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Simian Virus 40 T Antigen as a Carrier for the Expression of Cytotoxic T-Lymphocyte Recognition Epitopes

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Simian virus 40 (SV40) large T antigen can immortalize a wide variety of mammalian cells in culture. We have taken advantage of this property of T antigen to use it as a carrier for the expression of cytotoxic T-lymphocyte (CTL) recognition epitopes. DNA sequences corresponding to an *H-2D*^{b}-restricted SV40 T-antigen site I (amino acids 205 to 215) were translocated into SV40 T-antigen DNA at codon positions 350 and 650 containing *Eco*RI linkers. An *H-2K*^{b}-restricted herpes simplex virus glycoprotein B epitope (amino acids 498 to 505) was also expressed in SV40 T antigen at positions 350 and 650. Primary C57BL/6 mouse kidney cells were immortalized by transfection with the recombinant and wild-type T-antigen DNA. Clonal isolates of cells expressing chimeric T antigens were shown to be specifically susceptible to lysis by CTL clones directed to SV40 T-antigen site I and herpes simplex virus glycoprotein B epitopes, indicating that CTL epitopes restricted by two different elements can be processed, presented, and recognized by the epitope-specific CTL clones. Our results suggest that SV40 T antigen can be used as a carrier protein to express a wide variety of CTL epitopes.

CD8$^+$ cytotoxic T lymphocytes (CTL) are crucial in controlling viral infections (14, 57). CTLs recognize viral antigens in the form of peptide fragments of 8 to 10 amino acids which are bound in the grooves of major histocompatibility complex (MHC) class I molecules (5, 20, 33, 49, 51). These antigenic peptides are derived from nonstructural and structural viral proteins through proteolysis in the cytosolic compartment (15, 46, 56) and are translocated into the endoplasmic reticulum by TAP1/TAP2 transporters (2, 31, 36, 37, 50, 52), where the peptide, MHC class I molecule, and β-2 microglobulin form a trimolecular complex (48). This complex is then exported to the cell surface through the exocytic pathway (9, 32, 55) for recognition by CD8$^+$ CTL via the T-cell receptor (24, 25).

The identification of viral CTL epitopes that are presented by MHC class I molecules and recognized by the epitope-specific CTL suggests a strategy for designing effective immunogens for the induction of a CD8$^+$ T-cell immune response to provide protection against viral infection (14, 28, 29, 47, 49). One approach to generating such an immune response utilizes immunization with synthetic peptides corresponding to the CTL epitopes either by themselves or with adjuvants (1, 7, 13, 26, 35, 39). Another approach has been to express CTL epitopes in virus vectors such as vaccinia virus (3, 4), either as chimeric proteins (12) or as minigene products (21, 38, 53, 54). These chimeric proteins or minigene products have been demonstrated to induce CTL responses and, in most cases, provide in vivo protection (12, 28, 54).

To utilize CTL epitopes as immunogens, it is important to experimentally assess their immunogenic potential by using a vector system most likely to enhance the immunogenicity of the CTL epitopes. In this study, we explored an approach using simian virus 40 (SV40) large tumor antigen (T antigen) as a carrier protein to express CTL epitopes. T antigen, a 94-kDa nuclear protein, immortalizes cells in culture (19, 27, 30, 42) and is highly immunogenic, containing multiple B-cell and T-cell epitopes (43). SV40 T antigen contains at least one *H-2K*^{b}-restricted epitope (site IV) and three *H-2D*^{b}-restricted CTL epitopes (sites I, II/III, and V). Sites I (positions 207 to 215), II/III (223 to 231), and V (489 to 497) are recognized by CTL clones Y-1, Y-2 or Y-3, and Y-5, respectively (10, 40, 41). Site IV (404 to 411) is *H-2K*^{b}-restricted and is recognized by CTL clone Y-4 (43a). Cells immortalized by T antigen can grow continuously in culture and express high levels of T antigen. Immunization of mice with cells expressing T antigen results in the generation of both primary and memory CTL responses (43).

