Late Expression of a β Chemokine Homolog by Murine Cytomegalovirus

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A gene (HJ1) present in murine cytomegalovirus (MCMV) encodes an open reading frame (ORF) whose predicted amino acid sequence shows homology to the β (or C-C) class of chemotactic cytokines known as chemokines. The region of homology is located in the carboxyl-terminal portion of the 116-amino-acid ORF. A 0.9-kb RNA transcript corresponding to the HJ1 gene is expressed in MCMV-infected fibroblasts and macrophages as a member of the late kinetic class of virally encoded transcripts. A transcriptional start site is located between the third and fourth methionine residues in the ORF, predicting a primary amino acid structure, starting at the fourth methionine residue, which includes a possible signal peptide as well as conserved cysteine residues typical for mammalian β chemokines. The RNA transcript is polyadenylated, suggesting that it can undergo translation within the cytoplasm of an MCMV-infected cell. We speculate that expression of the chemokine homolog at late times in the MCMV replication cycle plays a role in MCMV pathogenesis, possibly by action as a chemotactic agonist or antagonist or by alteration of the activation or differentiation state of a susceptible cell such as a macrophage.

Human cytomegalovirus (HCMV) is a ubiquitous human pathogen which, like other members of the herpesvirus family, establishes a lifelong infection in its host. HCMV has the capacity to reactivate and cause significant morbidity and mortality in immunocompromised people (15, 35, 37, 44). Infection of mice with the closely related murine cytomegalovirus (MCMV) is used as an animal model for HCMV infection (reviewed in reference 42). The genomes of several members of the herpesvirus family, including HCMV (9), herpesvirus saimiri (HVS [2]), human herpesvirus 6 (HHV-6 [18]), and HHV-7 (30), contain sequences encoding proteins with homology to G-protein-coupled receptors for chemotactic cytokines known as chemokines. In addition, the HCMV-encoded US28 gene product (17, 29) and the HVS-encoded ECRF3 gene product (1) have been shown to encode functional chemokine receptors. Chemokines are small, inducible, secreted inflammatory cytokines which share a common structural motif of four conserved cysteine residues involved in disulfide bonding (reviewed in references 5, 7, 28, and 31). β chemokine family members lack an intervening amino acid found in the α chemokine family members between the first and second conserved cysteine residues. Other conserved residues within the β chemokine family members include a tyrosine residue between the second and third conserved cysteine residues, a phenylalanine residue between the third and fourth cysteine residues, and a tryptophan after the fourth cysteine residue (see reference 31). While the α chemokines primarily attract neutrophils, the β chemokine family members are chemoattractant for monocytes, lymphocytes, basophils, and eosinophils and are thought to play a role in activation, proliferation, and adhesion (reviewed in references 5, 7, 28, and 31).

Although the roles of chemokines and virally encoded chemokine receptors in herpesvirus pathogenesis have not been

elucidated, the presence of putative chemokine receptors in many of the herpesvirus family members suggests an important role in herpesvirus biology. This, coupled with the ability of both the HCMV- and HVS-encoded chemokine receptor homologs to bind known human chemokines and transmit a signal via calcium mobilization (1, 17, 29), suggests that herpesviruses have evolved mechanisms to utilize host chemokine signaling pathways in their life cycle or have developed mechanisms to counteract or influence the host inflammatory response to viral infection. Given this, we found it interesting that MCMV open reading frame (ORF) HJ1 showed homology to several mammalian chemokines when we searched for homologies using the BLASTP program (4). Sequencing of the HHV-6 genome also reveals potential chemokine homologs (18), indicating that the herpesvirus family members may influence this important inflammatory cytokine pathway via expression of homologs of both receptors and ligands.

ORF HJ1 of the MCMV *HindIII* J fragment encodes a β chemokine homolog. The predicted amino acid sequence of MCMV-encoded ORF HJ1 (42) in the GenBank database (accession number U10326) was used to search for homologous sequences in the database utilizing the program BLASTP (4) and found to be homologous to several members of the mammalian β chemokine family. The sequences of several known murine β chemokine family members were aligned with the predicted amino acid sequence encoded by ORF HJ1 by the program MegAlign (DNASTAR), and the resultant alignment is shown in Fig. 1. Although the ORF predicts a 116-amino-acid protein, which we have designated MCK-1 (for MCMV-encoded chemokine 1), only the carboxyl-terminal 81 amino acids (starting at amino acid residue 36) are aligned in Fig. 1 and contain the four conserved cysteine residues characteristic of β chemokine family members, as well as conserved phenylalanine, tyrosine, and tryptophan residues indicated in the figure. β chemokine family members typically share 28 to 45% amino acid homology (reviewed in reference 31). The carboxyl-terminal 81 amino acids of MCK-1 show close homology to murine β chemokines, with 24 to 28% amino acid identity and 47 to 50% amino acid similarity to macrophage

