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Identification, Analysis, and Evolutionary Relationships of the Putative Murine Cytomegalovirus Homologs of the Human Cytomegalovirus UL82 (pp71) and UL83 (pp65) Matrix Phosphoproteins

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We have identified three open reading frames (ORFs) in murine cytomegalovirus (MCMV), designated M82, M83, and M84, which likely encode homologs of the human cytomegalovirus (HCMV) UL82 and UL83 matrix phosphoproteins. These ORFs, in the *Hin*dIII C fragment of MCMV, are colinear with the UL82, UL83, and UL84 ORFs of HCMV. M82 encodes a 598-amino-acid (aa) protein with homology to UL82, M83 encodes an 809-aa protein with homology to UL82 and UL83, and M84 encodes a 587-aa protein with homology to UL83 and UL84. Analysis of transcription by Northern (RNA) blotting indicated that the M82 and M83 ORFs are transcribed as 2.2- and 5-kb mRNAs, respectively, at 24 to 48 h postinfection (p.i.), while M84 is transcribed as a 6.9-kb mRNA only at 8 h p.i. All transcripts appear to terminate at the same position 3′ of the M82 ORF. Of the products of the three ORFs, only M83 is strongly recognized by hyperimmune mouse serum. The M83 protein is a virion-associated phosphoprotein with an apparent molecular mass of 125 kDa. In MCMV-infected cells, it is detectable by Western blotting (immunoblotting) only at 48 h p.i. in the absence of phosphonoacetic acid, consistent with late gene expression. The M83 ORF is also expressed at high levels in cells infected by a recombinant vaccinia virus and yields a protein which is serologically cross-reactive and comigrates with the authentic MCMV protein in sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Human cytomegalovirus (HCMV) is the major viral cause of birth defects in newborns and poses serious problems in individuals with impaired immune function (24). The severe disease resulting from infection with this virus has provided a strong impetus for studies directed toward its prevention and treatment. Although there have been significant advances in our knowledge of the molecular biology of HCMV in vitro, investigation of the in vivo pathogenesis and immunology has been limited by the strict species specificity of the virus. To circumvent this limitation, cytomegaloviruses of other species, in particular murine cytomegalovirus (MCMV), have been used as models of the human infection (5, 24, 41, 67, 68).

In the last 15 years, our understanding of the cell-mediated immune response to MCMV infection has expanded significantly. Early work by Koszinowski and colleagues demonstrated that cytotoxic T lymphocytes (CTLs) generated in BALB/c mice were directed against both structural and nonstructural viral antigens, although a major fraction was specific for antigens expressed at immediate-early times during infection (29–31, 54, 55, 69). The dominant CTL class was directed against the 89-kDa IE1 (immediate-early 1) phosphoprotein, designated pp89. Vaccination of BALB/c mice with recombinant vaccinia viruses expressing this protein generated some protective immunity, as evidenced by increased resistance to the lethal effects of subsequent MCMV challenge (28). Protection was mediated entirely by $CD8^+$ lymphocytes. However, it is important to note that protective immune mechanisms in addition to $CD8^+$ antiviral effector cells are induced following MCMV infection (28, 53, 56–58).

Although the immune response to HCMV infection is less well defined, several recent findings have yielded important clues for deciphering key targets of the cell-mediated immune response. Particularly notable are studies showing that HCMV-seropositive individuals possess relatively low levels of $CD8^+$ CTL specific for both the HCMV IE1 72-kDa protein, a functional homolog of the MCMV pp89, and the structural glycoprotein B (3). The bulk of HCMV-specific CTLs are directed against a subset of viral antigens introduced into the host cell during viral penetration (20, 59). The HCMV UL83 (pp65) matrix phosphoprotein appears to be the major target of these CTLs, as well as a dominant target of proliferative responses by $CD4^+$ T lymphocytes (1, 17, 38). The clinical application of these findings has already generated much excitement. Although the absolute number of trials is still small, there is increasing evidence that matrix protein-specific CTLs can be isolated from HCMV-seropositive bone marrow donors, propagated in vitro, and adoptively transferred to immunodeficient bone marrow recipients, conferring protection against HCMV viremia and pneumonia (60, 70).

In addition to UL83, the HCMV virion contains at least three other proteins, designated UL32 (pp150), UL82 (pp71), and UL99 (pp28), which are localized to the matrix of the viral particle and induce strong humoral immune responses in infected individuals (26, 27, 33–35, 40, 45, 51, 72). Recognition of the key role played by this class of proteins in protective im-

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mune responses has provided a strong impetus for studies to determine whether similar mechanisms function in the murine model. If such is the case, studies of MCMV may yield insights into the properties of the matrix-specific immune response unobtainable in the more unwieldy experimental context of HCMV.

As a prerequisite for establishing the immunological role of MCMV matrix proteins, we initiated studies to identify the MCMV gene products homologous to the HCMV matrix proteins. The first such gene identified represented the MCMV homolog of the HCMV 28-kDa matrix phosphoprotein (UL99) (9). In this report, we used a similar strategy to identify and characterize MCMV open reading frames (ORFs) encoding likely homologs of the HCMV UL82 and UL83 matrix phosphoproteins. On the basis of the colinearity of recently identified MCMV genes with their HCMV counterparts, we selected subgenomic fragments of MCMV for sequencing (8, 9, 12, 41). In this way, we were able to identify a region of MCMV *Hin*dIII fragment C containing ORFs which displayed colinearity and significant homology with the UL86 major capsid protein, the UL85 putative capsid protein, and the UL82 and UL83 matrix phosphoproteins of HCMV (7, 8, 27, 39, 63, 65). Additionally, one ORF was identified in a position colinear with and possessing weak homology to the nonstructural HCMV UL84 protein (23). This ORF, however, possessed much stronger homology to UL83 than to UL84. We describe here the temporal kinetics and pattern of transcription from this region and analyze in detail the protein product of the MCMV M83 ORF, a UL82/UL83 homolog, which generates a strong humoral immune response during MCMV infection. In addition, we show that the M83 ORF can be efficiently expressed by a recombinant vaccinia virus.

MATERIALS AND METHODS

Cells and viruses. NIH 3T3 cells (ATCC CRL 1658) were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, glutamine (0.29 mg/ml), penicillin (200 U/ml), streptomycin (0.2 mg/ml), gentamicin (0.05 mg/ml) and amphotericin B (1.5 μg/ml).

The K181 strain of MCMV was used in all experiments. Salivary gland stocks of virus were prepared following intraperitoneal inoculation of female BALB/c mice as previously described (12). Uninfected salivary glands for mock infection were prepared in an identical manner. Tissue culture MCMV was prepared by infection of NIH 3T3 cells at a multiplicity of infection of 0.01 to 0.05. The infection was allowed to proceed until cytopathic effect was 100%, at which point the medium was changed. After 3 days, the supernatant was harvested and supplemented with dimethyl sulfoxide to a final concentration of 1%. Titers of the virus stocks were determined by plaque assay, and the virus stocks were stored at −70°C until use. MCMV was purified from extracellular tissue culture medium by density gradient centrifugation as previously described (9).