Recently, a library of T antigens in which *Eco*RI linkers were inserted into the coding region of T antigen at 50-codon intervals between codons 250 and 650 was generated (27). Two locations in the T antigen (residues 350 and 650) were considered ideal sites for inserting CTL epitopes, since these sites in the protein are not required for immortalization of primary cells in culture (45). We have translocated an *H-2D*^{b}-restricted T-antigen epitope, site I, from its original position between residues 205 and 215 to amino acid positions 350 and 650 of T antigen. In addition, an *H-2K*^{b}-restricted herpes simplex virus (HSV) type 1 glycoprotein B (gB) CTL epitope, residues 498 to 505 (6), was cloned into the same locations of T antigen (positions 350 and 650). The results show that the translocated SV40 T antigen site I and the HSV gB CTL epitopes in the chimeric T antigens, when expressed in immortalized C57BL/6 (B6) mouse kidney cells, were efficiently processed and were presented by the MHC class I molecules and recognized by the epitope-specific CTL clones.

The chimeric T antigens were generated by using two T-antigen constructs (27) which contain an *Eco*RI site immediately following codons 350 and 650 of T antigen, pPvu0:E350 and pPvu0:E650, respectively. Oligonucleotide pairs representing two strands of the SV40 T-antigen site I (residues 205 to 215) or the HSV gB (residues 498 to 505) CTL epitope sequence were designed such that when annealed, the small double-stranded fragment would have exposed at each end the sequence 5′-AATT. The annealed oligonucleotides were ligated with the *Eco*RI-linearized plasmids pPvu0:E350 and pPvu0:E650 by using T4 ligase (Fig. 1A), and the ligated

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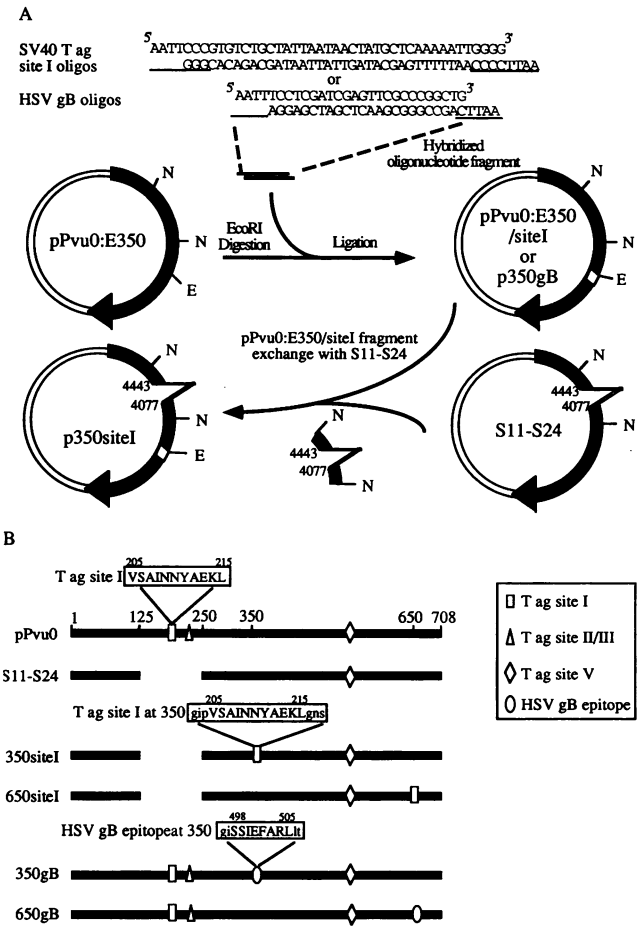

FIG. 1. Cloning strategy and resultant T-antigen constructs. (A) Two pairs of oligonucleotides representing SV40 T-antigen (Tag) site I (residues 205 to 215) or HSV gB epitope (residues 498 to 505) with *Eco*RI-compatible ends were synthesized and hybridized. The *Eco*RI-compatible sequences and adjacent nucleotides added to maintain the correct reading frame are underlined. The oligonucleotide fragments were inserted at the *Eco*RI site of pPvu0:E350. To remove the original site I sequence within the T-antigen construct which contains the site I sequence at amino acid position 350, the *Nde*I fragment within the construct was replaced with the corresponding fragment from S11-S24 T-antigen mutant, which has a deletion of nucleotides 4443 to 4077 including the site I sequence. The final constructs, p350site I and p350gB, are indicated. Constructs p650site I and p650gB were constructed in a similar manner. N, recognition site for the endonuclease *Nde*I; E, recognition site for *Eco*RI. (B) Constructs used in this study and positions of the CTL epitopes. The amino acid sequences of the CTL epitopes are listed in single-letter code. Uppercase letters refer to the CTL epitope amino acid sequences, and lowercase letters represent the flanking amino acids encoded by the *Eco*RI linker sequence.