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FIG. 1. Alignment of MCK-1 with β chemokine family members. The deduced amino acid sequence encoded by the MCK-1 ORF was aligned with those of five murine β chemokine family members by the program MegAlign (DNASTAR), using the CLUSTAL method with a PAM250 weight table (40). The amino acid residues are represented by the single letter amino acid code, with dashes indicating gaps in the alignment. Residues identical in all of the aligned chemokines are highlighted in black, while those identical to MCK-1 are boxed. Residues showing similarity, defined as acidic (D or E), basic (H, K or R), hydrophobic (A, F, I, L, M, P, V, or W), or polar (C, G, N, Q, S, T, or Y), are shaded. The four conserved cysteine residues are indicated with filled arrows above the alignment, and conserved tyrosine, phenylalanine, and tryptophan residues characteristic of β chemokine family members are indicated with open arrows. Lengths of the aligned sequences are indicated to the right. MCMV MCK-1, the predicted amino acid sequence of ORF-1 of the MCMV HindIII J fragment (accession number U10326) starting at the fourth ORF methionine residue; MIP-1 α , macrophage inflammatory protein 1 α , accession number P10855; MIP-1 β , macrophage inflammatory protein 1 β , accession number P14097; RANTES, regulated upon activation, normal T-cell expressed and secreted, accession number M77747; eotaxin, accession number U26426; MCP-1, monocyte chemoattractant protein 1, accession number M19681.

inflammatory protein (MIP)-1 α , MIP-1 β , regulated on activation, normal T-cell expressed and secreted (RANTES), eotaxin, and the amino-terminal portion of macrophage chemotactic protein 1 (MCP-1) aligned in Fig. 1. MCK-1 is homologous to many other mammalian β chemokines as well (not shown).

The region of MCMV DNA encoding MCK-1 was cloned from the strain of MCMV in use in our laboratory (strain Smith MSGV, ATCC VR-194) and sequenced. MCMV was isolated from infected NIH 3T12 fibroblast cells (ATCC CCL 164) as previously described (19), and virions were partially purified by sucrose density centrifugation (13). MCMV genomic DNA was isolated by proteinase K digestion in the presence of 0.5% sodium dodecyl sulfate and 1% sarkosyl, followed by phenol-chloroform extraction essentially as described previously (21). Using standard techniques (36), the DNA was digested with *Eco*RI and *Bam*HI and size separated via low melting temperature agarose gel electrophoresis, and the ~2-kb size fragments were ligated to similarly digested pGEM-1 (Promega). After transformation into *Escherichia coli*, transformants were screened by colony hybridization to oligonucleotide probe 1 (Table 1) complementary to the putative MCK-1-encoding DNA. Figure 2D shows the resultant plasmid, designated pHDLbr. To facilitate sequencing of the region immediately 5' to the MCK-1 ORF, plasmid pHDL-

JLsma-bam, containing the sequences from a unique *Sma*I site located 5' to the ORF through the *Bam*HI site, was generated by digestion of pHDLbr with *Sma*I and *Eco*RI and incubation with DNA polymerase large fragment (Klenow), followed by ligation and transformation. Sequencing of the cloned DNA (Sequenase; United States Biochemical Corporation) revealed the presence of the MCK-1 ORF and no nucleotide differences when compared with the sequence present in the database (data not shown).

TABLE 1. Oligonucleotide primers and probes utilized

Oligonucleotide	Sequence (5' to 3')
A ^a	GTCCGCGACGGTAGACACGACCAACAC
B ^a	GTGTTAGTGTATTCCGGCGCTCTGTACCGCG
C ^a	GAGCGTTCCCATTTTCAGATGAGATTTTATTGT
	GAATCAGTGAAACGTTAAGCAAAAACAT
1	AGAAGGTAGTGAAAACACCTCTCG
2	CGCGGATCCGTCGTGTCTACCGTCGCG
3	GGAATTCCTCACCTGACCAGACACAAGAG
4	CGCGGATCCACCATGGGAACGCTCTCTCGT

^a Letters refer to oligonucleotides indicated in Fig. 2C.

