

CYTOLYTIC T LYMPHOCYTE RECOGNITION OF THE  
MURINE CYTOMEGALOVIRUS NONSTRUCTURAL  
IMMEDIATE-EARLY PROTEIN pp89 EXPRESSED BY  
RECOMBINANT VACCINIA VIRUS

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The infection of the mouse with murine cytomegalovirus (MCMV)<sup>1</sup> serves as a model to study the biology of CMV infection. Similar to the situation in the immunocompromised patient after infection with human CMV (HCMV), immunodeficient mice develop interstitial pneumonia and succumb to generalized MCMV infection (1). Immunocompetent mice generate specific cytolytic T lymphocytes (CTL) (2) and prophylactic as well as therapeutic adoptive transfer of CTL into immunodeficient recipients limits viral spread in tissues and protects mice from fatal disease (1, 3).

We wish to define the antigens detected by protective CTL and the genes encoding them. As in other herpesviruses, MCMV is expressed in three sequential phases, immediate-early (IE), early, and late, which show coordinate regulation and temporal control (4). IE genes are the first viral genes expressed after infection, and IE proteins are required for the induction of early gene expression. CTL recognize at least one membrane antigen expressed already during the IE phase of infection (5). These IE-specific CTL comprise the majority of MCMV-specific CTL (6). Within the 235 kbp double-stranded linear DNA of the MCMV genome (7), abundant IE transcription originates from a region of ~12 kbp containing three transcription units: ie1, ie2, and ie3 (4, 8). Transcription unit ie1 is predominantly transcribed, and proteins encoded by this unit have a function in transcriptional activation (9, 10).

Transfection of the complete IE region or of the isolated transcription unit ie1 leads to antigen expression in transfected cells detected by IE-specific CTL (11, 12). Several differently spliced mRNAs are transcribed from ie1. A 2.75 kb mRNA transcribed from gene *ie1* contained in transcription unit 1 is translated into a nonstructural phosphoprotein of 89 kD, the major MCMV IE protein

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<sup>1</sup> *Abbreviations used in this paper:* IE, immediate-early; MCMV, murine cytomegalovirus; MEF, mouse embryo fibroblasts; orf, open reading frame; VAC, vaccinia virus.

pp89. Additional smaller *ie1* products, which are antigenically related to pp89, range in size from 31 to 67,000 kD (8). Gene *ie1* has been sequenced, and after mapping of exons, an open reading frame has been deduced (13). To prove the identity of the deduced *ie1* product with pp89 and to test whether the major IE protein is processed to a membrane antigen recognized by IE-specific CTL, we turned to the selective expression of gene *ie1* in eukaryotic cells. Here we report on the construction of the continuous coding sequence of *ie1* by oligonucleotide-directed mutagenesis, its integration into a vaccinia virus (VAC) vector, and on some biological properties of the resulting MCMV-*ie1*-VAC recombinant.

### Materials and Methods

*Site-directed Mutagenesis and Construction of VAC Recombinants.* The 1.5 kb Hind III–Sac I fragment of gene *ie1*, containing the 3' splice site of intron 1 and the introns 2 and 3, was cloned into M13mp19 for mutagenesis. Oligonucleotide-directed mutagenesis was performed according to a protocol by Zoller and Smith (14). 10 pmols of kinase-treated mutagenic primer were added to 1  $\mu$ g of single-stranded template DNA in 10  $\mu$ l TM-buffer (10 mM Tris HCl, pH 8, 10 mM MgCl<sub>2</sub>), incubated at 80°C, and allowed to cool down to room temperature for annealing. The annealed mixture was adjusted to a 20  $\mu$ l volume of 10 mM Tris HCl, pH 8, 10 mM MgCl<sub>2</sub>, 250  $\mu$ M dNTPs, 250  $\mu$ M rATP, and 5  $\mu$ M dithiothreitol. After addition of 10 U ligase (Boehringer Biochemicals, Mannheim, Federal Republic of Germany) and 1 U Klenow fragment of DNA polymerase (Bethesda Research Laboratories, Gaithersburg, MD), the sample was incubated overnight at 12°C for primer extension. The mixture was used for transformation of BMH 71-18 mut (mutator) L cells (15). BMH 71-18 (15) provided the feeder lawn, to minimize undesired mutagenesis by the transformed mutator strain. Plaques were picked and grown on LB agar plates (Difco Laboratories, Detroit, MI). The colonies were transferred to nitrocellulose filters and screened for mutagenized clones by hybridization using the end-labeled mutagenic oligonucleotides. Finally, the resulting mutagenesis was controlled by sequencing of DNA from the plaque-purified clones. The open reading frame of *ie1* was cloned into the Eco RI site of the VAC recombination vector pGS62 (16). Construction of recombinant VAC followed established procedures (17, 18) using the VAC strain WR and the temperature-sensitive mutant ts 7 of the VAC strain Copenhagen (19–21).