products were transfected into competent *Escherichia coli* DH5α. The recombinant plasmid DNA was purified by the alkaline lysis procedure and analyzed by restriction endonuclease digestion (34). The restriction endonuclease used to screen recombinant T-antigen constructs containing the site I insert was *Asn*I, which recognizes an ATTAAT sequence located within the site I epitope sequence, and the restriction endonuclease used to screen recombinants containing the gB epitope insert was *Eco*RI, because the oligonucleotides were designed such that the *Eco*RI site would be destroyed upon the insertion. The orientation and authenticity of the inserts were

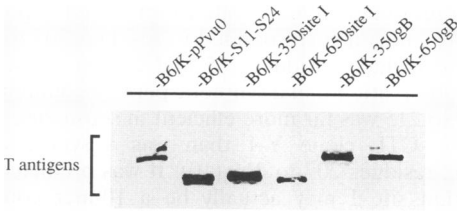

FIG. 2. Levels of T-antigen expression in immortalized cell lines. The same amount of protein from each cell lysate was first immunoprecipitated with PAb901 and then blotted onto nitrocellulose membrane and probed with PAb901. Binding of PAb901 to T antigen was detected by reacting the blot with goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase and then subjecting the blot to enhanced chemiluminescence treatment (27). B6/K-pPvu0 cells express a wild-type T antigen of about 94 kDa, whereas B6/K-S11-S24 cells express a mutant T antigen with a molecular mass of about 67 kDa as a result of deletion of 124 amino acids.

determined and confirmed by DNA sequencing as described previously (27). In constructing recombinant T antigens containing a relocated site I epitope, the original site I sequence was removed by fragment exchange with a construct containing the mutant T antigen, S11-S24. S11-S24 produces a T antigen which contains an in-frame deletion from amino acids 127 to 250. Therefore, the S11-S24 T antigen lacks the CTL recognition epitopes site I (205 to 215) and site II/III (223 to 231) (41). A fragment containing the deletion was released from the S11-S24 construct by digestion with *Nde*I and used to replace the corresponding fragment of the constructs that have the site I sequence inserted at positions 350 and 650 of T antigen (Fig. 1A).

Expression of chimeric T antigens in primary B6 cells. Primary adult B6 kidney cells or embryo fibroblasts were immortalized by the wild-type or chimeric SV40 T antigen as described previously (42). All recombinant T antigens (Fig. 1B) were capable of immortalizing B6 primary kidney cells and B6 embryo fibroblasts. Colonies of immortalized cells were picked and expanded into stable cell lines. All cell lines were found to synthesize SV40 T antigen when examined by indirect immunofluorescence with monoclonal antibodies PAb419 and PAb901 (45), which recognize the N and C termini, respectively, of T antigen (results not shown). The recombinant T antigen containing site I translocated to residues 350 or 650 was localized to the cytoplasm (data not shown) because of the deletion of the nuclear localization signal. The chimeric T antigen containing the HSV gB epitope at position 350 or 650 of T antigen was localized to the nucleus as expected. All of the cell lines used in the CTL assays were examined by Western blotting (immunoblotting) as described previously (27) with PAb901 and were found to contain comparable levels of T antigen. The expression of T antigen in one clonally derived immortalized B6 kidney cell line of each type is shown in Fig. 2. The lower molecular weight of the chimeric S11-S24 T antigen than of wild-type T antigen is due to the deletion of 124 amino acids. The B6 mouse embryo fibroblasts immortalized by these constructs were found to express similar levels of T antigen (data not shown).

Expression and recognition of *H-2D*^{b}-restricted site I translocated to T-antigen positions 350 and 650. The SV40 large T antigen contains three *H-2D*^{b}-restricted CTL epitopes, sites I, II/III, and V, which are recognized by the site-specific CTL clones Y-1, Y-2 or Y-3, and Y-5, respectively (10, 40, 41). Site I is located in the amino-terminal one-third of SV40 T antigen and corresponds to residues 207 to 215 (10). However, the