FIG. 2. Schematic representation of the MCK-1 ORF and its location in the MCMV genome. (A) Schematic representation of the MCMV genomic *Hind*III map, with the letters above indicating the identity of the fragments (27). (B) Expansion of the J and L fragments, showing the approximate locations of previously defined transcripts (42), with arrowheads indicating the direction of transcription. The approximate locations of several restriction enzyme sites are shown. (C) Expansion of the region surrounding the MCK-1 ORF, showing the approximate location of the four potential initiator methionine residues as well as the locations of several restriction enzyme sites. The locations of experimental probes are shown below, with the assay for which they were used indicated. Asterisks indicate the 5' ends of probes which were 5' end labelled with 32 P prior to experimental use, while arrows indicate the 3' ends of riboprobes. (D) Schematic representation of plasmid pHDJLbr. Restriction sites and locations are indicated. The solid segment represents the gene encoding resistance to ampicillin; the hatched segment represents the origin of replication. MCMV DNA is represented by the shaded segment, with the solid arrow representing the location and direction of transcription of the MCK-1-encoding HJ1 ORF.

Northern blot analysis demonstrated that RNA encoding MCK-1 is expressed in MCMV-infected cells. Strand-specific RNA probes, as well as DNA probes, were generated for Northern blot analyses. Figure 2 shows a schematic representation of the MCMV *Hind*III genomic map, the transcripts

known to be generated from this region (42), and the locations of the probes utilized. Plasmid pMCK-ORF was constructed for the generation of strand-specific RNA probes. DNA encoding the carboxyl-terminal 66 amino acids of MCK-1 was amplified from plasmid pHDJLsma-bam by PCR, utilizing primers 2 and 3 (Table 1) and Vent DNA polymerase (New England Biolabs; 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min). The resultant DNA was cloned into *Eco*RI- and *Bam*HI-digested pGEM-1, resulting in plasmid pMCK-ORF, which was utilized for transcription. Double-stranded DNA probe encompassing the carboxyl-terminal 81 amino acids of MCK-1 was generated by utilizing the same PCR cycle parameters with primers 3 and 4 (Table 1) and pHDJLsma-bam as template DNA.

Northern blot analyses (Fig. 3) utilizing these probes revealed that a 0.9-kb polyadenylated MCK-1-specific mRNA is expressed in NIH 3T12 fibroblast cells infected with MCMV. No signal was detected in RNA from uninfected cells with any of the probes (Fig. 3A, lanes 1), while an MCMV-specific 0.9-kb message was seen which hybridized to the antisense riboprobe and DNA probe specific for the MCK-1 ORF (Fig. 3A, lanes 2). The presence of a poly(A) tail indicates that the mature MCK-1-encoding mRNA is likely transported to the cytoplasm and is thus capable of generating MCK-1 in MCMV-infected cells. The lack of any detectable MCMV-specific RNA species with the sense riboprobe indicates that no major polyadenylated mRNA transcripts crossing the MCK-1 ORF are generated from the opposite viral DNA strand. The specificity of the probes is shown in Fig. 3B.

The MCK-1-encoding message is a member of the late kinetic class of virally encoded transcripts. Herpesvirus gene expression follows a temporal pattern of sequential expression of three classes of RNA (20). Immediate-early (IE) transcripts require only preformed host factors for expression and thus are expressed despite inhibition of host cell translation. Early (E) transcripts require the IE gene products for expression, whereas late (L) transcripts are expressed maximally after viral DNA replication has been initiated. The kinetic class of the MCK-1-encoding message was determined by Northern blot analysis of total RNA isolated from NIH 3T12 cells that were mock or MCMV infected. After infection, cells were enriched to contain RNA transcripts of the IE, E, or L class (23), using cycloheximide or phosphonoacetic acid, and Northern blot analysis was performed (Fig. 3C). No MCK-1-specific RNA was detected in mock-infected cells incubated under any of the conditions. An ~0.9-kb MCK-1-specific RNA species was detected in cells enriched for viral L transcripts, with no species detectable in cells grown in the presence of the viral DNA synthesis inhibitor phosphonoacetic acid. Larger species of heterogeneous size, including a predominant RNA of ~5 kb, were detected in RNA enriched for transcripts of the IE class, but no 0.9-kb MCK-1-specific message was detected. The structure of these larger IE transcripts containing sequences which hybridize to the MCK-1-encoding region remains uncertain. They may represent primary unprocessed transcripts, since they were not significantly enriched in the polyadenylated RNA fraction (Fig. 3A), although the abundance of IE transcripts in this polyadenylated fraction is unknown. However, several larger polyadenylated RNA species can be seen on longer exposures of Fig. 3A (not shown) as well as in a separate experiment (Fig. 4A), raising the possibility that larger processed transcripts do contain the MCK-1-encoding region of the MCMV genome. The relationship between the larger MCK-1-specific IE transcripts and polyadenylated RNA species (harvested from cells in the absence of inhibitors) is unknown. Further investigation into the identity of these tran-