*Immunoprecipitation.* Protein extracts were prepared of cells labeled with 100  $\mu$ Ci [<sup>35</sup>S]methionine for 2 h in methionine-free DMEM and solubilized in lysis buffer (10 mM Tris HCl, pH 7.6, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mg/ml ovalbumin, 1 mM methionine, 1 mM phenylmethylsulfonyl fluoride). IE protein was precipitated with murine antiserum to MCMV. SDS gel electrophoresis was carried out as previously described (9).

*Generation of Cytolytic Effector Cells.* For polyclonal CTL, splenocytes and mesenteric lymph node cells were obtained from BALB/c mice 2 wk after intravenous immunization with  $2 \times 10^7$  PFU of the VAC recombinant or of wild-type VAC. Lymphocytes ( $4 \times 10^6$  in 2 ml cultures) were restimulated in vitro for 5 d using  $10^6$  PFU of live MCMV per culture (strain Smith, VR-194; American Type Culture Collection, Rockville, MD) as antigen (2, 22).

Expression of MCMV IE membrane antigen was tested with CTL clone IE1, line 1.18-IL (22–24). This line was maintained in IL-2-supplemented medium without antigen.

*Target Cells and Cytolytic Assay.* The following target cells were used: (a) BALB/c mouse embryo fibroblasts (MEF). MEF were infected with MCMV under conditions that provided selective expression of IE genes as described before (4–6, 9). (b) Ltk<sup>-</sup> (C3H/An origin, haplotype H-2<sup>k</sup>) cells transfected with the H-2L<sup>d</sup> gene (line L/L<sup>d</sup>) or cotransfected with H-2L<sup>d</sup> and MCMV *ie1* (line L/*ie1*-L<sup>d</sup>/2) (12). (c) L/L<sup>d</sup> cells infected with MCMV-*ie1*-VAC or wild-type VAC. L cells were infected at a multiplicity of infection of 5 to 10 with wild-type VAC or with recombinant VAC 18 h before testing in the cytolytic assay.

A standard 3-h CTL assay was used (6, 22–24). In brief,  $10^3$  <sup>51</sup>Cr-labeled target cells

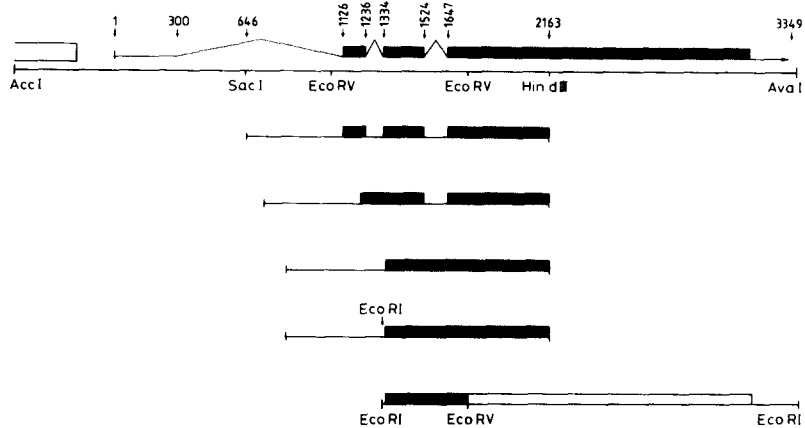


FIGURE 1. Alteration of gene *iel* structure. Gene *iel* was modified as described in Materials and Methods. The structural organization of the *iel* gene in the MCMV genome is depicted at the top. The coding sequences of exons are indicated by black bars. The location of the upstream MCMV enhancer (25) is indicated by an open box. The distance (in base pairs) from the transcription start site and the positions of exons, introns, and some cleavage sites are indicated. Mutagenesis was performed in three sequential steps shown below: First, intron 2 and then intron 3 were removed by using oligonucleotides comprising the flanking exon sequences of the respective intron. The following 20-mers were used for deletion: Intron 2, 5'-CAT CAG ACA AGG TGC CAG CT-3'; intron 3, 5'-CAT GCT GCA GTG AGG AGC GT-3'. The 26-mer 5'-TGA TGA TAA AGA ATT CTA TTT TTT TA-3', with the mismatches underlined, was used to introduce a new Eco RI site 22 bp upstream of the initiation codon by a two-basepair exchange. The restructured continuous coding sequence of *iel*, consisting of the mutagenized fragment (*black bar*) joined to the nonmutagenized large fragment of exon 4 (*open bar*), flanked by Eco RI sites, is shown at the bottom.

