The murine cytomegalovirus M73.5 gene, a member of a 3′ co-terminal alternatively spliced gene family, encodes the gp24 virion glycoprotein

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Abstract

We have identified a novel family of five 3′ co-terminal transcripts in murine cytomegalovirus (MCMV) arranged in a tail-to-tail orientation with respect to the MCMV glycoprotein H (gH) gene M75. They share the same exon 2 sequence but possess different exon 1 sequences. Two of these spliced transcripts (M73) encode the MCMV homolog of glycoprotein N (gN) entirely within exon 1. Two other transcripts designated M73.5 encode a previously described virion glycoprotein gp24 that shares its first 20 amino acids with gN, but which has another 64 amino acids encoded within exon 2. The fifth transcript, designated m60, has an 80-bp exon 1 near the MCMV oriLyt region 10.8 kb upstream of exon 2. Both MCMV M73.5 and m60 encode type II glycoproteins expressed at the cell surface. Their shared exon 2 coding sequences likely represent the highly conserved region of an as yet unidentified betaherpesvirus-specific glycoprotein species.

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Introduction

Murine cytomegalovirus (MCMV) is a member of the Betaherpesvirinae subfamily of Herpesviridae. Characteristic features of betaherpesviruses include slow replication cycle, restricted host range, and the establishment of latency in secretory glands, lymphoreticular cells, kidney, and other tissues (Mocarski, 1996). This subfamily includes three human viruses, including human CMV (HCMV), human herpesvirus-6 (HHV6), and human herpesvirus-7 (HHV7). Many animal species also harbor betaherpesviruses. Apart from MCMV, other representative animal betaherpesviruses include chimpanzee CMV (CCMV), rhesus CMV (RhCMV), rat CMV (RCMV), guinea pig CMV (GPCMV), and Tupaia herpesvirus of tree shrews (THV). Except for GPCMV, which has only been partly sequenced (Brady and Schleiss, 1996), all of these betaherpesviruses have been fully sequenced (Bahr and Darai, 2001; Chee et al., 1990; Davison et al., 2003; Gompels et al., 1995; Hansen et al., 2003; Megaw et al., 1998; Rawlinson et al., 1996; Vink et al., 2000).

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The strict species specificity of cytomegaloviruses has meant that many aspects of the pathogenesis of HCMV infection of humans cannot be directly investigated in animal models. Thus, the many parallels in HCMV and MCMV pathogenesis in their respective hosts (Stacek, 1990; Sweet, 1999) and the similarities in their molecular makeup have led to a widespread usage of MCMV as a model system for HCMV pathogenesis. The MCMV genome, which is 230-kb long, has been predicted to encode approximately 170 open reading frames (ORFs) (Rawlinson et al., 1996). In that analysis, a total of 64 genes were identified as having the potential to encode glycoproteins based on similarities to other herpesviruses or the presence of potential transmembrane regions and N-linked glycosylation sites within predicted ORFs.

The main structural virion glycoproteins of cytomegalovirus characterized to date include those forming the glycoprotein complexes gCI, gCII, and gCIII first identified in HCMV (Gretch et al., 1988). In HCMV, the gCI complex has been found to be a homodimeric complex (gB/gpUL55) involved in fusion. The gCII complex is a dimer comprising gM (gpUL100) and gN (gpUL73, respectively) (Mach et al., 1996). In that analysis, a total of 64 genes were identified as having the potential to encode glycoproteins based on similarities to other herpesviruses or the presence of potential transmembrane regions and N-linked glycosylation sites within predicted ORFs.

Despite the MCMV sequence having been determined for some time now, only a few structural virion glycoproteins have been characterized thus far. These include gB (Loh, 1991; Loh et al., 1988; Rapp et al., 1992; Xu et al., 1996), gH (Loh and Qualtiere, 1988; Rapp et al., 1994, 1993; Xu et al., 1992), gL (Xu et al., 1994), and gM (Li et al., 1995; Scalzo et al., 1995). Another virion glycoprotein designated gp24 was identified using the neutralizing monoclonal antibody (MAb) 6A1.21A (Loh, 1989). However, a HCMV homolog to gp24 has not yet been described.

In this study, we report on the identification and characterization of the M73/M73.5 family of transcripts and their gene products. During the screening of a MCMV cDNA library with a polyclonal antiserum to MCMV, we identified a cDNA clone, TS9, which represented a novel spliced transcript present in the MCMV genome. It is arranged in a tail-to-tail arrangement with respect to the M75 (gH) ORF and consists of two exons. Analysis by immunofluorescence, immunoprecipitation, and Western blotting has shown that the protein encoded by TS9 resembled the previously described virion glycoprotein gp24. Final confirmation of its identity was provided by the sequencing of tryptic peptides derived from gp24 that was immunoprecipitated from MCMV-infected cells by MAb 6A1.21A. Database analysis indicates that this glycoprotein is conserved in all betaherpesviruses. Further analyses of MCMV transcripts in this region revealed additional spliced transcripts that were 3′ co-terminal with the novel M73.5 mRNA. Firstly, the gene encoding MCMV gN (M73) is a spliced transcript with the gN ORF residing entirely within the first exon, and it also shares its second exon with M73.5. Secondly, longer versions of M73 and M73.5 transcripts with extended 5′ ends also exist. Finally, an additional 3′ co-terminal spliced transcript of this gene family was identified, which has its exon 1 located nearly 10.8 kb upstream near the MCMV OriLyt region, but which shares the same exon 2 with both M73 and M73.5, indicating a complex genomic pattern of transcription in this region.