FIG. 3. Northern blot analysis and identification of the 0.9-kb MCK-1-encoding mRNA as a member of the late kinetic class of virally encoded transcripts. NIH 3T12 fibroblasts were mock infected or infected with MCMV, and total RNA was harvested (RNAzolB; Teltest, Inc.). Polyadenylated mRNA species were purified by oligo(dT) column chromatography (mRNA purification kit; Pharmacia Biotech), and total or polyadenylated fractions were size separated by 1.2% agarose gel electrophoresis after formaldehyde denaturation (36). After transfer to nylon membrane (Hybond N+; Amersham), the RNA was sequentially probed (8) as indicated and the autoradiograms are shown. (A) Fibroblasts were mock infected or infected with MCMV (multiplicity of infection, 0.05), and total RNA was harvested after 5 days when cytopathic effect was extensive. Polyadenylated RNA fractions (0.25 μ g) were size separated and transferred to nylon membrane, and the RNA was sequentially probed with the antisense riboprobe, sense riboprobe, and double-stranded DNA probe. Antisense riboprobe, specific for the MCK-1 message (Fig. 2C, probe E), was generated by T7 RNA polymerase (Boehringer Mannheim) transcription, in the presence of [α -³²P]CTP, of *Bam*HI-linearized pMCK-ORF. Sense riboprobe, specific for transcripts originating from the opposite strand (Fig. 2C, probe F), was generated by utilizing SP6 RNA polymerase (Promega) and *Eco*RI-linearized pMCK-ORF. A DNA probe (Fig. 2C, probe D) encompassing the carboxyl-terminal 81 amino acids of MCK-1 was generated by random prime labelling (Megaprime kit; Amersham) in the presence of [α -³²P]dCTP of DNA generated by PCR (see text) utilizing pHDJLsma-bam as template with primers 3 and 4 (Table 1). Lanes: 1, poly(A)⁺ RNA from mock-infected cells; 2, Poly(A)⁺ RNA from MCMV-infected cells. The probes used are indicated at the top and shown schematically in Fig. 2C. Migration of 18S and 28S rRNA as well as size standards (in kilobases) is indicated. The asymmetric signal at ~2.8 kb seen in lane 2 with the antisense riboprobe and the DNA probe is an artifact which was not seen in a repeat experiment utilizing the DNA probe (Fig. 4). (B) Specificity of the probes used in panel A is shown by Northern analysis of 10 μ g of total RNA harvested from uninfected NIH 3T12 cells, spiked with the indicated copies of sense or antisense message produced with T7 or SP6 RNA polymerase utilizing *Bam*HI- or *Eco*RI-digested pHDJLbr, respectively. The probes utilized are indicated to the right. (C) Ten micrograms of total RNA from mock- or MCMV-infected NIH 3T12 fibroblasts (multiplicity of infection, 3) was subjected to Northern blot analysis utilizing the MCK-1-specific DNA probe (Fig. 2C, probe D). Prior to RNA harvest, RNA was enriched for each of the three kinetic classes. RNA was enriched for transcripts of the IE class by incubation in the presence of 50 μ g of cycloheximide per ml, with RNA harvest at 6 h. Transcripts of the E class were enriched by incubation of the cells for 24 h in the presence of 250 μ g/ml of the viral DNA synthesis inhibitor, phosphonoacetic acid. Enrichment of L transcripts was achieved by incubation, prior to RNA harvest, for 24 h in the absence of any inhibitors. The migration of the 0.9-kb putative MCK-1-encoding transcript is indicated with an arrow. RNA integrity was assessed by reprobing the same Northern blot for cyclophilin mRNA, a constitutively expressed message (12) detected by a rat cyclophilin cDNA probe. The efficacy of the inhibitors was assessed by probing for messages previously identified for the IE and E kinetic classes. A probe specific for the IE transcript encoding ie1 (22) was generated by PCR utilizing outer primers of a nested PCR previously described (33). A probe specific for the early transcripts encoding Sggl (25) was generated by digestion of pHDJLbr with *Eco*RI and *Stu*I (Fig. 2D). The resultant ~0.5 kb fragment contains sequences represented in both the 1.5- and 1.8-kb Sggl transcripts (Fig. 2B). IE, RNA from cells enriched for transcripts of the IE class; E, RNA from cells enriched for transcripts of the early kinetic class; L, RNA from cells grown in the absence of inhibitors to allow production of transcripts of the late kinetic class. Migration of RNA standards, with sizes indicated in kilobases, is shown to the right.