per well were added to the effector cells in 96-well tissue culture plates. The E/T ratio ranged from 3:1 to 50:1 for polyclonal CTL, and from 0.7:1 to 25:1 for the IE-specific CTL clone. Data are expressed as the mean percent specific lysis of four replicate cultures.

## Results

*Construction of a Recombinant VAC Containing the pp89 Open Reading Frame.* The gene *iel* encoding the major IE protein pp89 has a four-exon structure (13) (Fig. 1). The initiation codon of the pp89 open reading frame (orf) is located at position 1,130 in the second exon. Interrupted twice by introns 2 and 3, it is terminated at position 3,134 in the fourth exon. For selective expression of the pp89 orf in VAC, we had to construct the continuous pp89 coding sequence without introns. The introns 2 and 3 were removed by site-directed mutagenesis. To this end, the Hind III-Sac I fragment, which contains the 3' splice acceptor site of intron 1, the introns and exons 2 and 3, and a fragment of exon 4, was cloned into M13mp19. Single-stranded template DNA was isolated, and the orf was restructured by sequential excision of the introns 2 and 3. In addition, an Eco RI site was created 22 bp upstream of the initiation codon. The Eco RV fragment, which contained the mutagenized regions, was sequenced and then cloned into the Eco RV site of the 2 kb Ava I fragment encompassing the complete fourth exon. The 5' Ava I site is located in the third exon, and the 3' Ava I site is at position 3,349. Before, the Ava I fragment was

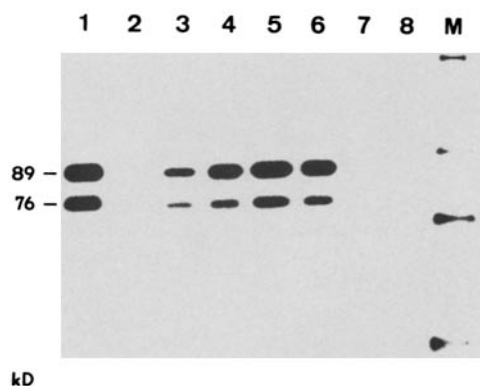


FIGURE 2. Immunoprecipitation of proteins synthesized after infection with MCMV-*ieI*-VAC. Lane 1, MEF infected with MCMV; lanes 2–6, L/L<sup>d</sup> cells infected with MCMV-*ieI*-VAC for 6, 8, 10, 14, and 16 h; lane 7, L/L<sup>d</sup> cells infected with wild-type VAC; lane 8, mock-infected L/L<sup>d</sup> cells; M, marker lane. Markers were myosin, 200; phosphorylase, 92.5; BSA, 69; and ovalbumin, 46 kD. Cells were infected at a multiplicity of infection of 20, and were labeled 2 h before the preparation of lysates.

filled up to blunt ends and cloned into the Sma I site of pUC12 in the orientation that placed the Eco RI site of the polylinker at the end of the fourth exon of *ieI*. After insertion of the mutagenized Eco RV fragment, the restructured intron-free gene *ieI* was now flanked by two Eco RI sites: a polylinker Eco RI site at the 3' end, and a new Eco RI site introduced by site-directed mutagenesis 5' of the orf. This Eco RI fragment was cloned into the Eco RI site of pGS62 downstream of the P7.5 VAC promoter. This construct (pGS62-pp89-orf) was used to prepare a recombinant VAC.