Recombinant DNA clones. Unless otherwise noted, enzymes were obtained from Bethesda Research Laboratories, Inc., and used as recommended by the manufacturer. A 14.2-kbp *Hin*dIII-*Stu*I subfragment of MCMV *Hin*dIII fragment C (39) was subcloned from pACYC184-H3C into *Hin*dIII- and *Sma*I-cleaved pGEM1 (Promega Biotech, Inc.) to form H3C14.2-GEM. A 2.2-kbp *Stu*I subfragment of the *Hin*dIII fragment C, representing the fragment adjacent to the 14.2-kbp *Hin*dIII-*Stu*I subfragment, was subcloned into *Sma*I-cleaved pGEM1 to form H3C2.2-GEM. A clone was selected in which the end of the 2.2-kbp fragment adjacent to the 14.2-kbp fragment was located proximal to the *Eco*RI site of pGEM1. H3C14.2-GEM was cut with *Pst*I, and a 2.1-kbp subfragment, encoding the N-terminal 669 amino acids of the M83 ORF, was isolated and subcloned into *Pst*I-cleaved pGEM1 to form M83(*Pst*)-GEM. The 5′ end of the putative M83 ORF was proximal to the *Xba*I site of pGEM1, while the 3′ end was proximal to the *Hin*dIII site. A 3-kbp *Kpn*I subfragment of H3C14.2-GEM, containing all of the M83 ORF except the N-terminal 69 amino acids, was isolated, blunt ended, and ligated into *Sma*I-cleaved pGEM1 to form the clone H3C3-GEM. A clone with the 3′ end of the putative M83 ORF proximal to the *Eco*RI site in the polylinker of pGEM1 was selected. M83(*Pst*)-GEM was cleaved with *Xba*I and *Hin*dIII, and a 2.1-kbp subfragment was isolated and ligated to *Xba*I- and *Hin*dIII-cleaved pGEX-KG (21) to form M83-GEX. H3C3-GEM was cleaved with *Eco*RI and *Pst*I, and a 1.1-kbp subfragment isolated and ligated to *Eco*RI- and *Pst*I-cleaved pGEM1, forming 3′M83-GEM. M83-GEX was cleaved with *Pst*I, and a 2.1-kbp subfragment was isolated and ligated to *Pst*I-cleaved 3′M83-GEM, forming M83(Whole)-GEM. The orientation of the *Pst*I fragment in M83(Whole)-GEM reconstructed the genomic orientation. M83(Whole)-GEM was cleaved with *Hin*dIII, *Xba*I, and *Dra*I (New England Biolabs, Inc.), and a 3.1-kbp fragment, containing the entire M83 ORF, was isolated. This fragment was either ligated directly to the *Hin*dIII- and *Xba*I-cleaved eukaryotic expression plasmid pcDNA3 (Invitrogen), yielding pcDNA3-M83, or blunt ended and ligated to the *Sma*I-cleaved vaccinia vector pSC11 (a gift of B. Moss), yielding pSC11-M83.

A 3-kbp *Eco*RV fragment of pACYC184-H3C, containing the entire M82 ORF, was isolated and ligated to *Sma*I-cleaved pGEM1, yielding H3C(RV)-GEM. The 5′ end of the M82 ORF was proximal to the *Eco*RI site of pGEM1. To place an *Eco*RI site just 5′ of the initiating codon of the M82 ORF, H3C(RV)-GEM was cut with *Eco*RI and *Bgl*II and a 5.3-kbp fragment, containing the vector and the entire M82 ORF 3′ of a *Bgl*II site 328-bp downstream from the initiating methionine codon, was isolated. To reconstruct the 5′ 0.33 kbp of the M82 ORF, PCR was performed, using as the template H3C(RV)-GEM and as primers 5′-M82 PCR (5′-GAA TTC ATG GCC GAG GAA TTT-3′) and 3′-M82 PCR (5′-AGG TGG CTG TCG TTC AGC TC-3′) (Oligos Etc.). Standard PCR mixtures, supplemented with 2 mM $MgCl_2$, were prepared with or without template DNA, using the materials in the GeneAmp PCR reagent kit (Perkin-Elmer) and following the manufacturer's recommendations. Twenty-five cycles of PCR were carried out (1 min at 94°C, 1 min at 50°C, and 1 min at 72°C, followed by 10 min at 72°C). A 471-bp product, synthesized only in the presence of template DNA, was digested with *Eco*RI and *Bgl*II, and a 0.33-kbp product was isolated and ligated to the 5.3-kbp *Eco*RI- and *Bgl*II-cleaved H3C(RV)-GEM, yielding M82-GEM. Both strands of the PCR product were sequenced. M82-GEM was then cut with *Eco*RI and *Xba*I, and a 2.5-kbp fragment, containing the entire M82 ORF, was isolated and ligated to *Eco*RI- and *Xba*I-cleaved pcDNAI/Amp (Invitrogen), yielding pcDNAI-M82. The 5′ end of the M82 ORF was proximal to the *Eco*RI site of pcDNAI/Amp. pcDNAI-M82 was next cut with *Eco*RI and *Xba*I, and a 2.5-kbp fragment was isolated and ligated to *Eco*RI- and *Xba*I-cleaved pcDNA3, yielding pcDNA3-M82. The 5′ end of the M82 ORF was proximal to the *Eco*RI site of pcDNA3.

H3C14.2-GEM was digested with *Hin*dIII and *Kpn*I, and an 8.2-kbp *Kpn*I subfragment was isolated and subcloned into *Kpn*I-cleaved pGEM4Z (Promega Biotech), yielding H3C8.2-GEM. A clone which had the 5′ end of the M86 ORF proximal to the *Hin*dIII site of the vector was selected. H3C8.2-GEM was cleaved with *Eco*47III (New England Biolabs) and *Not*I, and a 5.4-kbp subfragment, containing vector sequences, the M84 ORF, and some additional material 5′ of the M86 ORF, was isolated and recircularized, yielding pGEM-iM84. pGEM-iM84 was digested with *Bam*HI, and a 4.6-kbp subfragment, containing vector sequences and all of the M84 ORF 3′ from a *Bam*HI site 0.13 kbp downstream from the putative initiating methionine codon of the ORF, was isolated and recircularized to yield pGEM-iM84/2. To reconstruct the 5′ 0.13 kbp of the M84 ORF, PCR was performed, using as the template pGEM-iM84 and as primers 5′-M84 PCR (5′-CGG GAT CCT TCG ACA TGT CGG TCA ACG TTT ACT TGC CG-3′) and 3′-M84 PCR (5′-GAA GAT CTC GGT CTG GAG GAA TTT GGT CTC GTA GGG GC-3′) (Integrated DNA Technologies, Coralville, Iowa). Standard PCR mixtures were prepared as described above. Thirty cycles of PCR were carried out (1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, followed by 10 min at 72°C). A 150-bp product, synthesized only in the presence of the DNA template, was isolated, digested with *Bam*HI and *Bgl*II, and cloned into *Bam*HI-cut pGEM-iM84/2, yielding pGEM-M84. The *Bam*HI end of the PCR product was proximal to the vector, while the *Bgl*II end was proximal to the M84 ORF. This recreated the M84 ORF with a *Bam*HI site immediately 5′ of the initiating methionine codon. The internal *Bam*HI site of M84 was destroyed, and amino acid 44 of the encoded protein was changed from glycine to glutamate. Both strands of the PCR product were sequenced. A 2-kbp *Bam*HI-*Eco*RI subfragment of pGEM-M84 was then subcloned into *Bam*HI- and *Eco*RI-cut pcDNA3, yielding pcDNA3-M84.

The vaccinia virus vector pGS62-pp89 (a gift of U. Koszinowski), encoding the MCMV pp89, was cleaved with *Eco*RI, and a 1.8-kbp fragment was isolated and ligated to *Eco*RI-cleaved pcDNA3, yielding pcDNA3-pp89 (28, 69). The 5′ end of the pp89-encoding insert was proximal to the *Hin*dIII site of pcDNA3.

DNA sequencing and data analysis. Sequencing of the Smith strain of MCMV is described elsewhere (52). The M83 region of the K181 strain MCMV genome was sequenced from either M13 subclones of the region or denatured double-stranded DNA templates by the dideoxynucleotide chain termination method (64), using protocols and reagents supplied with the Sequenase version 2.0 DNA sequencing kit (United States Biochemical) and the T7 or SP6 promoter primer (Promega Biotech) or synthetic oligonucleotides (Oligos Etc.) as primers. Both strands were completely sequenced.

Analysis of sequence overlaps, open reading frames and homologies was done by computer, using the programs DNA Inspector IIe (Textco) and FASTA (49, 50). Analysis of phylogenetic relationships between ORFs was also done by computer using the programs inspect, Progressive Alignment, Tree, and noneg (11, 13, 14).

Preparation of recombinant GST and GST-M83 fusion proteins. The DH5α strain of *Escherichia coli* (Gibco BRL) was transformed with pGEX-KG (21) or M83-GEX as recommended by the manufacturer. Recombinant glutathione *S*-transferase (GST) and GST-M83 proteins were purified from transformed bacteria by affinity chromatography using glutathione-agarose beads as previously described (9).

Preparation of normal and hyperimmune mouse serum and anti-GST-M83 rabbit serum. Normal and hyperimmune anti-MCMV mouse sera were prepared as previously described (9). To generate immune rabbit serum against GST-M83, a naive female New Zealand White rabbit was bled from the ear vein, and serum was prepared and adsorbed from the blood as described below. After confirmation by Western blot (immunoblot) analysis of the animal's lack of reactivity against GST-M83, the rabbit was primed by intramuscular and subcutaneous injection of 350 μg of purified GST-M83 in Freund's complete adjuvant. Booster doses of 70, 175, and 270 μg of purified GST-M83 were given at weeks 3, 6, and 20, respectively, by intramuscular and subcutaneous injection in Freund's incomplete adjuvant. At week 22, blood was obtained and serum was prepared as described below. Serum obtained at 22 weeks was found to be strongly positive by Western blot analysis for reactivity against the GST-M83 protein and was used in the experiments described herein.