FIG. 4. Analysis of MCK-1 mRNA for sequences 5' and 3' of the ORF. NIH 3T12 fibroblasts were infected with MCMV (multiplicity of infection, 0.3), and total RNA was harvested (RNAzolB; Teltest, Inc.) 3 days after infection when cytopathic effect was extensive. Polyadenylated mRNA species were purified by oligo(dT) column chromatography (mRNA purification kit; Pharmacia Biotech). Five micrograms of total RNA or 0.5 μ g of the polyadenylated fraction was size separated by 1.2% agarose gel electrophoresis after formaldehyde denaturation (36). After transfer to nylon membrane (Hybond N+; Amersham), the RNA was sequentially probed with 32 P-labelled DNA probes for MCK-1, sequences 5' to the MCK-1 ORF, and sequences 3' to the ORF. (A) Autoradiogram obtained with the MCK-1 probe (Fig. 2C, probe D). (B) Autoradiogram obtained with the *Stu*I to *Eco*RI fragment (Fig. 2D and Fig. 3) located 5' to the MCK-1 ORF, which recognizes the Sgg1 1.5- and 1.8-kb transcripts. (C) Autoradiogram obtained with the *Bgl*II to *Bam*HI fragment (Fig. 2D) located 3' to the MCK-1 ORF. The type of RNA is indicated at the top, with migration of molecular weight standards indicated to the right. Probes utilized are indicated below each panel. The migration of the MCK-1, Sgg1 1.5-kb, and Sgg1 1.8-kb transcripts is indicated by arrows on the left.

scripts is required to predict the potential gene products they might encode.

In order to ensure RNA integrity and uniform loading, as well as appropriate enrichment for transcripts of the IE and E kinetic classes, the blot was sequentially hybridized with probes for cyclophilin mRNA, a constitutively expressed message (12), MCMV ie1 mRNA, a known IE transcript (22), and MCMV Sgg1 mRNAs, known E transcripts (25). The results indicated that the inhibitors successfully enriched for RNA transcripts of the appropriate classes, allowing the MCK-1 message to be classified as a member of the L kinetic class of MCMV transcripts. Similar analysis of RNA from MCMV-infected bone marrow macrophages differentiated in culture revealed that the MCK-1 transcript is a member of the L kinetic class in this cell type as well (data not shown). It is likely that a previously described 0.85-kb late transcript originating from this region (42) is identical to the 0.9-kb mRNA detected here with the MCK-1-specific probe.

The MCK-1-encoding message is distinct from nearby Sgg1 messages and contains sequences 3' to the MCK-1 ORF. Analysis of sequences surrounding the MCK-1 ORF reveals that the polyadenylation signal for the previously identified Sgg1 1.5- and 1.8-kb transcripts is located within the 5' end of the MCK-1 ORF (42), at the 30th amino acid residue of the ORF. To ensure that MCK-1 message is distinct from the Sgg1 messages, total and poly(A)⁺ RNA from MCMV-infected fibroblast cells were assessed by Northern blot analysis. The blot (Fig. 4A and B) was sequentially probed with the MCK-1-specific DNA probe encompassing the carboxyl-terminal 81 amino acid residues (Fig. 2C, probe D) and a *Stu*I to *Eco*RI fragment located 5' to the MCK-1 ORF which recognizes the 1.5- and 1.8-kb Sgg1 messages (Fig. 2B and D). The Sgg1 1.5- and less abundant 1.8-kb messages are clearly distinct and larger in size than the MCK-1 mRNA. There is no hybridization of the MCK-1 probe to the Sgg1 transcripts, indicating that the Sgg1 messages do not extend to include the carboxyl-terminal 81 amino acids of the MCK-1 ORF.