T-antigen residues containing site I transferred to position 350 or 650 of T antigen corresponded to residues 205 to 215. The decision to include residues 205 and 206 was based on our previous observation that a synthetic peptide containing amino acids 205 to 215 was far more efficient in sensitizing $H\text{-}2^b$ cells to lysis by CTL clone Y-1 than was a synthetic peptide containing residues 207 to 215 (10). It was predicted, but not proved, that site I may actually be a 10-mer consisting of residues 206 to 215. The original site I along with the neighboring site II/III sequence within the recombinant T antigens was removed by fragment exchange with a mutant T-antigen sequence, S11-S24, which has a deletion removing codons 127 to 250. Site II/III has been defined by T-antigen residues 223 to 231 (10). The processing, presentation, and recognition of the site I in these cell lines were examined by standard ^{51}Cr release assay as described previously (10, 41). Briefly, target cells were treated with gamma interferon for 48 to 72 h prior to the assay to increase the cell surface expression of MHC class I molecules on target cells and then labeled with 150 μCi of ^{51}Cr for 1 h, washed three times, and mixed with CTLs at designated effector/target cell ratios. After centrifugation of the CTL-target cell mixture at 400 × *g* for 5 min, the cultures were incubated for 5 h at 37°C in the presence of 5% CO_2. A 100-μl sample of supernatant from each cell mixture was counted to determine the amount of ^{51}Cr released from target cells. The percentage of specific lysis was calculated as $(E - S)/(M - S)$, where E represents the average counts per minute released from target cells in the presence of effector cells, S represents the spontaneous counts per minute released in the presence of medium only, and M represents the maximum counts per minute released in the presence of 5% sodium dodecyl sulfate. The results in Fig. 3 show that two clonal isolates immortalized with the wild-type T antigen (B6/K-pPvu0 Cl-1 and Cl-2) were recognized by $H\text{-}2D^b$-restricted CTL clones Y-1, Y-2, and Y-5. The lytic activity of CTL clone Y-5 is low compared with that of CTL clone Y-1. Site V is an immunorecessive site and has been mapped to SV40 T-antigen residues 489 to 497 (10). CTL clone Y-5 shows a consistently lower level of recognition in ^{51}Cr release assays in most cell lines (40). The cells (B6/K-S11-S24 Cl-1 and Cl-2) immortalized by S11-S24 were not recognized and lysed by CTL clones Y-1 and Y-2 as a result of the deletion of sites I and II/III, as expected, but were lysed by CTL clone Y-5, which recognizes residues 489 to 497 in the T antigen.

Cells (B6/K-350 site I Cl-1 and Cl-2) expressing chimeric T antigens containing site I at position 350 were recognized by CTL clone Y-1 (Fig. 3) at levels comparable to those in cell lines expressing wild-type T antigen. Again, site II/III was not recognized by the CTL clone, as expected because of the deletion of residues 127 to 250, a segment which includes site II/III (residues 223 to 231). These cells, however, remained susceptible to the lysis by CTL clone Y-5. Similar results were obtained when site I was translocated to T-antigen position 650. These results indicate that the T-antigen site I CTL epitope can be transferred to either position 350 or 650 in T antigen without affecting its processing, presentation, or recognition by the site-specific CTL clone.

Expression and recognition of the $H\text{-}2K^b$-restricted HSV gB epitope (498 to 505) transferred to T-antigen positions 350 and 650. Comparison of CTL clone Y-1's recognition of the site I CTL epitope at the original and relocated positions clearly indicated that transfer of this $H\text{-}2D^b$ epitope did not affect the efficient processing and presentation of this antigenic determinant. We then tested whether these two positions can be used to express and present an $H\text{-}2K^b$-restricted HSV gB CTL recognition epitope at amino acids 498 to 505. This epitope is