Results

Isolation of the M73.5 sequence from an MCMV cDNA library

Polyclonal hyperimmune antiserum to MCMV was used to screen a λgt11 cDNA library prepared from mRNAs isolated from MCMV-infected mouse embryo fibroblasts (MEFs) at 24 h after infection (Dallas et al., 1994). An insert from a positive clone (initially designated TS9) was amplified by PCR (polymerase chain reaction) and subcloned into pGEM11-ZF(+) (Fig. 1). A comparison of the 420-bp cDNA sequence with the complete sequence of the Smith strain of MCMV (Rawlinson et al., 1996) indicated that the transcript encoding this cDNA is spliced. The first 162 nucleotides of the transcript represent nucleotides 103,999–104,160 of the published MCMV sequence, a region that encodes MCMV M73 (gN), and the following 215 nucleotides represent nucleotides 105,879–106,093 followed by a 43-bp poly(A) tail. The transcript has a combined stop/polyadenylation signal at nucleotides 106,068–106,073. This transcript contains a 252-bp open-reading frame (ORF) encoding a putative protein of 84 amino acid residues with a predicted molecular weight of 8.8 kDa (Fig. 1). A potential N-linked glycosylation site (NAT) is located at amino acid residues 74–76. Interest-ingly, the first 20 amino acid residues of this protein are shared with the M73-encoded gN molecule, and therefore this novel glycoprotein is designated gpM73.5. Several features suggest gpM73.5 is a type II integral membrane protein. First, it lacks a hydrophobic N-terminal signal peptide sequence, and second, a potential N-linked glycosylation site (NAT) is present in the C-terminal domain, downstream of a potential transmembrane region spanning amino acid residues 36–53 (VIMVAMLVLYRAIVGF).
Fig. 1. The DNA and deduced amino acid sequence of the M73.5 cDNA. The first G nucleotide after the splice site is identified by an arrow. The first 20 amino acids of the M73.5 protein sequence shared with M73 are shown in bold. The combined stop codon and polyadenylation signal is indicated by a dotted underline. The amino acid sequences of two purified tryptic digest peptides derived from gp24 immunoprecipitated from MCMV-infected fibroblasts with MAb 6A1.21A are underlined. 

Transcription analysis of the M72–M75 region

Northern blot analyses

To investigate the size and kinetics of expression of the transcript from which the M73.5 cDNA was derived, Northern blot analysis was undertaken using total RNA isolated from uninfected MEFs and MCMV-infected MEFs at 4, 8, 16, 20, or 24 h (hr) post-infection (p.i.). RNA was also extracted from MEFs infected with MCMV in the presence of 250 μg/mL of PAA (phosphonoacetic acid) at 24 h p.i. RNA was run on 1.4% agarose gels following glyoxylation of samples. The Northern blot was sequentially probed with the full-length M73.5 cDNA probe, and with a series of probes specific for M72, M73, m74, exon 2 of the M73.5 ORF, and M75. The location of these probes in the MCMV genome is depicted in Fig. 2A. The blot was also probed with a M55 (gB) probe, which is a true late gene, and finally a mouse β-actin probe, to verify equivalent RNA loading.

Fig. 2B shows that the M73.5 cDNA probe detects a 0.85-kb transcript at 4 h p.i. (lane 2). At 8 h p.i., 0.75–0.9 and 1.1–1.2-kb transcripts were detected (lane 3). At 16 h p.i., the level of expression of these transcripts increased and additional transcripts were detected. These included mRNAs with discrete sizes of 3.8, 3.1, 2.6, 2.1, and 1.7 kb, and a transcript (or transcripts) ranging in size from 0.45 to 0.6 kb (lane 4). At later time points, all of these transcripts were still present, and there was also abundant expression of the 0.75–0.9- and 0.45–0.6-kb transcripts, as well as a smaller 0.3–0.4-kb transcript (lanes 5 and 6). Both the 0.75–0.9- and 1.1–1.2-kb transcripts were weakly detected in cells cultured in the presence of PAA (lane 7), suggesting that these transcripts may be leaky late transcripts with predominant expression at late time points. When the M72-specific probe was used, only the 3.8-, 2.6-, and 1.7-kb transcripts were detected (Fig. 2C). Use of a probe specific for the M73 ORF presented a pattern of transcription essentially identical to that with the full-length M73.5 cDNA probe (Fig. 2D), with the exception that the 0.3–0.4-kb transcript was absent. Other than the presence of a 3.1-kb transcript at 20 h p.i., a similar pattern of expression to that observed for the M72 probe was obtained with the m74-specific probe (Fig. 2E). The M73.5 exon-2-specific probe yielded an expression pattern essentially identical to that obtained with the full-length M73.5 cDNA probe (Fig. 2F), with the exception that the 1.7- and 2.6-kb transcripts were not detected. Using a probe specific for M75 (gH), only the 3.8-kb band was detected (data not shown). Probing of the blot with a 907-bp probe for M55 (gB), which is a true late gene, revealed increasing expression commencing from 16 h post-infection, and transcription was completely blocked in the presence of PAA (Fig. 2G). Finally, probing of the blot with a mouse β-actin probe confirms that essentially equal amounts of RNA sample were loaded in each lane (Fig. 2H).

These data indicate that the 0.45–0.6-, 0.75–0.9-, and 1.1–1.2-kb transcripts are likely to be spliced, with introns spanning the m74 ORF and exons present in the M73 region (exon 1) as well as the intergenic region between m74 and M75 (exon 2). The 1.7- and 2.6-kb transcripts span the region from M72 to m74, and the 3.8-kb transcript spans the region from M72 to M75. Lastly, the 3.1-kb transcript spans the region covered by the M73.5 cDNA, m74, M73.5 exon 2, and M73 ORF probes.

RACE-PCR and RT-PCR analyses

To gain further insights into the genomic organization of the abundantly expressed spliced transcripts, we initially performed 5’-RACE (rapid amplification of cDNA ends) PCR to define the 5’ ends of these transcripts using primers specific for the exon 2 sequence of the M73.5 cDNA and total RNA isolated at 24 h post-infection. The M73.5 specific primer TS9-Not1-Rev (see Table 1) was used for priming reverse transcription and then the TS91 and TS94 primers were used for primary and nested PCR, respectively. Fig. 3A (lane 2) shows that 2 major 5’-RACE PCR products approximately 0.3 and 0.65 kb in size were obtained, with a faint band also present at approximately 0.2 kb. These products were gel-purified, cloned into pGEM-T, and their nucleotide sequences were determined. Nucleotide sequences of uncloned, gel-purified PCR products were also determined.