**Expression of pp89 in Recombinant VAC.** Infection of MEF and of L cells with the isolated recombinant (MCMV-*ieI*-VAC) resulted in expression of nuclear antigen detected by immunofluorescence using monoclonal antibody specific for pp89 (9, 10), while cells infected with wild-type VAC showed no fluorescence (not shown). The size of the polypeptide expressed in MCMV-*ieI*-VAC-infected cells was determined by immunoprecipitation (Fig. 2). Murine antiserum to MCMV precipitated a polypeptide migrating at the position of 89 kD. In addition, a band was seen that migrated with an  $M_r$  of 76,000. The relative molecular masses of the larger and the smaller protein correspond to the size of MCMV pp89 and its 76,000  $M_r$  derivative, a posttranslational modification product (9). Thus, the results demonstrated that pp89 was correctly expressed by the VAC vector and that the polypeptide was properly modified and correctly transported to the cell nucleus. Despite that the P7.5 promoter of VAC is active at both, early and late times of infection, the kinetics of polypeptide synthesis showed that efficient pp89 expression in MCMV-*ieI*-VAC occurred mainly at the late stage of VAC replication.

**CTL Recognition of Antigens Induced by MCMV-*ieI*-VAC.** The CTL clone IE1 detects an antigen expressed at IE times of infection in association with the MHC class I molecule L<sup>d</sup> (11, 22–24). After transfection of transcription unit 1 into L cells, the epitope recognized by this CTL clone is detectable (12). This CTL clone was now used to test L/L<sup>d</sup> cells after infection with MCMV-*ieI*-VAC for expression of the epitope (Fig. 3). The specific cytolytic activity of the CTL clone IE1 is demonstrated by lysis of line L/ie1-L<sup>d</sup>/2 expressing both, H-2L<sup>d</sup> and products of transcription unit *ie1* of MCMV (Fig. 3, ■). L/L<sup>d</sup> cells infected with wild-type VAC (Fig. 3, ○) and mock infected cells (□) were not lysed. Specific lysis of L/L<sup>d</sup> cells infected with the MCMV-*ieI*-VAC (●) was comparable to that

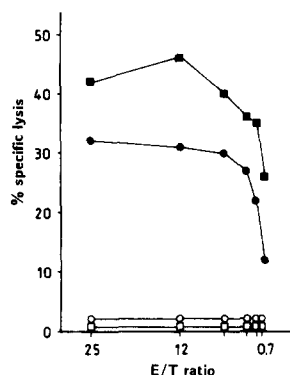


FIGURE 3. Lysis of MCMV-*iel*-VAC-infected cells by cloned CTL. Noninfected ( $\square$ ) L/L<sup>d</sup> cells or cells infected with either recombinant MCMV-*iel*-VAC ( $\bullet$ ) or wild-type VAC ( $\circ$ ) at a multiplicity of infection of 5–10 were used. After 18 h incubation, the cells were labeled for 1.5 h with <sup>51</sup>Cr and dispensed into 96-well microtiter plates (10<sup>3</sup> cells/well) containing graded numbers of CTL clone IE1. <sup>51</sup>Cr-labeled L/*iel*-L<sup>d</sup>/2 cells served as control target for the detection of the IE1 epitope ( $\blacksquare$ ).

of L/*iel*-L<sup>d</sup>/2. MCMV-*iel*-VAC-infected L cells that lacked L<sup>d</sup> were not recognized (not shown). Thus, pp89, the product of the *iel* gene in transcription unit 1, provides the epitope recognized by the CTL clone IE1.

**Recombinant VAC Primes for pp89-specific CTL.** BALB/c mice were infected with either wild-type or recombinant VAC. At day 14, cells from the spleen and lymph nodes were restimulated with MCMV in vitro. The secondary polyclonal CTL response was tested 5 d later (Fig. 4). CTL from MCMV-*iel*-VAC-primed mice were able to lyse syngeneic MCMV-infected fibroblasts selectively expressing IE gene products after MCMV infection (IE-MEF), L/L<sup>d</sup> cells infected with MCMV-*iel*-VAC, and also line L/*iel*-L<sup>d</sup>/2 (Fig. 4, top row,  $\bullet$ ). IE antigen expression in target cells is usually low, and specific lysis, even when the cloned CTL line is used rarely exceeds 40%. In the experiment shown, the lytic activity of MCMV-*iel*-VAC-primed polyclonal CTL on target cells expressing the *iel* gene was comparable to that of the cloned CTL line. Uninfected MEF, wild-type VAC-infected L/L<sup>d</sup> cells, and noninfected L/L<sup>d</sup> cells were not recognized, demonstrating the specificity of the CTL activity (Fig. 4, bottom row,  $\bullet$ ). Lymphocytes from wild-type VAC-sensitized mice restimulated in vitro with MCMV failed to lyse any of the target cells ( $\circ$ ). MCMV-*iel*-VAC- and wild-type VAC-primed mice generated VAC-specific CTL after restimulation with VAC (not shown).