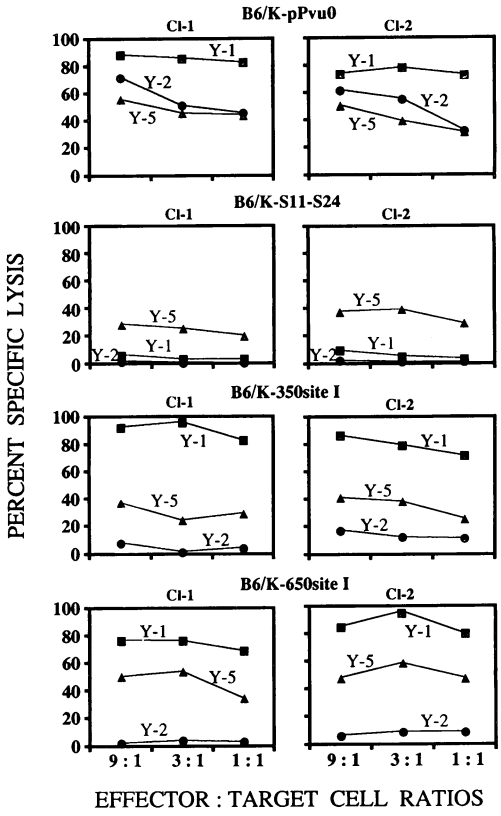

FIG. 3. Effect of translocation of SV40 T-antigen site I to positions 350 and 650 in T antigen on recognition by the site I-specific CTL clone. B6/K-pPvu0, B6/K-S11-S24, B6/K-350site I, and B6/K-650site I cells were labeled with ^{51}Cr and reacted with T-antigen site-specific CTL clones Y-1, Y-2, and Y-5 for 5 h at the indicated effector/target cell ratios. Cl-1 and Cl-2 refer to the two independent clonal isolates.

recognized by HSV CTL clone 2D5 (6). The data in Fig. 4 show that CTL clone 2D5 can recognize cells immortalized by T antigens containing the gB epitope irrespective of its location at amino acid 350 or 650. These cells were also susceptible to lysis by the CTL clone Y-1 and thus served as positive internal control for the processing of T antigen. It should be noted that the sequences flanking the gB epitope within the recombinant T antigens are different from those of site I transferred to the same locations (Fig. 1B), and neither change in flanking sequences affects the processing, presentation, and recognition of the transferred CTL determinants. Our results show that CTL epitopes restricted by two different restriction elements can be expressed from the same location in T antigen and efficiently presented to epitope-specific CTLs.

The demonstration that CTLs recognize 8- to 10-amino-acid-long peptides presented by the MHC class I molecules has led to efforts to utilize CTL epitopes to induce CTL responses and immune protection against viral infections (28, 29, 35, 49, 51, 54). Immunization of hosts with synthetic peptides themselves or in combination with adjuvants has been shown to induce the generation of CTL (1, 7, 13, 26, 35, 39). Alternatively, the CTL epitopes have been presented by using virus vectors, among which vaccinia virus has been used most effectively (3, 4, 12, 28, 53, 54). In addition, CTL epitopes expressed within heterologous proteins have been shown to be processed, presented, and recognized by epitope-specific CTL (8, 11). In this study, we investigated the possibility of using

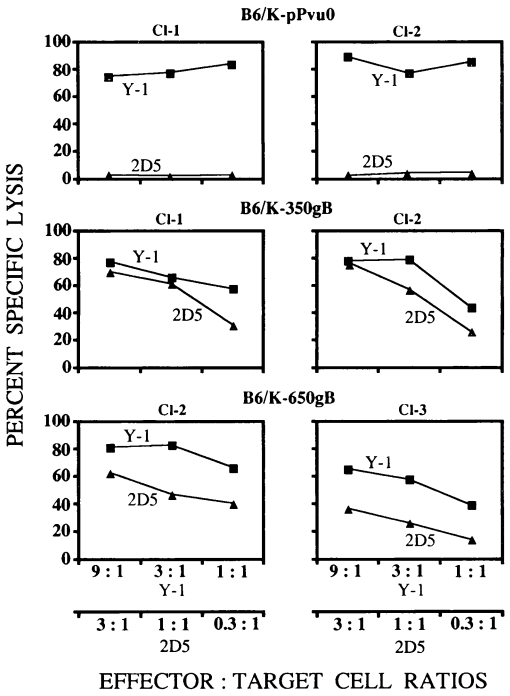

FIG. 4. Recognition of the HSV gB epitope expressed at amino acid positions 350 and 650 of T antigen by the HSV gB epitope-specific CTL clone. B6/K-pPvu0, B6/K-350gB, and B6/650gB cells were labeled with ^{51}Cr and incubated with site-specific CTL clones 2D5 and Y-1 for 5 h at the indicated effector/target cell ratios.

SV40 T antigen as a carrier protein to express CTL epitopes. There are several advantages to using T antigen to deliver CTL epitopes. T antigen is a nonstructural nuclear multifunctional protein which is well marked antigenically; as a result, the integrity of the protein in the cells expressing the chimeric protein can be precisely determined by using monoclonal antibodies with defined specificities (43, 45). The SV40 T-antigen-coding sequence has undergone close scrutiny with respect to the regions that are dispensable for its immortalization function (45), and multiple cloning sites which can be used for the introduction of CTL epitopes have been inserted into T antigen (27). T antigen can immortalize primary mouse and other mammalian cells in culture and thus acts as a dominant marker for the selection of transfectants (19, 27, 30, 42). The cells immortalized by T antigen express high levels of the protein.