The 300-bp fragment represents part of a spliced transcript in which the first exon spans nucleotides 103,985–104,160 and the exon 2 fragment spans nucleotides 105,879–106,093, terminating at the binding site for probe 20.
the primer TS94. This is consistent with a M73.5 transcript that initiates at position 103,985 with splice donor and acceptor sites at 104,160 and 105,879, respectively. The larger 650-bp fragment corresponds to a spliced transcript with exon 1 spanning nucleotides 103,985–104,548 and exon 2 spanning nucleotides 105,879 to 106,093. Inspection of the sequence of this 5’-RACE PCR product indicates that the M73 (gN) ORF is present in its entirety within exon 1 of this transcript, suggesting that the MCMV M73 gene encoding gN is also spliced. Thus, like the M73.5 transcript, the M73 transcript also initiates at position 103,985, but has a different splice donor site at 104,548. Finally, a plasmid clone corresponding to the faint band of approximately 200 bp was sequenced and found to represent a spliced mRNA with exon 1 spanning nucleotides 94,984–95,063, and exon 2 spanning nucleotides 105,879 to 106,093. Thus, this transcript likely initiates at nucleotide 94,984 near the MCMV origin of lytic replication, and has an intron of over 10.8 kb. As no other genes have been designated for the region between m59 and M69 (Rawlinson et al., 1996), this putative MCMV transcript has been designated m60. The putative m60 protein comprises eight amino acids encoded within exon 1 and 64 amino acids from exon 2, which are shared with M73.5.
Table 1

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Primer sequence (5’–3’)</th>
<th>Genomic coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>M72-Seq5</td>
<td>AGCTGGGCTGCGGACGTCG</td>
<td>103,893–103,875 (C)</td>
</tr>
<tr>
<td>M72-Seq8</td>
<td>GCGCCTGCTCCCCACCTTG</td>
<td>103,766–103,784</td>
</tr>
<tr>
<td>M73A-EcoRV-For</td>
<td>ccacgatattCTCTGCGGACGAC</td>
<td>104,096–104,116</td>
</tr>
<tr>
<td>M73ForBamHI</td>
<td>tagtagCTCATAGGCGGGAAGGC</td>
<td>104,096–104,116</td>
</tr>
<tr>
<td>M73A-Not1-Rev</td>
<td>ccctgtgcgccGGCTCAATATTC</td>
<td>104,523–104,501 (C)</td>
</tr>
<tr>
<td>M73AreElTag</td>
<td>agttgatattATATCTCTTAGCT</td>
<td>104,516–104,499 (C)</td>
</tr>
<tr>
<td>M73B</td>
<td>GAGGATCGGTGAGCACCCCGCA</td>
<td>105,881–105,900</td>
</tr>
<tr>
<td>TS9-EcoRV-For</td>
<td>cagttgatattATATCTCTAGCT</td>
<td>105,881–105,900</td>
</tr>
<tr>
<td>TS9-Not1-Rev</td>
<td>cctgtgcgccCTCTTAGCTCAA</td>
<td>106,075–106,055 (C)</td>
</tr>
<tr>
<td>M73CreElTag</td>
<td>agttgatattATATCTCTAGCT</td>
<td>106,069–106,049 (C)</td>
</tr>
<tr>
<td>M73D-Ori-FBH1</td>
<td>tagagactCTATGGGCGGAGAC</td>
<td>95,036–95,058</td>
</tr>
<tr>
<td>Ori-TS9-For1</td>
<td>CAGGCTGAACCCATGCATCG</td>
<td>95,984–95,003</td>
</tr>
<tr>
<td>TS91</td>
<td>CATCTTGAATCCGAGCTGGTC</td>
<td>105,983–105,960 (C)</td>
</tr>
<tr>
<td>TS94</td>
<td>CTGGTGACACACCCACACATC</td>
<td>105,962–105,943 (C)</td>
</tr>
</tbody>
</table>

a MCMV-derived sequences are shown in upper case. Underlined sequences denote restriction sites introduced into the oligonucleotide.

b The genomic coordinates are based on the published nucleotide positions given for the Smith strain of MCMV (Rawlinson et al., 1996). C = complementary strand.

To define the 3’ ends of the spliced transcripts that apparently share exon sequences in the m74-m75 intergenic region, 3’-RACE PCR was performed using the M73A-EcoRV-For or Ori-TS9-For1 primers (see Table 1) together with an oligo-dT17-adaptor primer. With the M73A-EcoRV-For/oligo-dT17-adaptor primer combination, PCR products migrating at approximately 300 and 700 bp were obtained (Fig. 4A, lane 14). These PCR products were again cloned into the pGEM-T vector and sequenced. The 300-bp fragment is likely derived from the spliced M73.5 transcript. It spans the exon 1 sequence between the primer-binding site at 104,096 and the splice donor site at 104,160, continues through the entire exon 2 sequence (nucleotides 105,879–106,093), and ends with a poly(A) tail. The larger 700-bp fragment is likely derived from the spliced M73.5 transcript. It spans the exon 1 sequence between the primer-binding site at 104,096 and the splice donor site at 104,160, continues through the entire exon 2 sequence (nucleotides 105,879–106,093), and ends with a poly(A) tail. 3’-RACE PCR performed with the Ori-TS9-For1 primer and oligo-dT17-adaptor generated PCR products of approximately 300, 700, and 800 bp (Fig. 4B, lane 2). Therefore, our analysis thus far suggests that the 0.3–0.4, 0.45–0.6, and 0.75–0.9 kb spliced transcripts detected by Northern analysis likely correspond to mRNAs encoding the m60, M73.5, and M73 proteins, respectively.