Sera were prepared, adsorbed, and purified as described by Harlow and Lane (22) and stored at −20°C. All sera were adsorbed twice with an acetone powder derived from an induced culture of pGEX-KG-containing *E. coli* DH5α. After adsorption, hyperimmune mouse and rabbit anti-GST-M83 sera were further purified by ammonium sulfate precipitation. Purified sera were stored as 50% glycerol stocks at −20°C.

Production of recombinant vaccinia viruses. Recombinant vaccinia viruses expressing M83 and β-galactosidase proteins were constructed by using the vaccinia virus vector plasmids pSC11-M83 and pSC11, respectively, the WR strain of vaccinia virus as the parent virus (ATCC VR-119), and methods previously described (6, 9). The resulting recombinant viruses were designated M83-*vacc* and pSC11-*vacc*, respectively.

Analysis of infected cell RNA. Polyadenylated RNA was isolated from uninfected NIH 3T3 cells or at 8 h (early), 24 h (midpoint), or 48 h (late) after infection of confluent NIH 3T3 cells at a multiplicity of infection of 2 PFU per cell. Total RNA was isolated by using the RNAgents total RNA isolation system (Promega) or the Total RNA kit (Qiagen) according to the manufacturers' recommendations. Polyadenylated RNA was isolated from the total RNA preparations by using either the PolyATtract mRNA isolation system (Promega) or the Oligotex mRNA isolation kit (Qiagen) according to the manufacturers' recommendations, subjected to electrophoresis through 1 or 1.2% agarose-formaldehyde gels (15), transferred to a Nytran (Schleicher & Schuell) membrane, and probed with ^{32}P-labeled oligonucleotide or double-stranded DNA probes. Four oligonucleotides were synthesized (Integrated DNA Technologies) for use as probes: 5′-M86 (5′-GCA GCA GCT CCG TCG CCG TCC AGT TCT CCC-3′), 5′-M85 (5′-CCC CGG TCG TGA GGC GCT GCT CGA ACG TGA-3′), 5′-M83 (5′-AGT GCC ACC TGG GTG AGG GGA GCG ACG CCG-3′), and 3′-M82 (5′-TAC CGC CGG GAA ATT GGT GAA TGC TTC GTG-3′). Oligonucleotide probes were labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP (ICN). An 837-bp *Bam*HI-*Sma*I subfragment of pcDNA3-M84 and a 682-bp *Eco*RI-*Pst*I subfragment of H3C2.2-GEM, designated M84 and M82, respectively, were isolated and labeled by random hexamer priming with [α-^{32}P]dCTP (ICN), using the the Random Primers DNA labeling system (Gibco) according to the manufacturer′s recommendations.

Preparation of infected cell proteins. Proteins from mock-infected NIH 3T3 cells or those infected with salivary gland-passaged MCMV K181 strain were prepared at the same time points and in the presence of the same drugs as previously described (9). These protein extracts were used to study the kinetics of M83 protein expression.

In the experiment in which vaccinia virus-expressed proteins were compared with proteins in MCMV-infected cells, MCMV-infected NIH 3T3 cell lysates were prepared at 48 h postinfection (p.i.). Proteins from these cells, as well as uninfected NIH 3T3 cells, were then harvested as described previously (71). Protein extracts from vaccinia virus-infected cells were prepared in an identical manner at 75 h after infection of NIH 3T3 cells with M83-*vacc* or pSC11-*vacc* at a multiplicity of infection of 5.

Protein electrophoresis, Western blot analysis, and in vitro transcription-translation. Proteins were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) essentially as described by Laemmli (32). Ten percent mini-SDS-polyacrylamide gels were run, using the Mini-PROTEAN II system (Bio-Rad). Following electrophoresis, gels were transferred to nitrocellulose for Western analysis. Proteins were visualized by staining the nitrocellulose with amido black (Sigma N-9002) and photographed. The blots were then subjected to Western analysis using the ECL system (Amersham) according to the manufacturer's recommendations. Unless otherwise noted, primary mouse and rabbit antisera were used at a 1:1,000 dilution and horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin antiserum (Amersham) was used as secondary antiserum at a 1:3,000 dilution.

Plasmids pcDNA3, pcDNA3-M82, pcDNA3-M83, pcDNA3-M84, and pcDNA3-pp89 were used as substrates for in vitro transcription-translation reactions, using the TNT Coupled Reticulocyte Lysate system (Promega) according to the manufacturer's recommendations. T7 RNA polymerase was used for transcription. Reactions were carried out either in the presence or in the absence of [^{35}S]methionine (Amersham SJ1015). Labeled reactions were separated on 10% mini-SDS-polyacrylamide gels, stained for total proteins with Coomassie brilliant blue, treated with Fluoro-Hance (Research Products International, Mount Prospect, Ill.) according to the manufacturer's recommendations, dried, and exposed to autoradiography film without an enhancing screen. The products of unlabeled reactions were used for Western analysis.

In vivo metabolic labeling with ^{32}P and ^{35}S. In vivo metabolic labeling of uninfected and MCMV-infected NIH 3T3 cells with ^{32}P was carried out as previously described (9). Additionally, uninfected and MCMV-infected NIH 3T3 cells were metabolically labeled with ^{35}S, using the same procedure as for ^{32}P labeling except that the medium, Dulbecco's modified Eagle's medium, lacked methionine and cysteine (Gibco BRL) and was supplemented with 10% fetal bovine serum and 1,500 μCi of ^{35}S Translabel (ICN) per dish.

Immunoprecipitation and SDS-PAGE were carried out as previously described (9) except that rabbit anti-GST-M83 serum was used for immunoprecipitation and a mini-SDS–10% polyacrylamide gel was used for separation. For ^{35}S-labeled preparations, the gels were treated with Fluoro-Hance prior to fluorography.

Nucleotide sequence accession numbers. The GenBank accession number for the nucleotide sequence of the M83 region of the K181 strain of MCMV is U65003. The M82-M86 region of the Smith strain of MCMV has been assigned GenBank accession number U68299.

RESULTS

Genomic localization of MCMV homologs of the HCMV UL82 and UL83 proteins. The genomes of MCMV and HCMV show striking colinearity, which we and others have used to identify and clone the MCMV homologs of HCMV genes of interest (Fig. 1A) (8, 9, 12, 41). This strategy led us to target the *Hin*dIII C fragment of the MCMV genome as a likely site for the MCMV homologs of the HCMV UL82 and UL83 matrix proteins. Using the *Hin*dIII site at the junction of the MCMV *Hin*dIII C and G fragments as a reference, we identified five ORFs in the region of the Smith strain genome (52) starting 3,487 bp from the *Hin*dIII site and extending 15,035 bp from the *Hin*dIII site (Fig. 1B). These ORFs are all encoded by the same DNA strand, with the direction of transcription proceeding away from the border of the *Hin*dIII C and G fragments. The ORF furthest downstream from the *Hin*dIII C/G junction is 598 amino acids and shows homology to the HCMV UL82 protein. On the basis of this homology and its relative position within the MCMV genome, this ORF is designated M82. The next upstream ORF, 809 amino acids in length, shows homology to the HCMV UL82 and UL83 proteins but is designated M83 on the basis of its relative position within the MCMV genome. Upstream of M83 is an ORF consisting of 587 amino acids. This ORF is designated M84 on the basis of its relative position in the MCMV genome but, as discussed below, shows substantial homology to both the HCMV UL83 and UL84 proteins. The next ORF upstream from M84 encodes a 311-amino-acid protein with substantial homology to the HCMV UL85 and is designated M85. Finally, the most upstream ORF is designated M86 and encodes a potential product of 1,353 amino acids with homology to the HCMV UL86 major capsid protein (52).

Analysis of M82, M83, and M84 ORF homologies and evolutionary relationships. To determine the relationships between the M82, M83, and M84 ORFs and HCMV proteins, the nonredundant protein database of GenBank was screened for sequences related to the MCMV ORFs, using the FASTA analysis program and scoring with the PAM250 scoring matrix (49, 50). Table 1 summarizes the results of these analyses. For M82, this analysis revealed a significant relationship with UL82. The match between these two proteins was the closest identified by FASTA in the database.

