speck and Baltimore, 1987]. Studies on the pathogenesis of murine retroviruses have demonstrated that a crucial determinant of viral leukemogenicity is the transcriptional enhancer found within the U3 region of the LTR [Speck *et al.,* 1990; DesGroseillers and Jolicoeur, 1984; Morrison *et al.,* 1995]. Our lab has previously characterized the AK7 MuLV as having only one copy of the transciptional enhancer found in the U3 region as depicted in Fig. 1B [White *et al.,* 1993]. Interestingly, the AK7 virus differs in its single enhancer copy from AKR623 by two nucleotides [Fig. 1B] within the binding site for the transcription factor TEF-I [Lovmand *et al.,* 1990; Davidson *et al.,* 1988]. In this study, we have constructed a chimeric AKR623 retrovirus where we replaced its normal tandem direct repeat sequences with the single enhancer copy derived from the AK7 MuLV. If AK7-infected cells are inefficiently recognized by AKR/Gross MuLV-specific CTL solely due to the single copy of the transcriptional enhancer, then it would be predicted that the AKR623/7 LTR chimeric virus would similarly be inefficiently recognzied. Testing SC.K^b cells infected with this chimeric retrovirus against AKR/Gross MuLV-specific CTL has demonstrated that recognition by AKR/Gross MuLV-specific CTL occurs at levels intermediate to that of AKR623 and AK7-infected SC.K^b cells [Figs. 5A and 5B]. This experiment was performed four times and included the use of allogeneic CTL directed against H-2^b as an internal control for K^b expression levels [data not shown]. While variable susceptibilities to anti-H-2^b allogeneic CTL were sometimes observed, even when normalizing for the differential levels of allogeneic CTL susceptibilites, the overall interpretation that AKR623/7 LTR-infected SC.K^b cells are at lysis levels intermediate to that of AKR623- and AK7-infected cells by AKR/Gross MuLV-specific CTL remains consistent. When H-2K^b expression levels were monitored by flow cytometry, it was found that both AKR623- and AK7-infected SC.K^b cells expressed H-2K^b at essentially equivalent levels [Fig. 6].

FIG. 5. The AKR623/7 LTR chimeric virus is recognized by anti-AKR/Gross MuLV-specific CTL at a level intermediate to that of SC.K^b cells infected by AKR623 or AK7 MuLV. The AKR623/7 LTR viral chimera was used here to study the role of the AK7-derived enhancer on KSPWFTTL presentation. The AKR623/7 LTR viral chimera is essentially the AKR623 virus with its enhancer regions replaced with the single enhancer region derived from the AK7 LTR as detailed under Materials and Methods. SC.K^b cells infected with the AKR623/7 LTR chimeric retrovirus were used in a standard chromium release assay with AKR/Gross MuLV-specific CTL raised by B.GV tumor priming and either AKR.H-2^b.SL1 [A] or E♂G2 [B] tumor restimulation. A is a representative experiment while B depicts the averaged data of three other independent experiments. Target cell spontaneous release values ranged from 16 to 38%. These data were also analyzed for lytic units [LU]. The lysis curves corresponded to the following: (A) SC.K^b cells infected with AKR623, 57 LU/10^6 cells; SC.K^b cells infected with AKR623/7 LTR, 41 LU/10^6 cells; (B) SC.K^b cells infected with AKR623, 22 LU/10^6 cells; SC.K^b cells infected with AKR623/7 LTR, 4 LU/10^6 cells. In both A and B, the lysis of SC.K^b and SC.K^b cells infected with AK7 corresponds to less than 1 LU/10^6 cells.