Finally, the 1.1–1.2-kb transcript detected by Northern analysis is likely to be spliced, and may represent mRNAs with alternate initiation sites further upstream. Thus, additional 5’-RACE PCR analyses were performed with primers upstream of the primary initiation site at 103,985. The TS9-Not1-For primer (see Table 1) was used for priming the reverse transcription. Then, the TS91 and M72-Seq5 primers (Table 1) were used for primary and nested PCR, respectively, yielding a nested 5’-RACE PCR product of 200 bp (Fig. 3B, lane 2). Sequencing of this product indicated that it initiated from 103,700. To verify that mRNAs initiating at 103,700 also extend through the M73/M73.5 exon 2 sequences, RT-PCR and 3’-RACE PCR were performed using the M73A-EcoRV-For and TS9-Not1-Rev primers, three PCR products were obtained, with the 300- and 700-bp products corresponding to the M73.5 and M73 transcripts, respectively (Fig. 4A, lane 10). The 600-bp product corresponded to a nonspecifically amplified mouse sequence. RT-PCR with Ori-TS9-For1 and TS9-Not1-Rev primers yielded a single product of approximately 300 bp corresponding to the m60 transcript (Fig. 4B, lane 2).
In summary, data from the 5′- and 3′-RACE PCR and RT-PCR experiments indicate that transcription from the M72-M75 region is complex and supports the existence of at least 5 novel 3′ co-terminal spliced MCMV transcripts that share the same splice acceptor sites, but differ in their 5′-exons, splice donor sites, and transcription initiation sites. The nucleotide sequence coordinates of each of these characterized transcripts are summarized in Fig. 2A.

Characterization of the proteins encoded by the MCMV spliced transcripts

The detection of proteins encoded by these spliced MCMV transcripts was facilitated initially by the construction of expression plasmids in which c-myc tags were attached to the C-termini of each of the three putative ORFs (m60, M73/gN, and M73.5). Each plasmid construct was transfected into COS-7 cells and protein expression monitored by Western blotting using the anti-c-myc monoclonal antibody 9E10. Fig. 5A shows that the M73 (lane 3), M73.5 (lane 2), and m60 (lane 4) gene products were expressed as approximately 15, 14, and 8 kDa proteins, respectively. Furthermore, a 16-kDa protein and a series of fainter 22–26-kDa bands were also detected in cells transfected with the gpM73.5 expressing plasmid (lane 2). The fact that hyperimmune serum to MCMV detected the 14- and 15-kDa proteins (Fig. 5B, lanes 2 and 3), indicated that the M73.5 and M73 gene products are expressed as immunogenic proteins during virus infection in vivo.

The subcellular locations of these proteins were initially determined by confocal microscopy in COS-7 cells trans-

Fig. 3. Characterization of the M73 family of spliced transcripts by 5′-RACE PCR analysis. Mapping of 5′-ends of transcripts by 5′-RACE PCR was performed on total RNA isolated from MCMV-infected MEFs at 24 h p.i. Primary 5′-RACE PCR was performed with gene-specific primer TS91 (see Table 1) and nested PCR was performed using the gene specific primers TS94 (A) and M72-Seq5 (B) on first-strand cDNA generated plus (lane 2) or minus (lane 3) reverse transcriptase during the initial reverse transcription step.

Fig. 4. Characterization of the M73 family of spliced transcripts by RT-PCR and 3′-RACE PCR analysis. RT-PCR and 3′-RACE PCR were performed on first-strand cDNA prepared from poly(A)+ RNA isolated from MEF infected for 24 h. RT-PCR analysis was performed on first-strand cDNA using the primer combinations M72-Seq8 (A, lanes 2–5), M73A-EcoRV-For (A, lanes 10–13), or Ori-TS9-For1 (B, lanes 2–4) and TS9-Not1-Rev. RT-PCR was performed on templates prepared with (A, lanes 2 and 10; B, lane 2) or without (A, lanes 3 and 11; B, lane 3) reverse transcriptase during the initial reverse transcription step. Water alone controls are shown (A, lanes 5 and 13; B, lane 4). Control PCR on MCMV genomic DNA was performed with M72-Seq8 (A, lane 4), or M73A-EcoRV-For (A, lane 12) and TS9-Not1-Rev. 3′-RACE PCR mapping was performed on first-strand cDNA using the primer combinations M72-Seq8 (A, lanes 6–8), M73A-EcoRV-For (A, lanes 14–16), or Ori-TS9-For1 (B, lanes 5–7) and (dT)17-adaptor primer. PCR was performed on templates with (A, lanes 6 and 14; B, lane 5) or without (A, lanes 7 and 15; B, lane 6) reverse transcriptase during the first-strand cDNA synthesis. Water alone controls are shown (A, lanes 8 and 16; B, lane 7).
fected with expression plasmids (described in the previous paragraph) because of the high transfection efficiency in these cells (Fig. 6A). All three c-myc-tagged fusion proteins (m60, M73, and M73.5) were detected within the cytoplasm of permeabilized, transfected cells with the anti-c-myc MAb 9E10 (Figs. 6b–d). Interestingly, the M73.5 and m60 proteins (Figs. 6f–g), but not the M73 protein (Fig. 6h), could be detected on the cell surface of nonpermeabilized, transfected COS-7 cells. As the MAb 9E10 recognizes the c-myc tag at the C-termini of the m60 and M73.5 fusion proteins, their detection on the cell surface of nonpermeabilized cells supports the concept that they are type II glycoproteins.