FASTA was also used to screen this database with the M83 ORF. The database was first screened by using the entire 809-amino-acid ORF. Homology with the HCMV UL82 was detected. In this search also, the score assigned by FASTA for this relationship with UL82 was the highest detected in the database.

We noticed upon examination of homologies detected in the

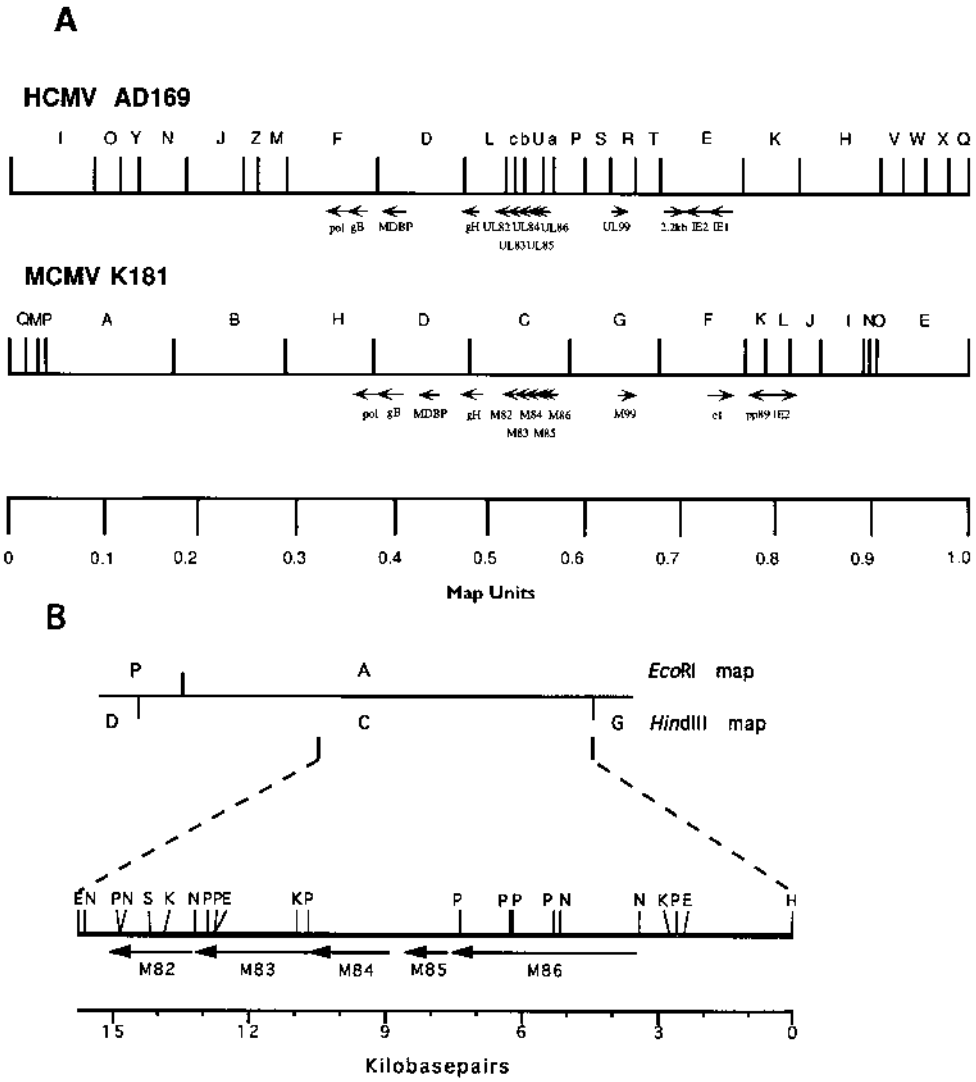

FIG. 1. (A) Comparison of the *Hin*dIII restriction maps of strain AD169 HCMV and strain K181 MCMV (39, 65). The approximate positions and transcriptional directions of a number of representative homologous genes are indicated to demonstrate the relative colinearity of the two genomes. The locations of the M82 to M86 ORFs are indicated within the MCMV *Hin*dIII map. The MCMV M82 to M86 and HCMV UL82 to UL86 ORFs occur in numerical order as diagrammed. pol, DNA polymerase; gB, glycoprotein B; gH, glycoprotein H; 2.2kb, HCMV 2.2-kb early transcription unit; M99, MCMV UL99 homolog; e1, MCMV early gene 1; pp89, MCMV major immediate-early gene; IE1, IE2, immediate-early genes 1 and 2; MDBP, major DNA-binding protein. (B) Detailed localization of the M82 to M86 ORFs within a 15.7-kbp *Hin*dIII-*Eco*RV subfragment of the *Hin*dIII fragment C. Arrows indicate the relative positions, sizes, and directions of the putative M82 to M86 ORFs. The relative positions of important restriction endonuclease cleavage sites are also indicated. E, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; N, *Not*I; P, *Pst*I; S, *Stu*I. Distances in kilobase pairs from the junction of the *Hin*dIII C and G fragments are indicated. The nucleotide positions for the various Smith strain ORFs are as follows (52): M82, 115714 to 117507; M83, 117617 to 120043; M84, 120085 to 121845; M85, 122192 to 123124; and M86, 123202 to 127260. All are on the complementary strand, with the genome in the prototype orientation.

first search with M83 that much of the homology to UL82 was localized to a repetitive acidic regions of the protein, located between amino acids 425 and 447 and between amino acids 538 and 558 (Fig. 2). To eliminate the influence of these charged repetitive regions, we repeated the search, using only the amino-terminal 400 residues of the protein. With these parameters, FASTA detected homology with the HCMV UL83 protein. While UL83 yielded the second-highest score in this search, the best match was with UL82, in agreement with the results of the previous search using the entire M83 ORF.

Searching the database by FASTA for proteins with homology to M84 detected a number of HCMV homologs. Surprisingly, the highest score was assigned to the match with the HCMV UL83. Homology at a lower level was identified with

TABLE 1. Results of FASTA analyses

MCMV ORF	Homolog[a]	% Identity	Overlap[b]	Score[c]	k-tuple
M82	UL82	18.6	360	182	2
M83[d]	UL82	21.2	444	233	2
M83[e]	UL82	22.4	343	210	2
M83[e]	UL83	17.3	323	192	2
M84	UL83	20.6	325	208	1
M84	UL84	21.7	189	130	1
M84	U54	19.1	444	198	1

[a] All homologs are from HCMV (7) except U54, which is from human herpesvirus 6 (19, 36).
[b] Length of amino acid overlap identified by FASTA.
[c] Optimized FASTA score.
[d] The entire 807 amino acids of the K181 M83 ORF were used in this search.
[e] The N-terminal 400 amino acids of the K181 M83 ORF were used in this search.

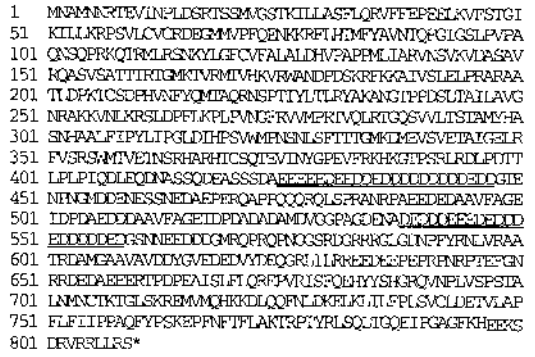

FIG. 2. Derived amino acid sequence of the M83 ORF of the Smith strain of MCMV. Extended stretches of acidic residues are underlined.

UL84, as we had predicted on the basis of the colinearity of the two genomes. However, the 189-amino-acid overlap identified by FASTA in its comparison of M84 and UL84 was considerably shorter than the 325-amino-acid overlap identified for the comparison of M84 and UL83. The program also identified fairly strong homology with U54 of human herpes virus 6, a homolog of the HCMV UL82 and UL83 proteins (19, 36).

The results of these comparisons indicated to us that M82 was clearly related to the HCMV UL82. M83 appeared to share homology with both the HCMV UL82 and UL83, with the similarity to UL82 being somewhat greater. M84 appeared to be a homolog of the HCMV UL83, although it also showed modest homology with its positional homolog, UL84.