Analysis of the sequences just 3' to the MCK-1 ORF reveal no polyadenylation signal. In order to determine if sequences 3' to the ORF are included in the 0.9-kb MCK-1 message, the same Northern blot (Fig. 4C) was probed with a *Bgl*II to *Bam*HI fragment (3' probe) from plasmid pHDJLbr (Fig. 2D). A predominant RNA species of ~0.9 kb was detected with this probe in both total and poly(A)⁺ RNA, and the pattern of poly(A)⁺ transcripts detected was identical to that seen with the MCK-1 ORF-specific probe (compare Fig. 4A and C). An additional ~0.35-kb transcript, not seen with the MCK-1-specific probe, was detected with the 3' probe in total RNA harvested from MCMV-infected cells but was absent in the polyadenylated fraction. These results indicate that the mature polyadenylated MCK-1 mRNA includes sequences 3' to the MCK-1 ORF.

The MCK-1 mRNA 5' transcriptional start site was determined and predicts a primary protein structure of 81 amino acids. Although message encoding MCK-1 is expressed which is likely to be translated within the cytoplasm of an infected cell at late times after infection, the primary protein structure is uncertain. Of the four potential initiator methionine residues, sequences surrounding the first and fourth best fit the consensus for likely use in translation initiation (24). Initiation at the first methionine residue would result in a protein with primary structure not likely to contain a signal peptide sequence and in addition would contain an amino-terminal portion not homologous to those of mammalian chemokines. However, translation initiation at the fourth methionine residue would predict a protein with homology to chemokines, including a potential cleavable signal peptide, with cleavage occurring after a leucine located 15 residues downstream from the fourth methionine residue (26, 28a, 43). Mammalian β chemokines are synthesized as preproteins containing cleavable signal peptides (reviewed in reference 5). Therefore, we further defined the 5' end of MCK-1 mRNA in order to see which of the four potential initiator methionine residues are contained in the message. Primer extension analysis was carried out (41) with oligonucleotide primers A and B (Table 1) complementary to MCK-1 mRNA, as indicated in Fig. 2C. The extension products were analyzed by denaturing acrylamide electrophoresis, and the sizes of the products mapped the location of the transcriptional start site to an area near the third and fourth methionine residues (data not shown). Figure 5A shows the results of primer extension analysis with primer A designed to more finely map the transcriptional start site. Extension products mapped the transcription start site(s) to a 6-nucleotide (nt) region located between the position of the third and fourth

FIG. 5. Analysis of the MCK-1 mRNA transcriptional start site. (A) Autoradiogram of primer extension analysis utilizing 3.25 μ g of poly(A)⁺ RNA or 10 μ g of total RNA isolated from MCMV- or mock-infected fibroblasts, respectively. RNA was hybridized with ³²P 5' end-labelled primer A (Table 1 and Fig. 2C) for 1 h at 65°C, and then extension was carried out at 42°C in the presence of Superscript II reverse transcriptase (Gibco, Life Technologies). Sequencing reactions (Sequenase; United States Biochemicals) of plasmid pHDJLbr, utilizing ³²P-labelled primer A, were analyzed in parallel to primer extension products in order to map the transcriptional start site. RNA utilized in the extension reaction is indicated above. G, A, T, and C indicate dideoxy analogs included in sequencing reactions of MCMV DNA. The sequence of the relevant portion of the ladder is indicated along with the complementary nucleotides that would be represented in the mRNA. (B) Autoradiogram of S1 nuclease analysis carried out with 0.5 μ g of poly(A)⁺ RNA isolated from mock- or MCMV-infected cells. RNA was hybridized overnight at 37°C to 4 ng of probe C (Table 1 and Fig. 2C) which had been labelled at the 5' end with ³²P. S1 nuclease (Promega) was then added at a concentration of 250 and 400 U/ml (final concentration, 225 or 360 U/ml), and after incubation at 37°C for 30 min, the protected products were analyzed by denaturing acrylamide gel electrophoresis. The source of poly(A)⁺ RNA is indicated above, along with the concentration of S1 nuclease added to the reaction. Chemical cleavage of the same probe was run in lane G+A. The sequence of the probe is indicated, along with the corresponding complementary bases at major sites of protection. Because chemical cleavage occurs on either side of the depurinated base, the ladder generated with the 5'-end-labelled probe represents fragments one base shorter than the location of the G and A nucleotides. (C) The amino-terminal residues of the MCK-1 ORF and the corresponding MCMV DNA sequence are indicated. Amino acid residues are shown using the single letter amino acid code. The methionine residues are shown in bold, with the likely initiator methionine (fourth methionine of the ORF) boxed. The sites of likely transcription initiation, as determined by primer extension and S1 nuclease analyses are underlined, as is the location of a possible TATA element.