To confirm our findings in cells that are normally permissive for MCMV replication, we carried out similar experiments in Balb/3T3 fibroblasts (Fig. 6B). In addition, cells were infected with MCMV 6 h before they were processed for immunofluorescence. Thus, detection of the M112-113 gene products, a family of early proteins (also known as E1 proteins) localized exclusively to the nuclei of infected cells (Bühler et al., 1990; Ciocco-Schmitt et al., 2002), could be used as an internal control for the permeability of transfected cells. Meanwhile, the m60, M73, and M73.5 proteins detected in these cells could only be derived from the transfected cDNA because late genes are not expected to be expressed at this stage of the MCMV infection. As shown in Fig. 6B, the c-myc-tagged m60 and M73.5 fusion proteins were detected by MAb 9E10 on the cell surface (panels b and c) of nonpermeabilized cells as well as the cytoplasm of permeabilized cells (panels f and g). While the characteristic nuclear fluorescence of E1 proteins in permeabilized cells (panels e to i, k) was readily detectable by rabbit antibodies raised against these proteins, the inability of the antisera to detect these nuclear proteins in nonpermeabilized cells (panels a to d, j) demonstrated convincingly that the c-myc tag at the C-terminus of the fusion proteins detected by 9E10 must indeed be present on the surface of these cells, thereby confirming our previous conclusion that both m60 and M73.5 encode type II glycoproteins. The detection of the c-myc-tagged M73 protein on the cell surface of transfected Balb/3T3 cells by 9E10 (Fig. 6B, panel d) would seem to imply that M73 encodes a type II glycoprotein and the expression/topology of the protein may differ between Balb/3T3 and COS-7 cells. However, we felt that this conclusion is premature because of the small number of cells positive for 9E10 as a result of the low efficiency of transfection in Balb/3T3 cells and the lack of additional evidence on the subcellular location of wild type M73 expressed during MCMV infection.

To better characterize these related gene products, a MAb designated CF24 (IgG2a isotype) was specifically raised against gpM73.5 by immunizing C57BL/6J mice with the M73.5-c-Myc fusion protein. The specificity of this MAb was assessed by Western blot analysis of lysates of COS-7 cells transfected with plasmids expressing the c-myc-tagged M73.5, M73, or m60 proteins, or untagged gpM73.5 (Fig. 5C). Monoclonal antibody CF24 showed strong reactivity against both the c-myc-tagged and unmodified M73.5 proteins (lanes 2 and 5, respectively) with very weak cross-reactivity against the M73 protein (lane 3), and no reactivity against the m60 protein (lane 4). The lack of reactivity of MAb CF24 with m60 and its very weak cross-reactivity with M73 suggest that CF24 may recognize an epitope spanning the exon 1/2 boundary of M73.5. It is interesting to note that the MAb CF24 detected a broad 22–26-kDa band rather than a series of distinct bands in that size range when unmodified M73.5 was transiently expressed (compare lanes 2 and 5), suggesting that the addition of the C-terminal c-myc tag not only increases the sizes of the M73.5 gene products, but may also affect the
glycosylation process in COS-7 cells. Finally, CF24 also reacts with M73.5 proteins in MCMV-infected cells, detecting a 14-kDa band and a broad 22–28-kDa band (Fig. 5D, lanes 2 and 3).

The M73.5 gene encodes the gp24 virion glycoprotein

The characteristics of the M73.5-encoded gene products are reminiscent of a previously described MCMV virion...
protein, gp24, defined by reactivity with MAb 6A1.21A (Loh, 1989). For example, gp24 was found in virus-infected cells as an 18.4-kDa precursor that matured into a 22–26-kDa glycoprotein which was incorporated into MCMV virions. The gp24 protein was also found on the surface of virus-infected cells and possessed at least one N-linked glycosylation site (Loh, 1989; Loh et al., 1991). To investigate whether M73.5 encodes the gp24 glycoprotein, the amino acid sequence of the latter was determined.

The gp24 glycoprotein and its 18.4-kDa precursor were immunoprecipitated from MCMV-infected fibroblasts with MAB 6A1.21A and purified on a 12% SDS-polyacrylamide gel as described in Materials and methods. As gp24 co-migrated with the light chain of the precipitating antibody, only the 18.4-kDa band was excised from the gel and subjected to tryptic digestion in gel as described in Materials and methods. As gp24 co-immunoprecipitated with the precipitating antibody, the amino acid sequence of the latter was determined.

M73.5 encodes the gp24 MCMV virion glycoprotein. 3T3-L1 fibroblasts infected with MCMV (lanes 1, 3, and 4) or COS-1 cells transfected with the plasmid pSV-gp24 (lanes 2 and 5) were radiolabeled with [35S]methionine, solubilized in immunoprecipitation buffer and precipitated with either MAb 8B6.21A (lanes 1 and 2) or MAb 6A1.21A (lanes 3–5) as described in Materials and methods. The precipitated proteins were either digested with Endoglycosidase F (lanes 4 and 5) or left untreated (lanes 1–3) before separation on a 12% SDS-polyacrylamide gel and visualization by autoradiography. The numbers on the left represent the sizes of molecular weight markers. The abbreviations above each lane indicate whether the proteins were derived from transfected (Tf) or infected (Inf) cells.

Perhaps the addition of N-linked carbohydrates to gp24/gpM73.5 is inefficient in COS-1 cells in the absence of virus infection. Finally, minor differences in size estimates between Figs. 5 and 7 may be accounted for by the use of different molecular weight markers and/or types of polyacrylamide gels.

As M73.5 and m60 share exon 2 sequences, we investigated if the gpM73.5-specific MAB 6A1.21A was able to recognize the m60 fusion protein expressed in permeabilized, MCMV-infected, pcDNAtag-m60-transfected cells (Fig. 6B, panel i). The reactivity of 6A1.21A with these cells indicates that the epitope recognized by this MAB is located at the C-terminal half of the m60 and M73.5 proteins. Furthermore, the reactivity of 6A1.21A on unpermeabilized, infected cells (Fig. 6B, panel j) suggested that wild-type gpM73.5/gpM73.5 synthesized during MCMV infection likely reflects their type II glycoprotein configuration, and the presence of the c-myctag in the fusion proteins did not affect their topology. Uninfected Balb/3T3 cells showed no reactivity with 6A1.21A (data not shown).