To confirm these hypothetical relationships and evaluate their significance, we constructed a tree describing the phylogenetic lineage of these proteins. Analysis of the sequences with the program inspect confirmed the relationships identified above (data not shown) (11, 13, 14). With confirmation of the hypothesized relationships, we used the program Progressive Alignment to determine the best possible region and alignment for the three HCMV and three MCMV ORFs. This program selected 107-amino-acid overlaps from the six proteins and calculated similarity scores and percent identity within the overlaps (Fig. 3A). Branching order and branch distances were next calculated by the program Tree, with the program noneg correcting for negative branch distances (Fig. 3B). The resultant tree indicated that a common ancestor protein gave rise to M82, M83, and M84 and to UL82, UL83, and UL84. UL84 appeared to be least related to the others, having been the first to diverge from the putative ancestor protein. Among the proteins derived from the putative cytomegalovirus matrix protein ancestor, M82 and M83 were most closely related to each other, with their closest HCMV homolog being UL82. M84 was most closely related to UL83. On the basis of this and our earlier analyses, we conclude that the likely MCMV homolog of the HCMV UL83 protein is M84. The M83 and M82 proteins appear to represent the murine homologs of the HCMV UL82 protein, although the M83 is also related to the HCMV UL83.

Transcriptional pattern in the M82-M86 region of *Hin*dIII fragment C. To examine the pattern of transcription within this region of the MCMV genome, we first examined the nucleic acid sequence of the Smith strain (52) for the presence of appropriate transcription control consensus sequences. TATA sequences at positions consistent with their utilization for initiation of transcription in this region are present at appropriate positions upstream of the five ORFs (Fig. 4A). Polyadenylation signals are present at positions immediately before the start of

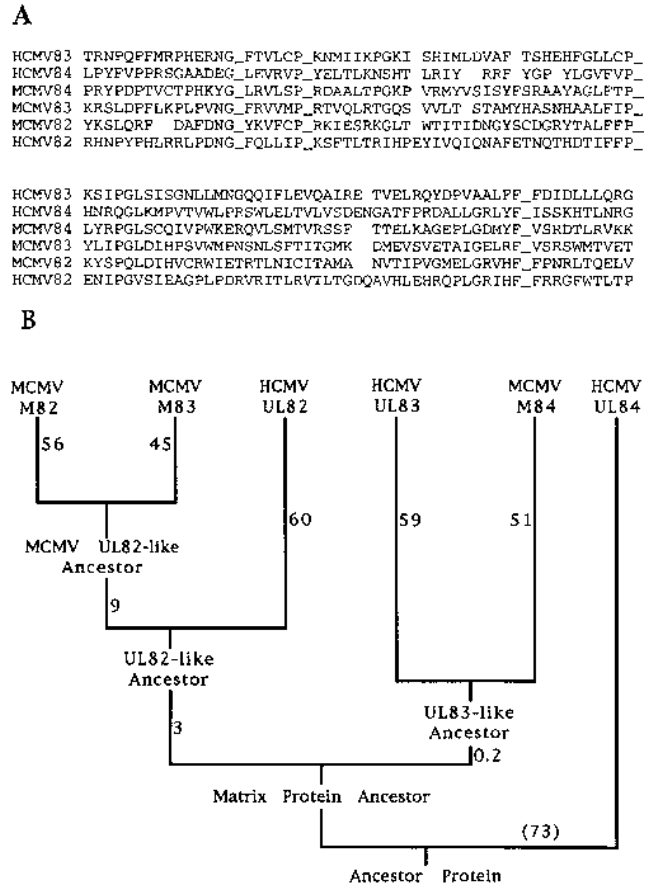

FIG. 3. Derived phylogenetic relationships between the MCMV M82, M83, and M84, and HCMV UL82, UL83, and UL84 proteins. (A) Alignment of regions identified by Progressive Alignment (11, 13, 14). This program selected the following residues for alignment: in HCMV, 268 to 374 for UL82, 257 to 360 for UL83, and 409 to 510 for UL84; in MCMV, 272 to 373 for M82, 259 to 361 for M83, and 253 to 356 for M84 (23, 63). Gap penalty was set at a value of 8, and the PAM250 scoring matrix was used. (B) Phylogenetic tree derived by Tree from the alignments identified in panel A after correction for negative branch distance values by noneg. Branch distances in arbitrary units of evolutionary distance are indicated.

the M84 ORF, in the middle of the M82 ORF, and just downstream from the M82 ORF.

We next analyzed transcription in this region by Northern (RNA) analysis. mRNA was harvested from uninfected or MCMV-infected NIH 3T3 cells at 8, 24, and 48 h p.i. Northern blots were prepared and probed with either oligonucleotide or restriction fragment DNA probes (Fig. 4B). The oligonucleotide probe 5′M86, complementary to nucleotides at the 5′ end of the M86 ORF, detected transcripts of 5.7 and >9.5 kb at 24 and 48 h p.i. The second oligonucleotide probe, designated 5′M85, is complementary to the coding strand at the 5′ end of the M85 ORF. It hybridized to four transcripts of 1.35, 5.7, 8, and >9.5 kb at 24 and 48 h p.i. A double-stranded DNA probe, designated M84, represented a restriction fragment encoding the amino-terminal half of the M84 ORF. It hybridized faintly to a transcript of 6.9 kb present only at 8 h p.i. and also to transcripts of 8 and >9.5 kb at 24 and 48 h p.i. Probing Northern blots with oligonucleotide probes complementary to the coding strand in the same region as the M84 probe yielded identical results (data not shown). A third oligonucleotide probe, designated 5′M83, was located at the 3′ end of the M84

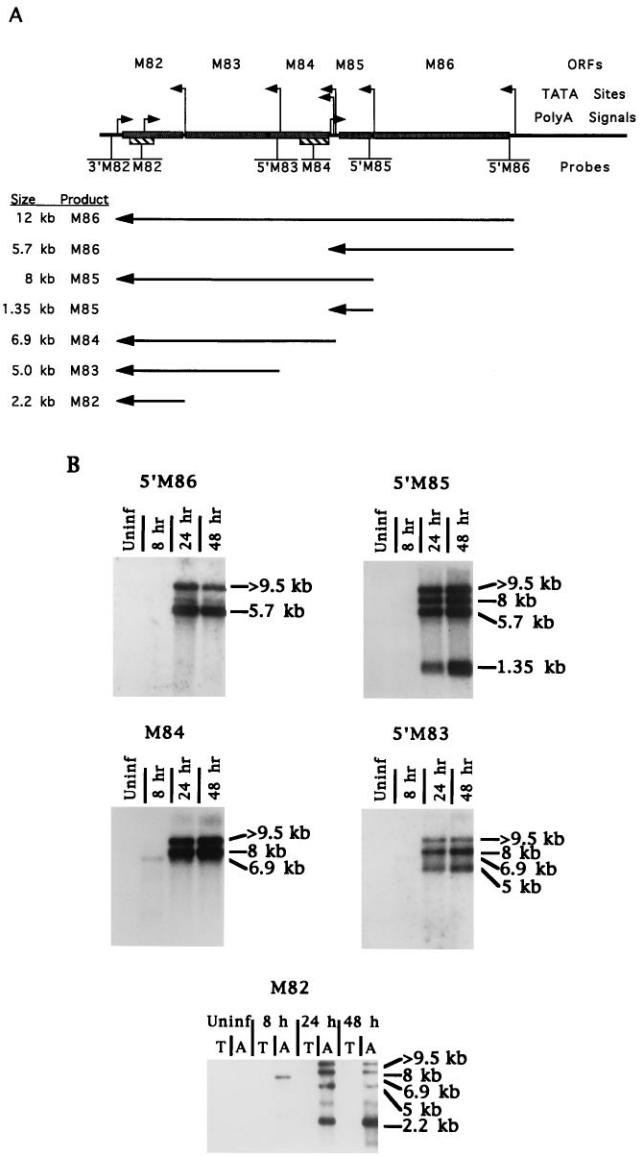

FIG. 4. Northern analysis of M82-M86 region of MCMV *Hin*dIII fragment C. (A) Diagram of the M82-M86 region of the MCMV *Hin*dIII fragment C, with the positions of the various ORFs, TATA sites, polyadenylation signals (PolyA Signals), probes, and putative transcripts indicated. The relative positions of TATA signals are indicated, with arrows indicating the direction of transcription. Polyadenylation signals are indicated with arrows pointing in the direction opposite the direction of transcription. The relative positions of oligonucleotide probes complementary to the coding strand are indicated and labeled (5′M86, 5′M85, 5′M83, and 3′M82). The relative locations and approximate sizes of double-stranded DNA probes are indicated by hatched boxes and labeled (M84 and M82). The relative positions, sizes, and predicted products of the various transcripts are indicated. (B) Northern blots were prepared from polyadenylated or total and polyadenylated (M82; indicated by T or A above the appropriate lane) RNA from uninfected NIH 3T3 cells (Uninf) or MCMV-infected NIH 3T3 cells at 8, 24, and 48 h p.i. The blots were probed with either an oligonucleotide probe complementary to the region at the 5′ end of the M86 ORF (5′M86), at the 5′ end of the M85 ORF (5′M85), or just 5′ of the beginning of the M83 ORF (5′ M83) or with double-stranded DNA probes complementary to regions within the M84 and M82 ORFs (labeled M84 and M82, respectively).