ORF methionine residues, and no extension products were present in RNA isolated from mock-infected cells. This 6-nt region is ~30 to 35 nt downstream from a TA-rich region, indicated in Fig. 5C, which may function as a TATA element.

Because primer extension analysis cannot precisely map the transcriptional start site when RNA splicing has occurred in the 5' end of the transcript, the start site was verified by nuclease protection. Figure 5B shows the results obtained

when oligonucleotide probe C (Table 1), which crosses the putative transcriptional start site (Fig. 2C), was utilized in S1 nuclease protection assays (45). No probe was protected from S1 nuclease digestion when RNA isolated from mock-infected cells was utilized for protection. In contrast, RNA isolated from MCMV-infected cells was able to protect several probe fragments from S1 nuclease-mediated digestion. The sizes of the protected fragments, as determined by comparison to fragments generated by chemical cleavage of probe C (36), are in agreement with the primer extension data, indicating that splicing of the 5' end of the MCK-1-encoding transcript does not likely occur. Whether the heterogeneity of products seen in the primer extension and S1 nuclease protection assays represents incomplete extension and digestion, respectively, or is reflecting true heterogeneity of the 5' end of the MCK-1 message is uncertain. Identical S1 nuclease protection results were obtained in three separate experiments utilizing two independently isolated RNA preparations. Although the exact nucleotide(s) of the start site(s) cannot be determined from these experiments, the fact that two different methods mapped the start of the message to this region supports our conclusion that the MCK-1 message starts between the third and fourth methionine residues of the MCK-1 ORF. Although not detected in these assays, alternative sites of transcription initiation may also exist. However, the region of the MCMV genome located between the third and fourth methionine residues of the MCK-1 ORF (Fig. 5C) is clearly a predominant site of transcription initiation. Thus, a virally encoded protein product containing a potential signal peptide, with high homology to the β chemokine class of chemotactic cytokines, is predicted to be produced at late times after infection of murine cells with MCMV.

In summary, we have found that MCMV contains an ORF whose predicted protein, MCK-1, bears a striking resemblance to members of the mammalian β chemokine class of chemotactic cytokines. RNA potentially capable of encoding this protein is expressed as a member of the late kinetic class of virally encoded transcripts in MCMV-infected fibroblasts and macrophages. We have mapped the site of transcription initiation to a region between the third and fourth methionine residues of the ORF, thus predicting a protein with features typical of members of the β chemokine class, including a potentially cleavable signal peptide which would target the protein to the secretory pathway. Since cells of the macrophage/monocyte lineage are capable of chemotaxis toward a variety of mammalian β chemokines, perhaps production of MCK-1 serves to attract this important cell type which has been implicated in spread of MCMV in the host (6, 11, 39) as well as a site of latent infection (32). Alternatively, since macrophages are important players in the innate resistance to MCMV infection (19, 34, 38), perhaps MCK-1 can act as an antagonist to host chemokine action which might be involved in generation of the normal inflammatory response to viral infection. MCMV has been noted to encode a sequence homologous to that of G-protein-coupled receptors (16a). The potential of MCMV encoding a functional chemokine receptor also raises the possibility that MCK-1 is acting in an autocrine fashion in order to provide an optimal intracellular milieu for some aspect of the virus life cycle. Host chemokine receptors have recently been found to be involved in the penetration of cells by human immunodeficiency virus (3, 10, 14, 16), indicating that manipulation of the mammalian chemokine signalling pathway is not unique to herpesviruses. The fact that many of the herpesvirus family members have been found to encode sequences homologous to those of chemokines and/or chemokine receptors indicates that intervention with, or subversion of, this mamma-

lian signalling pathway is likely to play a fundamental role in herpesvirus pathogenesis. Understanding the role of MCK-1 in MCMV pathogenesis will provide insight into the pathogenesis of other herpesvirus family members in their respective host species.

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