Genetic polymorphisms in M73 and M73.5

Previous sequence analyses of the HCMV UL73 ORF have revealed that this protein appears to be highly variable among clinical isolates (Dal Monte et al., 2001; Pignatelli et al., 2001; 2003), suggesting that either the UL73-encoded gN molecule is subjected to substantial immunological pressure for the selection of variant sequences or that these gN variants may have distinct functions. To determine whether the MCMV M73 (gN) homolog and the M73.5
protein are equally highly polymorphic, we performed sequence analyses of these genes from a panel of MCMV field isolates (Booth et al., 1993). The M73 ORF and M73.5 exon 2 sequences were amplified by PCR and pooled PCR products from replicate PCR reactions were subjected to sequence analysis. Fig. 8A shows that in a subset of approximately one-third of the field isolates tested, the M73 gene exhibited a modest degree of polymorphism from the laboratory strain K181 in the central portion of the protein. However, the extent of variation was less than that for HCMV UL73 and does not support the notion of extensive M73 genotypes as observed for HCMV UL73. Unlike M73, the exon 2 region amino acid sequence of the M73.5 protein is very highly conserved (Fig. 8B). A subset of 5 of only the 18 field isolates tested exhibited an additional glycine residue at position 80 of the protein sequence.

The M73.5/m60 ORF encoded within exon 2 is conserved among betaherpesviruses

The existence of spliced M73.5 and m60 genes had not been predicted previously during analysis of the complete MCMV sequence (Rawlinson et al., 1996). Hence, it is of considerable interest to determine whether protein coding sequences similar to M73.5 exist in other herpesviruses. To this end, the protein sequence of M73.5 was used to search nucleotide databases using the TBLASTN algorithm (Altschul et al., 1990). Significant homologies exist between the protein sequence of M73.5/m60 encoded within exon 2 and translated protein sequences present in all betaherpesviruses that have been sequenced to date (Fig. 9). The level of sequence identity varied from 30% to 46%. Furthermore, these deduced protein sequences are all conserved in their location between betaherpesvirus homologs of m74 (gO) and M75 (gH). However, similar homologous sequences were not identified in any alphaherpesviruses or gammaherpesviruses. Thus, our data suggest the existence of a hitherto unidentified betaherpesvirus-specific glycoprotein.

A number of other striking similarities are present between the MCMV M73.5 gene exon 2 coding sequence and other homologous betaherpesvirus sequences. First, all of the betaherpesvirus M73.5 ‘exon 2’ sequence homologs are bounded at their 5' ends by potential splice acceptor sites (Senapathy et al., 1990) (Table 2). Second, the sequences are flanked at the 3' ends by consensus polyadenylation signals that overlap, or are near, the putative stop codons (Table 2). Third, the predicted amino acid sequences exhibit remarkably similar hydrophilicity profiles, characterized by conserved hydrophobic stretches, consistent with membrane spanning domains, followed by a relatively hydrophilic C-terminal tail (data not shown). In addition, a highly conserved potential fusogenic domain is present in the M73.5 sequence.
conserved arginine residue corresponding to residue 48 in MCMV M73.5 is located within the putative membrane-spanning domain (Fig. 9). Finally, each sequence contains at least one consensus N-linked glycosylation site (N-X-S/T) in the hydrophilic C-terminal region of the putative gene product (Fig. 9). Overall, these similarities strongly suggest that all betaherpesviruses encode spliced gene products homologous to M73.5, which may play an important role in the betaherpesvirus life cycle.

**Discussion**

The replication of herpesviruses in host cells is dependent on suites of proteins that are conserved among all herpesviruses. However, there are proteins that are only expressed in certain herpesvirus subfamilies and these play critical roles in the replication strategies of viruses of that particular subfamily. In this study, we have analyzed a family of spliced transcripts expressed in murine cytomegalovirus. Two of these spliced mRNAs (M73) encode gN, which is conserved in all herpesviruses (Davison, 2002), whereas the other spliced transcripts (M73.5 and m60) encode proteins that appear to share strong sequence homologies to a putative glycoprotein species present in all betaherpesviruses that have been sequenced to date. Furthermore, we have shown the M73.5 gene encodes the structural viral glycoprotein gp24 recognized by the neutralizing antibody 6A1.21A (Loh, 1989).

Northern blot analysis utilizing probes spanning the region M72–M75 indicates that this region is transcriptionally complex. All probes detected a 3.8-kb transcript, and probes from M72 to m74 detected a 2.6-kb transcript. These transcripts possibly correspond to the 5.0- and 3.1-kb transcripts reported previously (Rapp et al., 1994) using probes specific for gH (M75) and dUTPase (M72). Rapp et al. (1994) concluded that the 5.0-kb transcript corresponds to the gH transcript that terminates at a polyA signal downstream of dUTPase (M72). The 2.6-kb transcript may represent a polycistronic mRNA encompassing the M72 and m74 ORFs, which are encoded on the complementary strand (Rawlinson et al., 1996). The precise origin of the 1.7-kb late transcript was not defined, but this was detected with probes spanning from

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**Table 2**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Splice acceptor</th>
<th>Location</th>
<th>Stop codon</th>
<th>Stop codon Location</th>
<th>PolyA signal (AATAAA) Location</th>
<th>Accession number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>THV</td>
<td>CTCTTCTGTCTCTCGCAG</td>
<td>120,538–120,557</td>
<td>TAA</td>
<td>120,744–120,746</td>
<td>120,792–120,797</td>
<td>AF281817</td>
<td>Bahr and Darai, 2001</td>
</tr>
<tr>
<td>GCMV</td>
<td>TCTCTTCCTTTTGTAG</td>
<td>2730–2731a</td>
<td>TAG</td>
<td>2719–2717b</td>
<td>2459–2454b</td>
<td>U49361</td>
<td>Brady and Schleiss, 1996</td>
</tr>
<tr>
<td>RhCMV</td>
<td>TGGCTCCCCCCTACG</td>
<td>98,726–98,745</td>
<td>TGA</td>
<td>98,973–98,975</td>
<td>98,975–98,980</td>
<td>YA168194</td>
<td>Hansen et al., 2003</td>
</tr>
<tr>
<td>HCMV</td>
<td>TTCTCTTCCTACGAG</td>
<td>107,514–107,533</td>
<td>TAA</td>
<td>107,727–107,729</td>
<td>107,727–107,734</td>
<td>X17403</td>
<td>Chee et al., 1990</td>
</tr>
<tr>
<td>HHV6</td>
<td>GTATTTTCTTTTTATAG</td>
<td>77,823–77,842</td>
<td>TAA</td>
<td>78,013–78,015</td>
<td>78,021–78,026</td>
<td>X83413</td>
<td>Gompels et al., 1995</td>
</tr>
<tr>
<td>HHV7</td>
<td>TATTCATTTCCTACGAG</td>
<td>74,845–74,864</td>
<td>TAA</td>
<td>75,036–75,038</td>
<td>75,183–75,188</td>
<td>AF037218</td>
<td>Megaw et al., 1996</td>
</tr>
</tbody>
</table>

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A consensus splice acceptor sequence is NTNYYYYYYYYYYYNACGG (Senapathy et al., 1990).
M72 through to the intergenic region between m74 and M75.