ORF, just 5′ of the M83 ORF. It hybridized to transcripts of 5, 8, and >9.5 kb at 24 and 48 h p.i. With longer exposures, hybridization to a 6.9-kb transcript was also observed at 8 h p.i. A double-stranded DNA probe, designated M82, represented a 682-bp *Stu*I-*Pst*I fragment from the 3′ end of the M82 ORF. This probe detected a 6.9-kb transcript at 8 h p.i. and transcripts of 2.2, 3.5, 5, 8, and >9.5 kb at 24 and 48 h p.i., with the 3.5-kb transcript likely arising from the antisense strand. Oligonucleotide probes complementary to the M82 ORF detected identically sized transcripts, other than the 3.5-kb species (data not shown). Finally, an oligonucleotide probe, designated 3′M82, which was complementary to a region 3′ of the most downstream polyadenylation signal, failed to hybridize with any transcripts (data not shown).

On the basis of the location of transcriptional control sequences in relation to the various ORFs and the transcript sizes observed in Northern analysis, we deduced a likely transcription pattern for this region (Fig. 4A). The initiation of 5.7- and 12-kb transcripts, the latter of which appeared as >9.5 kb in Northern blots, is directed by the TATA box located 5′ of the M86 ORF, with termination occurring at the polyadenylation signals just 5′ of the M84 ORF and 3′ of the M82 ORF, respectively. These transcripts likely encode the M86 gene product and are synthesized with kinetics consistent with late gene expression. Transcripts of 1.35 and 8 kb are directed by the TATA box located at the 3′ end of the M86 ORF, just 5′ of the M85 ORF, and extend to the polyadenylation signals just 5′ of the M84 ORF and 3′ of the M82 ORF, respectively. These transcripts, which exhibit late gene kinetics, appear to encode the M85 gene product. A 6.9-kb transcript is directed by either of two TATA boxes located just 5′ of the M84 ORF, extending to the polyadenylation signal located 3′ of the M82 ORF. This transcript likely encodes the M84 ORF product and is detectable only at 8 h p.i., consistent with early gene kinetics. However, we cannot exclude the possibility that the M84 protein is also translated from the >9.5- or 8-kb late transcripts which initiate upstream of the M86 and M85 ORFs, respectively. A 5-kb transcript with late kinetics is directed by the TATA box located 5′ of the M83 ORF, within the M84 ORF. It extends to the polyadenylation signal 3′ of the M82 ORF and encodes the M83 protein. Finally, a transcript of 2.2 kb is directed by the TATA box just 5′ of the M82 ORF and extends to the polyadenylation signal 3′ of this ORF. This transcript likely encodes the M82 gene product and displays late gene kinetics.

Although a potential polyadenylation signal is present in the middle of the M82 ORF (Fig. 4A), data from S1 nuclease protection experiments indicated that this site is not utilized (data not shown). Instead, the polyadenylation signal 3′ of this ORF is used by all transcripts that extend to it. This finding agrees with the results of Northern analysis, which showed that the oligonucleotide probe 3′M82 failed to hybridize to any transcripts.

Humoral immunogenicity of the M82, M83, and M84 ORF products during natural MCMV infection. We next assessed the development of humoral immune responses against the M82, M83, and M84 ORF protein products in MCMV-infected mice (Fig. 5). These ORFs and the DNA encoding the MCMV pp89 immediate-early protein were cloned into the plasmid pcDNA3, allowing expression of the encoded proteins by coupled in vitro transcription-translation. The proteins were produced either in the presence or in the absence of ^{35}S-labeled amino acids, allowing direct detection of the labeled translation products or Western analysis of the unlabeled products.

In the labeled reactions, pcDNA3-M82 yielded species of 77 and 61 kDa, with the 77-kDa product being somewhat larger than the 67 kDa predicted by the M82 primary amino acid sequence (Fig. 5A). pcDNA3-M83 yielded major products with calculated molecular masses of 113 and 97 kDa. While larger

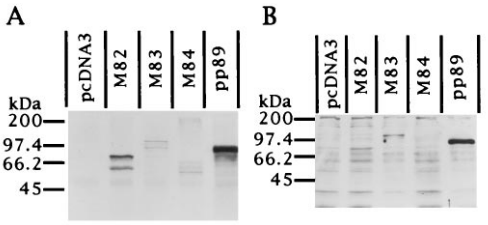

FIG. 5. Humoral responses against M82, M83, and M84 proteins in infection of BALB/c mice with MCMV. Plasmids containing ORFs encoding the M82, M83, M84, and pp89 proteins of MCMV and the plasmid vector pcDNA3 alone were translated in vitro with (A) or without (B) ^{35}S-labeled methionine. The translation products were then separated by SDS-PAGE. Gels containing labeled preparations were then stained, dried, and fluorographed. Gels containing unlabeled preparations were transferred to nitrocellulose and assayed by Western blot analysis with BALB/c mouse anti-MCMV hyperimmune serum as the probe as described in Materials and Methods. The ORF translated from each substrate plasmid is indicated above each lane. The positions of molecular mass markers are indicated.

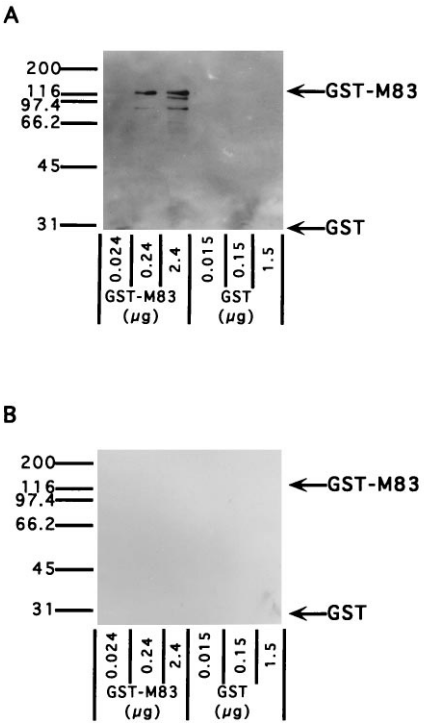

FIG. 6. Humoral immunogenicity of the MCMV M83 following infection of BALB/c mice. Various amounts of purified GST or GST-M83 were separated on mini-SDS–10% polyacrylamide gels and analyzed by Western blotting, using a 1:200 dilution of either mouse anti-MCMV hyperimmune serum (A) or normal mouse serum (B). The sera had been previously adsorbed with an acetone powder made from pGEX-KG-containing *E. coli* DH5α following induction. The positions of GST and GST-M83 fusion proteins and molecular mass markers (in kilodaltons) are indicated.

than the 91 kDa predicted by the amino acid sequence, the size of the larger species approximates that of the authentic protein, as we demonstrate below. pcDNA3-M84 yielded three major species of 66, 61, and 56 kDa. The largest species approximates the 65-kDa size predicted by the amino acid sequence. Finally, pcDNA3-pp89 yielded a single major product of approximately 89 kDa.

In parallel with the labeled translations, the same plasmids were used in unlabeled in vitro transcription-translation reactions to allow examination of the humoral immunogenicity of the ORF products in natural infection. A Western blot of the unlabeled in vitro-translated proteins was probed with anti-MCMV hyperimmune serum (Fig. 5B). As expected, the major 89-kDa species produced from pcDNA3-pp89 was strongly recognized by hyperimmune serum. Of the three putative matrix protein ORFs, only the major product of the M83 ORF, at 113 kDa, was clearly detectable. There was an increased band intensity in the M82 lane at a position approximating the location of the 77-kDa species, but comigrating immunoreactive species are present in the pcDNA3 negative control. Normal mouse serum failed to detect any of these proteins (data not shown). We cannot exclude the presence of antibodies directed against M82 or M84, but such antibodies are likely to be present only at low levels, if at all.