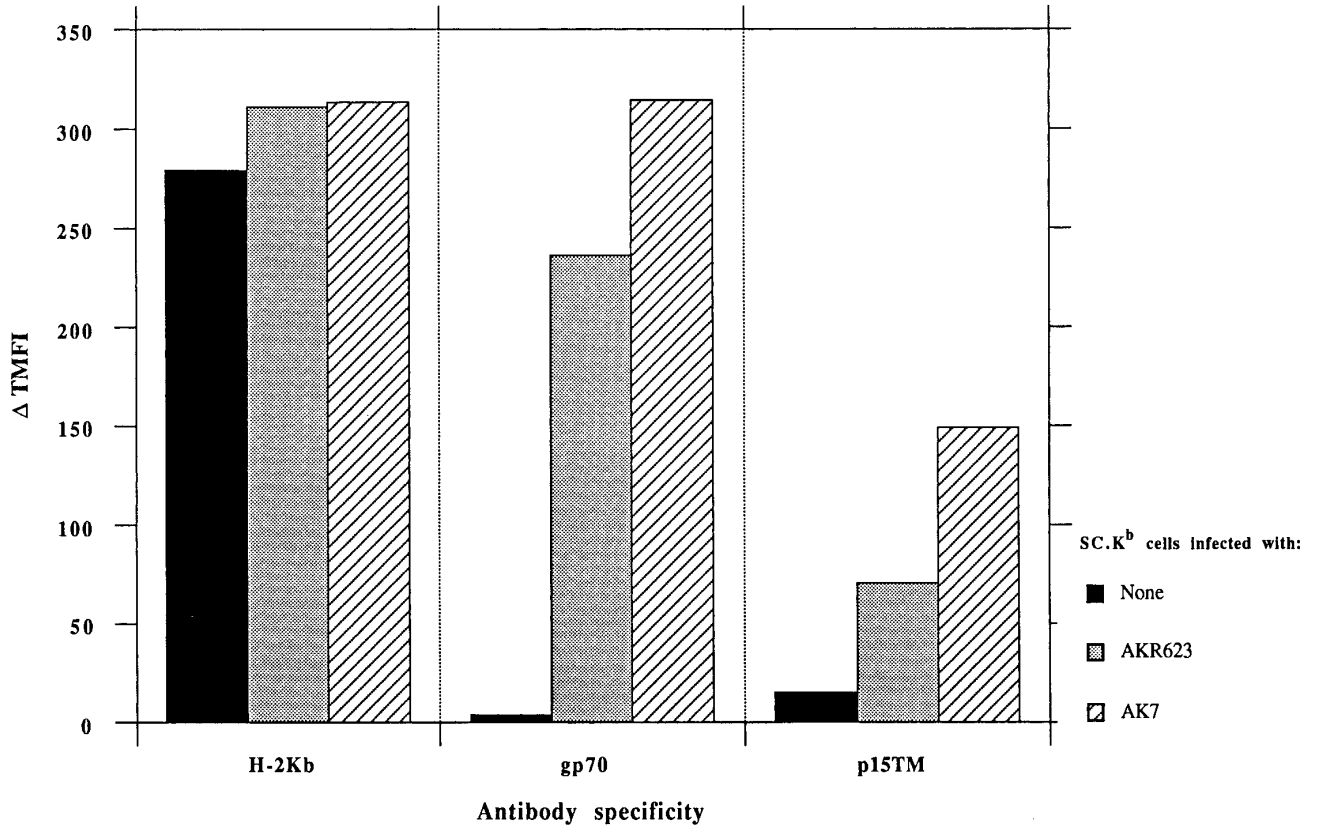

FIG. 6. The CTL restriction element, H-2K^b, and the viral envelope proteins gp70 and p15TM are expressed either equivalently or at somewhat greater levels in SC.K^b cells infected with the AK7 virus in comparison to cells infected by the AKR623 virus. Cell surface antigen expression was analyzed by indirect immunofluoresence as described under Materials and Methods. The results are displayed as the difference of the total mean fluoresence intensity [TMFI] values of the indicated experimental mAbs from which the background fluoresence TMFI of the matched isotype control was subtracted.

Collectively, these experiments suggest that because the AK7-derived transcriptional enhancer does not fully abrogate the ability of AKR623/7 LTR-infected cells to be recognized by AKR/Gross MuLV-specific CTL, the presence of only one copy of the transcriptional enhancer was sufficient for the processing and presentation of at least some of the KSPWFTTL CTL determinant. The observation that the AKR623/7 LTR chimeric was generally not as efficiently recognized as AKR623-infected cells, however, indicates that the introduction of the AK7-derived enhancer had a negative impact upon the ability to be recognized by AKR/Gross MuLV-specific CTL. Consequently, these data support the hypothesis that the deficiency of one copy of the AK7-derived transcriptional enhancer is an important, albeit incomplete, explanation for the inefficient levels of recognition of AK7-infected cells by AKR/Gross MuLV-specific CTL.