The Northern blot analyses also revealed that probes specific for M73 and the intergenic region between m74 and M75 detected a 1.1-kb leaky late transcript in addition to the abundant 0.45–0.6- and 0.75–0.9-kb transcripts. The 5′-RACE PCR and 3′-RACE PCR analyses performed indicate that the 1.1-kb transcript is a longer version of the M73 transcript that initiates from an upstream site. The RACE-PCR data indicate that a longer version of the M73.5 transcript also initiates from the upstream initiation site at 103,700. Both the upstream (103,700) and downstream (103,985) transcription sites have potential TATA box and cap signal elements. The expression of the MCMV M73 (gN) gene as a spliced transcript raises the question of cap signal elements. Studies conducted so far to characterize the (gN) gene as a spliced transcript raises the question of cap signal elements. The expression of the MCMV 103,700. Both the upstream (103,700) and downstream transcription also initiates from the upstream initiation site at M73.5.

PCR data indicate that a longer version of the transcript that initiates from an upstream site. The RACE-2002; Shiba et al., 2000). and Muggeridge, 1998; Cohen et al., 2001; Koshizuka et al., 2001, 2003). The lower level of variability in MCMV M73 may mean that the MCMV gN glycoprotein is not subjected to the same selective pressures as HCMV UL73. Similarly, the strong sequence conservation of the gpM73.5 protein among MCMV strains implies that either variation in this protein is constrained due to structure or function, or that it is not subjected to extensive immunological pressure from neutralizing antibodies. However, as the MCMV M73.5 cDNA was originally identified by screening of a cDNA expression library with anti-MCMV hyperimmune serum, this indicates the M73.5 protein is immunogenic during viral infection. Furthermore, as the 6A1.21A MAb is a neutralizing antibody, this suggests that gpM73.5/gp24 should be subjected to immunological pressure.

As shown in this study, MCMV possesses a family of spliced 3′-co-terminal transcripts that share a common exon 2 sequence in the intergenic region between m74 and M75. The translated exon 2 coding sequence of the M73.5/m60 transcripts is highly conserved among all of the betaherpesviruses sequenced to date. Further analyses of the role of the M73.5/m60 proteins in MCMV replication by disruption mutagenesis, together with the site-directed mutagenesis of the conserved residues and motifs such as the charged residue in the transmembrane domain of this protein, or the unique N-linked glycosylation site, will help us understand the role of this conserved protein(s) during betaherpesviral replication.

Materials and methods

Cells

Primary mouse embryo fibroblasts (MEFs) were prepared by trypsin dispersion of 15–17-day-old embryos from outbred ARC(S) mice (Animal Resources Centre, Murdoch,
Western Australia) as described previously (Chalmer et al., 1997). MEFs were cultured in minimal essential medium (MEM; Invitrogen Australia) supplemented with 10% fetal calf serum (FCS; Invitrogen), 100 U/mL penicillin, and 40 \( \mu \text{g/mL} \) gentamicin. COS-7, COS-1, mouse 3T3-L1, and Balb/3T3 cells were cultured in Dulbecco’s MEM (DMEM, Invitrogen) supplemented with 10% FCS, 100 U/mL penicillin, and 40 \( \mu \text{g/mL} \) gentamicin. For transfection of COS-7 cells, OPTI-MEM 1 was used as the culture medium (Invitrogen). Hybridomas were grown in RPMI-1640 (Invitrogen) supplemented with 10% FCS, 100 U/mL penicillin, and 40 \( \mu \text{g/mL} \) gentamicin.

Viruses and polyclonal antisera

Viruses used in this study were the K181, Smith, and Vancouver laboratory strains of MCMV, together with a panel of strains isolated from wild mice (Booth et al., 1993). These were the G2, G3C, G4, G5, G6, K4, K7, K10, K17A, K17B, K29, N1, W2, W3, W4, W5, W6, W7, W8, W9, W9077, and WE6 strains. Tissue culture passaged stocks were propagated by in vitro infection of MEFs at a multiplicity of infection (moi) of 0.1 and harvested when cultures showed 100% cytopathic effect. Virus stocks were quantified by standard plaque assay on MEFs (Scalzo et al., 1990). The preparation of a hyperimmune MCMV-specific antiserum was described previously (Dallas et al., 1994). The preparation of a hyperimmune MCMV-specific polyclonal antiserum to MCMV was as described previously (Dallas et al., 1994).

Screening of the MCMV cDNA library and subcloning

The preparation of a \( \lambda \)gt11 cDNA library from poly(A)\(^+\) RNA from MCMV-infected cells at 24 h post-infection and screening for MCMV DNA positive cDNA clones has been described previously (Dallas et al., 1994). MCMV DNA positive lambda clones were spotted onto lawns of \textit{Escherichia coli} Y1090 cells and screened with the polyclonal hyperimmune MCMV-specific antiserum essentially as described (Huyhn et al., 1985). Positive clones were amplified by PCR (Rasmussen et al., 1989) using \( \lambda \)gt11 forward and reverse primers (Promega) and then cloned into the pGEM11-Zf(+) plasmid vector.