## Discussion

In our previous studies on MCMV IE antigen recognition by CTL (5, 6, 11, 22–24), we detected that nonstructural viral IE proteins (7–10) serve as dominant target antigens for the cellular immune response. In further studies, we were able to associate IE antigen expression detected by CTL with the expression of transcription unit *iel* (12).

Because transcripts from *iel* are differently spliced and translated into proteins of different size, results obtained by transfection of MCMV DNA sequences could not identify the gene product of *iel* that leads to the expression of a target antigen. We have analyzed the structural organization and the sequence of the dominantly expressed gene *iel* in transcription unit *iel* that gives rise to the 2.75 kb mRNA translated into pp89 (13). Based on this analysis, the *iel* coding region was restructured by site-directed mutagenesis. This strategy was preferred to

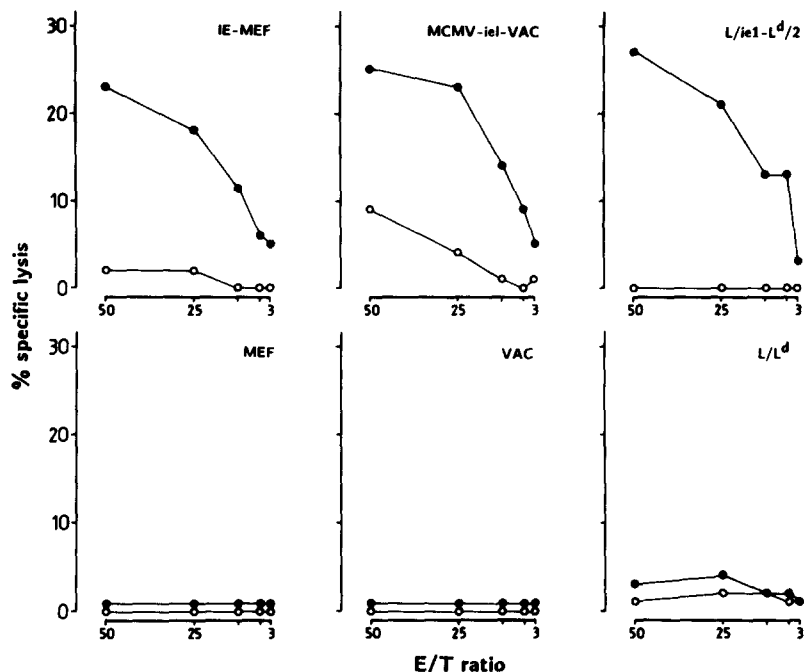


FIGURE 4. Priming for an *iel*-specific CTL response by MCMV-*iel*-VAC. Splenocytes and lymph node cells were obtained from BALB/c mice 2 wk after intravenous infection with  $2 \times 10^7$  plaque-forming units of MCMV-*iel*-VAC (●) or wild-type VAC (○). After restimulation in vitro with MCMV for 5 d, CTL activity and specificity was determined. Target cells used: BALB/c MEF, infected with MCMV in the presence of cycloheximide (50  $\mu$ g/ml), which was replaced after 3 h by actinomycin D (5  $\mu$ g/ml) to achieve selective and enhanced synthesis of IE proteins (*IE-MEF*), or noninfected cells (*MEF*); L/L<sup>d</sup> cells infected with the VAC recombinant (*MCMV-iel-VAC*) or infected with wild-type VAC (*VAC*). L cells doubly transfected with L<sup>d</sup> and MCMV-*iel*1 (*L/iel*1-L<sup>d</sup>/2) and L cells expressing only the L<sup>d</sup> molecule (*L/L*<sup>d</sup>). In this experiment, specific lysis by CTL clone IE1 (E/T ratio 20:1) of MCMV-*iel*-VAC-infected L/L<sup>d</sup> cells was 20%.

cDNA cloning, because at the IE stage of infection a second 2.75 kb RNA is transcribed, which shares coding regions with the mRNA encoding pp89 but terminates downstream in transcription unit *ie3* (8). The intron-free continuous coding sequence of the *iel* gene in the VAC recombinant encoded a protein indistinguishable from pp89 with regard to size, posttranslational processing, and intracellular transport, thereby confirming the data on the structural organization of *iel* (13). Thus, the VAC system could be used to selectively express a single gene product from a complex transcription unit and to test some of its properties.