Consistent with the idea that multiple enhancers may be more effective at increasing transcription rates than a single enhancer copy [Lovmand *et al.,* 1990; Graves *et al.,* 1985; Boral *et al.,* 1989; Hallberg and Grundstrom, 1988; Losardo *et al.,* 1989; Etzerodt *et al.,* 1990], it was also observed that infection by AK7 spreads more slowly than AKR623 [White *et al.,* 1993]. Previous studies, however, examining the relative antiviral CTL susceptibilities of AKR623 versus AK7-infected SC.K^b cells have demonstrated that when the infection by AK7 was allowed to proceed until the cell surface viral antigen display reached steady-state and equivalent levels as AKR623-infected SC.K^b cells, that AK7 was still inefficiently recognized by AKR/Gross MuLV-specific CTL [White *et al.,* 1993]. Indeed, relative to the present studies, Fig. 6 clearly demonstrates that by flow cytometric analysis, AK7 causes the accumulation of at least as much of the viral proteins *env,* gp70, and p15TM when compared to AKR623. Rather than quantitatively lower overall levels of expression of either the *env* or p15TM proteins as the source for peptide 12, it is thus conceivable that the inefficient recognition of AK7-infected SC.K^b cells by AKR/Gross MuLV-specific CTL is partly due to either a slower rate of viral envelope translation by AK7-infected cells and/or a slower rate of degradation that would result in lower KSPWFTTL production. Implicit in this explanation is the concept that the mature p15TM protein or envelope precursor that is destined to become cell surface and virion-associated gp70/p15TM is not the only, or perhaps the primary, source for peptide 12. Whatever the identity of the important polypeptide precursor for peptide 12,

the measurement of mature p15TM or the *env* precursor under steady-state conditions may not provide an accurate assessment of its total availability for processing into peptide 12 for presentation by MHC class I. The recently proposed model by Yewdell *et al.* (1996) that defective ribosomal products [DRiPs], as byproducts of protein synthesis, can be a significant source of antigenic peptides supports our concept that the net [LTR driven] rate of production of a precursor, other than mature virion proteins, is what is limiting. Because of the partial effect observed with the chimeric LTR virus, however, the data are also suggestive that other mechanisms may exist. Although speculative, it is possible that multiple sequence variations may act in a cooperative mechanism to affect proteolytic and/or nonproteolytic processing steps necessary for the display of KSPWFTTL by H-2K^b.

Although the exact mechanisms causing the poor presentation of the immunodominant KSPWFTTL epitope are thus unresolved, it was important to determine whether AK7 interfered globally with the presentation of other class I-presented epitopes. As an incisive system for comparison, we tested whether AK7 interfered with the presentation of another H-2K^b-restricted T cell epitope by employing recombinant vaccinia virus vectors encoding for either full-length ovalbumin [OVA] or the minimal T cell epitope [SIINFEKL] to infect SC.K^b cells chronically harboring AK7 or AKR623. Using the SIINFEKL specific, CD8$^+$ T hybridoma RF33.70, we were able to measure SIINFEKL presentation through its production of IL-2 upon antigen stimulation. In two initial experiments the results, obtained from an IL-2 bioassay, demonstrated that relative to the presentation of exogenous SIINFEKL peptide which does not require processing, AK7 does not significantly impair the ability of SIINFEKL to be processed from OVA and presented by H-2K^b at the cell surface [data not shown]. Although there was some variation in the stimulation of RF33.70 cells by SC.K^b, SC.K^b cells infected by AK7, and SC.K^b cells infected by AKR623 in the two experiments, there were no differences that were consistent or of sufficient magnitude to account for the very poor recognition of AK7-infected cells by AKR/Gross MuLV-specific CTL. Thus, there was no evidence for global or *trans* effects by AK7 on the processing and presentation of other class I presented epitopes.

In summary, AK7 represents an interesting mechanistic phenomenon. Although AK7 encodes for an immunodominant CTL epitope, cells infected by this MuLV are very inefficiently recognized. AK7 thus provides an opportunity for future investigations on how retroviruses may evade efficient CTL recognition.

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