Using this knowledge, we have identified at least two locations in T antigen, positions 350 and 650, which appear to be suited for the expression of CTL epitopes restricted by *H-2K*^{b} and *H-2D*^{b} epitopes. The transfer of the T-antigen CTL site I epitope (residues 205 to 215) to positions 350 and 650 of T antigen and expression of the HSV gB epitope (amino acids 498 to 505) in the same locations did not interfere with the processing, presentation, and recognition of these epitopes by the respective CTL clones. In this respect, our results appear to be similar to those reported by others (8, 22) who demonstrated that the flanking sequence changes surrounding the epitope do not affect its processing. It should be noted that T-antigen site I transferred to different locations in T antigen carries at least one additional residue, 205, flanking the amino terminus of CTL site; therefore, expression of this site may not be influenced by the residues flanking this site at positions 350 and 650. However, in other instances, epitope location and flanking sequence have been shown to influence antigen processing and presentation. Del Val et al. (11) have shown that an *H-2L*^{d}-restricted CTL epitope of the murine cytomegalovirus immediate-early (IE1) protein was not efficiently processed upon transfer to amino terminus of the hepatitis B virus core antigen (HBVc), which was expressed in a recombinant vaccinia virus. The defect in processing of this epitope from the amino-terminal region of HBVc was related to the decreased amounts of natural epitope peptide generated in vivo. This defect could be overcome by inserting polyalanine linkers surrounding the epitope at the unfavorable site. The processing of the same epitope, when expressed at another location in HBVc or translocated to another position within the murine cytomegalovirus IE1 protein, was not affected (11). Eisenlohr et al. (17) have shown that the addition of two residues, 157 and 158, with deletion of arginine at position 156 to the influenza virus *H-2K*^{d}-restricted CTL epitope, amino acids 147 to 155, and delivered through a recombinant vaccinia virus resulted in defective presentation of this epitope to CTL. This negative effect on antigen processing could be reversed by expressing this epitope, along with the negatively influencing residues, either in the context of full-length nucleoprotein (17) or in cells expressing a membrane dipeptidyl carboxypeptidase angiotensin-converting enzyme (16). Hahn et al. recently reported abolition of recognition of the minimum influenza virus hemagglutinin *H-2K*^{d}-restricted epitope (204 to 213) by an amino acid substitution (Val→Gly) at position 214 (23). The results from these studies appear to show that flanking residues in some cases do affect antigen processing, and the residues which negatively influence antigen processing are close neighbors of the CTL epitope.

It should be mentioned that T-antigen CTL site I (amino acids 205 to 215) relocated to residues 350 and 650 is an 11-mer, longer than the 10-mer predicted on the basis of the *H-2D*^{b} binding motif identified by Falk and coworkers (18). Although the minimum site I determinant includes residues 207 to 215, we have previously shown that an 11-mer synthetic peptide corresponding to T-antigen residues 205 to 215 is recognized at a much higher efficiency by CTL clone Y-1 than is the 9-mer synthetic peptide consisting of residues 207 to 215 (10). It is possible that other locations in T antigen have negative influences on antigen processing.

The *H-2K*^{b}-restricted HSV gB epitope, in contrast, matches the MHC binding motif (18, 51). Although the immediate flanking sequences surrounding the gB epitope encoded by the *Eco*RI linker sequence are different from those of the transferred site I determinant, neither showed any negative effect on antigen processing and presentation. Therefore, it appears that T-antigen locations at residues 350 and 650 act as universal acceptor locations for the expression of CTL epitopes restricted by various MHC alleles. The results obtained in this study show that epitopes restricted by both *H-2D*^{b} and *H-2K*^{b} can be expressed from these two locations in T antigen. The expression of the HSV gB epitope recognized by CTL clone 2D5 in T antigen will allow us to determine its immunogenic potential in vivo and its ability to induce long-term T-cell memory. Preliminary results indicate that B6 cells carrying the chimeric T antigen can induce the generation of HSV gB epitope-specific CTL and activate memory T cells (5a). The use of SV40 T antigen as a carrier for foreign CTL epitopes also will allow one to determine the genetic stability of sequences encoding CTL epitopes.

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