To confirm the presence of M83-specific antibodies in infected mouse serum, serum from MCMV-infected or uninfected BALB/c mice was adsorbed with an acetone powder derived from a culture of pGEX-KG-containing *E. coli* induced to produce large amounts of GST protein (22). This removed any fortuitous immunoreactivities against GST. The adsorbed sera were then used to probe Western blots containing various amounts of purified preparations of GST or GST-M83, the latter of which encodes a fusion protein containing the N-terminal 669 amino acids of M83. As expected, both normal and hyperimmune mouse sera showed no reactivity against GST, confirming that any antibodies detected were directed against M83 epitopes of the fusion protein (Fig. 6). Hyperimmune mouse serum demonstrated dose-dependent immunoreactivity against GST-M83, recognizing immunoreactive species of 121, 114, and 82 kDa (Fig. 6A). These proteins are somewhat larger than the predicted size of 94 kDa, likely because of the presence of strongly acidic regions encoded by the ORF. In contrast, uninfected mouse serum detected no immunoreactive species (Fig. 6B). We conclude that BALB/c mice are capable of mounting a humoral response against the MCMV M83 protein and do so in the course of MCMV infection.

Analysis of the derived M83 amino acid sequence. In view of the strong humoral response generated against the M83 protein following MCMV infection, we characterized the protein product of this ORF in detail. The derived amino acid sequence of this protein exhibits a number of interesting details (Fig. 2). The 809-amino-acid ORF of the Smith strain of MCMV, which encodes a protein with a predicted molecular mass of 90,837 Da, has an unusually high content of charged amino acids, comprising 30% of the protein (245 of 809 residues). Of particular note is the presence of two stretches of 23 and 21 contiguous acidic residues (residues 425 to 447 and 538 to 558, respectively). Within the M83 protein are a number of potential protein kinase target motifs, including those for protein kinase C, casein kinases I and II, and tyrosine kinases (37). Additionally, amino acid residues capable of phosphorylation (serine, threonine, and tyrosine) comprise a large fraction (114 of 809 amino acids) of the ORF. These may serve as target sites for other protein kinases.

We have sequenced both strands of the M83 ORF of the K181 strain of MCMV (12). The M83 of K181 encodes an 807-amino-acid protein with a calculated molecular mass of 90,578 Da. Comparison of the derived amino acid sequence with that of the Smith strain reveals relatively strong sequence conservation, with differences being limited essentially to the acidic regions of the protein. The K181 M83 possesses an insertion of a glutamate between residues 424 and 425 and a deletion of residues 544 to 546 (Glu-Glu-Asp). The K181 M83

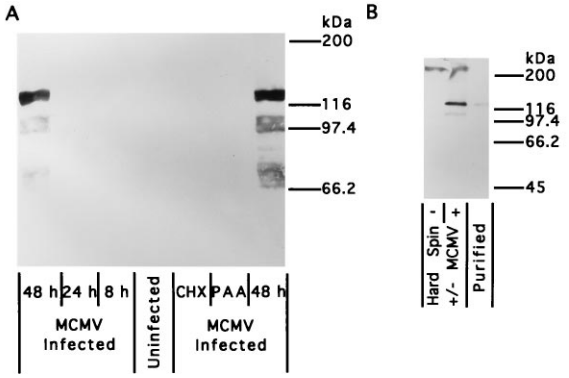

FIG. 7. Properties of the M83 protein. (A) Gene class and time course of expression. NIH 3T3 cells were infected with the K181 strain of MCMV with or without additional metabolic inhibitors. Cells infected in the absence of inhibitors were harvested at 8, 24, and 48 h p.i. Cells were also infected in the presence of cycloheximide for 8 h, washed to remove the drug, and harvested at 12 h p.i. (CHX) or were infected in the presence of phosphonoacetic acid and harvested at 48 h p.i. (PAA). The proteins were separated on an SDS–7.5% polyacrylamide gel and analyzed by Western blotting, using rabbit anti-GST-M83 antiserum as probe. Positions of molecular mass markers are indicated. (B) Virion association. Supernatant from MCMV-infected or uninfected NIH 3T3 cells was pelleted by ultracentrifugation (Hard Spin ± MCMV). Pelleted material from infected cell cultures was subjected to further purification by centrifugation through a sucrose gradient (Purified). The resulting preparations were separated on mini-SDS–10% polyacrylamide gels and subjected to Western analysis, using rabbit anti-GST-M83 antiserum as the probe. Positions of molecular mass markers are indicated.

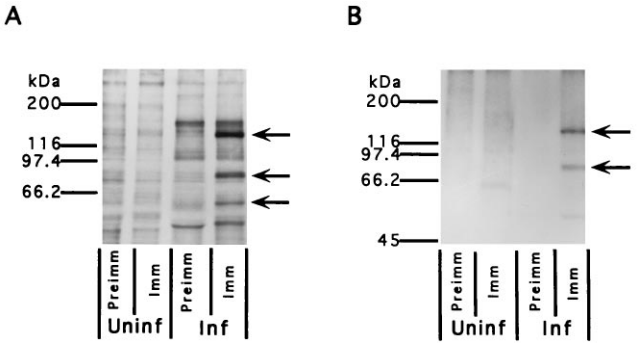

FIG. 8. In vivo labeling with ^{35}S (A) and ^{32}P (B). Uninfected (Uninf) and MCMV-infected (Inf) NIH 3T3 cells were labeled at 46 to 48 h p.i. with ^{35}S or ^{32}P. Total proteins were harvested from these cells and immunoprecipitated with either preimmune (Preimm) or immune (Imm) rabbit anti-GST-M83 antiserum. Immunoprecipitated material was separated on mini-SDS–10% polyacrylamide gels and fluorographed (^{35}S) or autoradiographed (^{32}P). Arrows indicate the sizes of proteins specifically precipitated from infected cell extracts by anti-GST-M83 antiserum. Positions of molecular mass markers are indicated.

has substitutions of a glutamate for an aspartate at residue 549, an aspartate for a glutamate at residue 551, and an aspartate for a glutamine at residue 562.

Pattern of protein expression, virion association, and in vivo phosphorylation state of the MCMV M83. The kinetics of expression of the M83 protein were examined by Western analysis of MCMV-infected NIH 3T3 cells harvested at 8, 24, or 48 h p.i., using rabbit anti-GST-M83 antiserum as the probe. Immunoreactive proteins of approximately 125, 105, and 70 kDa are detectable by Western blotting only at 48 h p.i. (Fig. 7A). This finding suggests that the M83 ORF is a member of the late class of viral genes. In the same experiment, NIH 3T3 cells were infected with MCMV either in the presence of cycloheximide, limiting protein expression to viral immediate-early genes, or in the presence of phosphonoacetic acid, limiting protein expression to viral immediate-early and early genes. Species immunoreactive with anti-GST-M83 antiserum are detectable only at 48 h p.i. in the absence of metabolic inhibitors, thereby classifying the M83 ORF as a member of the true late class of viral genes. Such a classification agrees with the pattern of transcription of this gene, with its putative 5-kb mRNA being expressed only at 24 to 48 h p.i. The absence of detectable protein at 24 h p.i., despite the presence of the M83 transcript at this time, is likely due to the level of protein expression being below the limit of detection of the Western blot.

To investigate the virion association of the M83 proteins, we purified virions from the tissue culture supernatant of MCMV-infected NIH 3T3 cells. Virions were first pelleted by centrifugation and then purified further by sucrose gradient sedimentation (9). When analyzed by Western blotting using anti-GST-M83 antiserum as the probe, a major immunoreactive protein of 125 kDa and a minor one of 105 kDa are detectable in the material pelleted from infected tissue culture supernatant (Fig. 7B). The 125-kDa band was also detected when the virus was further purified by sucrose gradient sedimentation. These data indicate that the M83 protein is virion associated, with the 125-kDa species being the major form of the protein in the virus particle.

To examine the in vivo phosphorylation state of the M83 gene product, uninfected or MCMV-infected NIH 3T3 cells were labeled at 46 to 48 h p.i. with ^{35}S or ^{32}P, and total proteins were harvested and subjected to immunoprecipitation with either the anti-GST-M83 antiserum or preimmune serum. SDS-PAGE and fluorography of ^{35}S-labeled proteins revealed the production of proteins of 125, 70, and 60 kDa that are specifically immunoprecipitated by the anti-GST-M83 serum from virally infected cells (Fig. 8A). Immunoprecipitation from ^{32}P-labeled cells indicated that only the 125- and 70-kDa species are detectably phosphorylated (Fig. 8B). We conclude that the M83 protein exists in vivo in a phosphorylated state, consistent with the presence of numerous potential protein kinase target motifs within the primary amino acid sequence.