The formal proof for our previous postulate (6) that the dominant nonstructural, regulatory IE protein of MCMV, pp89, gives rise to a CTL response and is detected by CTL after infection of cells, represents the important information of this contribution. Although new for a herpesvirus, the recognition of nonstructural viral proteins by CTL is, however, not without precedent. Murine CTL can recognize the nonstructural large T antigen in SV-40-transformed murine cells (26), and recently it was reported that CTL can detect the nonstructural NSI protein of influenza virus (27). The mechanism by which proteins that lack

signal sequences for transport to the plasma membrane and that do not possess transmembrane domains are presented to serve as antigens for CTL is a matter of current investigation. There is some evidence for intracellular protein degradation and membrane presentation of peptides in association with class I gene products (28, 29). Our finding that a monoclonal antibody that detects a sequential epitope in the pp89 molecule fails to detect the antigen at the plasma membrane (our unpublished observation) also suggests fragmentation of pp89.

The possible role for glycoproteins in immunity to herpesvirus has been demonstrated by the biological activity of recombinant VAC expressing herpes simplex virus type I glycoprotein D (30, 31). Expression of virus-encoded proteins that do not contribute to the virion structure is restricted to cells infected with live virus. The fact that nonstructural proteins are also detected by CTL could offer, among other arguments, an explanation for the well-known superior efficiency of live over inactivated vaccines. Because the MHC-restricted function of CTL in principle prevents detection of free virions, the recognition of viral structural proteins by CTL also requires cell membrane expression of the antigen. Therefore, it should make no fundamental difference for the potential protective function of a CTL clone whether it detects a structural or a nonstructural viral polypeptide. Demonstration of CTL with specificity for a nonstructural protein does not predict whether such CTL contribute to protection or cause immunopathology. For the MCMV model, our results indicate so far that CTL populations enriched for CTL with selective specificity for IE antigen(s) can have protective effects *in vivo* (32). These findings did not allow us to conclude which of the IE proteins contributes to protective immunity. The VAC recombinant containing the *ie1* gene now provides for the first time the possibility of testing the *in vivo* function of CTL sensitized against a single herpesviral IE protein.

The MHC class I genes have an immune response gene function in dictating the restriction specificity of virus-specific CTL and determining the responder phenotype of a given mouse strain. For certain virus models, a nonresponder or low responder status has been described that reflects either the complete inability to generate specific CTL or the inability to generate CTL that recognize viral antigens in association with the nonresponder allelic form of one of the three class I molecules (reviewed in 33 and 34). Cytomegaloviruses have very large genomes and the highest potential coding capacity of all known animal viruses. Because MCMV is a natural pathogen of the mouse, it is questionable whether mouse strains that are total nonresponders to this virus exist at all. This may be different with regard to the response to single gene products expressed by genetically engineered recombinant vaccines. Such information is relevant for the design of vaccines. In the BALB/c strain (haplotype H-2<sup>d</sup>), which was mainly used in our studies so far, at least a substantial fraction of CTL recognize products encoded by transcription unit *ie1* in association with the H-2L<sup>d</sup> molecule (12). Further experiments using the VAC recombinant will show whether vaccination with MCMV-*ie1*-VAC can protect mice against the challenge with a lethal dose of MCMV and whether this effect is restricted to certain MHC haplotypes.

### Summary

The murine immediate-early (IE) protein pp89 is a nonstructural virus-encoded phosphoprotein residing in the nucleus of infected cells, where it acts as transcriptional activator. Frequency analysis has shown that in BALB/c mice the majority of virus-specific CTL recognize IE antigens. The present study was performed to assess whether pp89 causes membrane antigen expression detected by IE-specific CTL. Site-directed mutagenesis has been used to delete the introns from gene *ie1*, encoding pp89, for subsequent integration of the continuous coding sequence into the vaccinia virus genome. After infection with the vaccinia recombinant, the authentic pp89 was expressed in cells that became susceptible to lysis by an IE-specific CTL clone. Priming of mice with the vaccinia recombinant sensitized polyclonal CTL that recognized MCMV-infected cells and transfected cells expressing pp89. Thus, a herpesviral IE polypeptide with essential function in viral transcriptional regulation can also serve as a dominant antigen for the specific CTL response of the host.

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