We believe that the 125-kDa form of the M83 protein is the major product of this ORF. The discrepancy in size between the apparent molecular mass by SDS-PAGE and the calculated molecular mass of about 91 kDa can be accounted for by the extensive acidic regions in the M83 amino acid sequence and also by phosphorylation of the protein, both of which will tend to retard the protein's migration in SDS-PAGE. Lower molecular mass products are possibly the result of either proteolytic degradation or posttranslational processing of the protein.

Construction of recombinant vaccinia viruses expressing the M83 ORF. In preparation for studies of the protective efficacy of M83 in experimental vaccination, we constructed a recombinant vaccinia virus (M83-*vacc*) expressing this protein. Additionally, we constructed a recombinant vaccinia virus expressing β-galactosidase (pSC11-*vacc*) as a negative control. The products of these vaccinia viruses were analyzed by Western blotting of infected NIH 3T3 cells, alongside uninfected or MCMV-infected NIH 3T3 cells. Probing a Western blot of M83-*vacc*-infected NIH 3T3 cells with the anti-GST-M83 antiserum revealed production of a major immunoreactive product that comigrates with a MCMV-specific protein of about 125 kDa (Fig. 9). Similar results were obtained with the anti-MCMV hyperimmune serum (data not shown). Additionally, a number of smaller comigrating products are evident in both cases, albeit in lower relative amounts in M83-*vacc*-infected cells. No immunoreactive species is detectable in extracts of cells infected with pSC11-*vacc*, the β-galactosidase-expressing

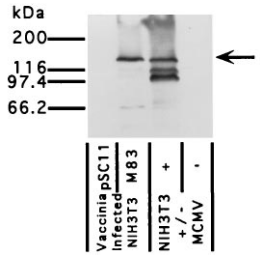

FIG. 9. Expression of M83 protein by recombinant vaccinia virus. Lysates of NIH 3T3 cells uninfected or infected with the recombinant vaccinia virus M83-*vacc* or pSC11-*vacc* or MCMV were separated on mini-SDS–10% polyacrylamide gels and transferred to nitrocellulose for Western analysis using rabbit anti-GST-M83 antiserum as the probe. The arrow indicates the position of the major species present in both M83-*vacc*- and MCMV-infected cells. Positions of molecular mass markers are indicated at the left.

negative control virus. Differences in relative amounts of different species in the MCMV-infected cells, compared with those in other blots (Fig. 7A), are likely attributable to the different extraction protocols used.

Production of a protein by M83-*vacc* that comigrates with and is serologically cross-reactive with the major 125-kDa band in MCMV-infected cells supports the contention that M83-*vacc* expresses the authentic M83 protein. It also provides additional evidence for lack of splicing within the M83 transcript, since vaccinia virus, with its cytoplasmic replication scheme, cannot mediate splicing (42, 43). The vaccinia virus-expressed product comigrates with the authentic M83 protein despite the fact that the latter exhibits aberrant migration in SDS-PAGE, indicating that the two proteins must possess similar biophysical properties. We cannot exclude the possibility of a small splicing event which alters the size of the protein at a level undetectable in this analysis but maintains the reading frame.

DISCUSSION

We show here that the MCMV genome has continued to maintain its global colinearity with the HCMV genome with respect to the apparent MCMV homologs of the HCMV UL82 and UL83 proteins. However, the local organization of the MCMV homologs appears to be somewhat different. As predicted, the MCMV M84 ORF shows some homology to its colinear HCMV counterpart, UL84. Unexpectedly, however, M84 shows even stronger homology to the HCMV UL83 matrix protein. The relationship of M84 to matrix proteins is further supported by its relatively strong homology with U54 of human herpesvirus 6, which also has homology with UL82 and UL83 and is thus believed to be a matrix protein (19, 36). Moreover, our analysis of the phylogenetic relationship of these proteins provides additional evidence for the close relationship between M84 and UL83.

The HCMV UL84 is a nonstructural early protein which has been shown to interact with the HCMV immediate-early protein IE86 (23, 66). UL84 is also required in the complementation assay for replication of a plasmid containing the HCMV origin of replication (25, 48). Interestingly, the 6.9-kb transcript, which likely encodes the M84 product, is detectable only at 8 h p.i., consistent with early gene expression. This parallels the transcription pattern of UL84. UL83 shows somewhat different kinetics of expression, being an early-late gene (10, 16). As we noted earlier, M84 could potentially be synthesized from the 8- or >9.5-kb transcripts synthesized at 24 to 48 h p.i., potentially making it a member of the early-late or true late gene class. This could perhaps occur through a mechanism involving internal initiation of translation, a mechanism that has been identified for other cytomegalovirus proteins, including the UL32 (pp150) matrix phosphoprotein (2). Generation of an M84-specific antiserum should allow more precise definition of the temporal expression kinetics and localization. On the basis of homology to known structural components of HCMV, we predict that M84 is virion associated. At present, we can only speculate that M84 may represent a hybrid protein, serving functions for MCMV carried out by both of its apparent HCMV homologs.

MCMV has two apparent homologs of the HCMV UL82, M82 and M83, possibly due to a duplication event at some point during the evolutionary history of MCMV after it diverged from HCMV. It is important to note that these relationships are not absolute, as M83 shows detectable homology to the HCMV UL83, indicating that M83 may represent a type of hybrid between the HCMV UL82 and UL83 proteins. Our analysis of the phylogenetic relationships of the various MCMV and HCMV proteins nevertheless supports the close relationship between M82, M83, and the HCMV UL82.

When we initiated our studies of the transcription pattern for the M82 to M86 ORFs, we found that the presence of overlapping transcripts of various sizes, some quite large, interfered with attempts to analyze mRNA expression through the use of primer extension and nuclease protection assays. However, Northern analysis, coupled with analysis of the DNA sequence for transcriptional control consensus sequences, allowed us to decipher a likely pattern of transcription. There is good agreement in our transcriptional model between the predicted and observed sizes of the transcripts, suggesting an absence of large-scale splicing events. However, we cannot exclude the possibility of small splicing events, which would not be reflected as detectable changes in transcript size in Northern analysis. Such a splicing event has been observed in UL83 of the Towne, but not of the AD169, strain of HCMV (46, 47). In that case, however, the splice maintains the predicted reading frame.

Of the three putative MCMV matrix proteins, only the M83 product is the target of an easily detectable humoral immune response, as are its putative HCMV counterparts, the HCMV UL82 and UL83 (4). Although antibodies directed against MCMV matrix proteins, like those against HCMV matrix proteins, are likely nonneutralizing and nonprotective, their detection may be useful as a serological marker of the infection (18, 61). If the immune response against these matrix proteins can be protective, we predict that it will be so through the induction of cellular responses, specifically through CTLs.

The M83 gene product shows a number of properties consistent with its proposed identity as an MCMV homolog of the HCMV UL82 protein (62). Specifically, it is virion associated and phosphorylated in vivo. While neither of these properties clearly identifies this protein as being the MCMV homolog of the HCMV UL82 protein, they are both properties of matrix proteins of HCMV. Additionally, M83 is a true late protein, as are all known HCMV matrix components other than UL83 (10, 16, 26, 40, 44, 63). These properties of M83 support its putative identification.

When we compared the amino acid sequences of the M83 proteins of the Smith and K181 strains of MCMV, we found that the differences were minor and limited to the repetitive acidic regions of the protein. However, the variability is sufficient for potential use as a strain-specific marker in experiments involving the introduction of viruses of both strains into the same tissue culture or experimental animal.

In summary, we have analyzed the potential protein coding

content of a portion of the MCMV *Hin*dIII C fragment, containing ORFs corresponding to the apparent MCMV homologs of the HCMV UL82 and UL83 matrix phosphoproteins. MCMV has three apparent matrix protein ORFs: M84, encoding an HCMV UL83/UL84 homolog; M83, encoding a UL82/UL83 homolog; and M82, encoding a UL82 homolog. We found that only M83 is the target of an easily detectable immune response in natural infection, and it exhibits kinetic properties, virion association, and in vivo phosphorylation consistent with its identification as a matrix protein. These results provide a starting point for detailed characterization of the immunological roles played by matrix proteins in MCMV infection. By analogy, such analyses may provide us with deeper insights into the immunology of HCMV, as have previous studies in the mouse model.

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