DNA sequencing and analysis

DNA sequencing was performed on purified plasmid DNA or on purified PCR products using the Applied Biosystems Big-Dye version 3.0 or 3.1 sequencing kit according to the manufacturer’s instructions. In all cases, DNA sequences were determined on both strands. Overlapping sequence contigs were assembled using Vector NTI v.5.3.0 (InforMax Inc., Bethesda, MD). Sequence data were analyzed using the GCG suite of programs on WebANGIS (http://www.angis.org.au/WebANGIS). Predicted protein sequences were compared to nucleotide sequence databases using the TBLASTN algorithm (Altschul et al., 1997).

Preparation of RNA

Total RNA was isolated from MEFs infected with K181 MCMV strain at a moi of 5. Total RNA was extracted using RNAwiz reagent (Ambion, Austin, TX) at 4, 8, 16, 20, and 24 h post-infection. Total RNA was also extracted from uninfected MEFs and from flasks of MEFs infected for 24 h in the presence of 250 \( \mu \text{g/mL} \) of phosphonoacetic acid, an inhibitor of DNA synthesis. Total RNA was treated with DNA-Free reagent (Ambion) to remove contaminating DNA. Poly(A)\(^+\)-selected RNA was prepared from MCMV-infected MEFs at 24 h p.i. using the Poly(A)Pure kit (Ambion).

Northern hybridizations

Total RNA samples (10 \( \mu \text{g} \)) were glyoxylated, electrophoresed on 1.4% agarose gels in 10 mM NaH\(_2\)PO\(_4\) buffer (Sambrook et al., 1989), and then transferred to Hybond XL membrane (Amersham). Membranes were prehybridized for 1 h in Church–Gilbert buffer at 65 °C, before overnight hybridization at 65 °C with \( ^{32}\text{P} \)-labeled DNA probes in fresh buffer. DNA probes were labeled with [\( ^{32}\text{P} \)]-dATP by random priming according to the manufacturer’s instructions (DECAprime II, Ambion). After hybridization blots were washed at 65 °C once for 15 min with 5 \( \times \) SSC buffer/0.1% SDS, two times with 2 \( \times \) SSC buffer/0.1% SDS, and once with 0.1 \( \times \) SSC buffer/0.1% SDS followed by autoradiography.

RT-PCR and 5’ and 3’ RACE-PCR analyses

RT-PCR was performed on poly(A)\(^+\) RNA prepared from MEFs at 24 h after MCMV infection. Following first-strand cDNA synthesis with oligo-dT\(_{17}\)-adaptor primer [5’-GACTCGACGTCGACATCGA-(T)\(_{17}\)-3’ (Frohman et al., 1988)], RT-PCR was performed with either the M73A-EcoRV-For, M72-Seq8, or Ori-TS9-For1 forward primers and the TS9-Not1-Rev primer (see Table 1). PCR products were gel purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA) and then sequenced.

To map the 5’-ends of transcripts, 5’-RACE PCR was performed according to the manufacturer’s instructions using the 5’-RACE system, Version 2 (Invitrogen, Carlsbad, CA) and total RNA derived from MCMV-infected MEFs at 24 h p.i. The initial reverse transcription step was performed using the TS9-Not1-Rev primer (see Table 1). The primary and nested PCR reactions were performed using the TS91 and TS94, or TS91 and M72-Seq5 gene-specific primers, respectively. 5’-RACE PCR products were gel-purified, cloned into pGEM-T (Promega) and then sequenced.

To map 3’-ends of transcripts, 3’-RACE PCR was performed essentially as described (Frohman et al., 1988) using the oligo-dT\(_{17}\)-adaptor primer for first-strand cDNA synthesis using poly(A)\(^+\) RNA. PCR was then performed using either the M73A-EcoRV-For, Ori-TS9-For1, or M72-Seq8 primers (see Table 1) and the oligo-dT\(_{17}\)-adaptor
primer. PCR products were gel purified and then sequenced, or cloned into pGEM-T and then sequenced.

DNA manipulations

To generate C-terminally c-myc-tagged proteins, a derivative of pcDNA-3 (Invitrogen) with a c-myc encoding sequence inserted at the EcoRI site was constructed. This was created by first annealing oligonucleotides 5'-AATTCCACGTAAGCAGCTGATATCCGAAGGAGCTTGAACCT-3' and 5'-AATTAGTTCAAGGCTCTCCTGAGATATCAGCTCTGCTACGGT-G-3'. The annealed oligonucleotide pair was then ligated into the EcoRI site of pcDNA-3. This vector, designated pcDNAtag, retains a single EcoRI site to facilitate directional cloning, as the site downstream of the stop codon after the c-myc sequence was disrupted in the oligonucleotide pair. Plasmid constructs expressing the M73, M73.5, and m60 ORFs in-frame with a C-terminal c-myc tag were constructed by first amplifying the respective ORFs by RT-PCR using the M73forBamHI and M73AreveITag, M73forBamHI and M73CrevE1Tag, and M37D-Ori-FBH1 and M73CrevE1Tag primer combinations, respectively (see Table 1). Following digestion of these PCR products with BamHI and EcoRI, they were ligated into BamHI and EcoRI-digested pcDNA-tag vector to give the appropriate constructs. The inserts were sequenced to verify that no sequence errors were introduced during PCR.

To construct the expression plasmid pSV-gp24, the 2.77-kb NruI–BglII fragment was excised from the EcoRI-P fragment of the MCMV (Vancouver strain) genome and inserted between the BamHI and BglII cloning sites of the plasmid vector pM1 (Sadowski et al., 1992). In particular, the BglII site on pM1 was filled in with the Klenow fragment of DNA polymerase I to facilitate ligation with the NruI end of the insert. Thus, the shared ATG translation start codon for the M73 and M73.5 ORFs was placed approximately 70 bp downstream from the SV40 early promoter, allowing the expression of both proteins. Exon 2 sequences can be removed from pSV-gp24 by digestion with XbaI followed by self-ligation. The sequence of the NruI–BglII fragment was verified by nucleotide sequencing and was identical to the published sequence for the Smith strain of MCMV (Rawlinson et al., 1996).

Transfections

For analysis of protein expression by Western blot, COS-7 cells were cultured in 75-cm² flasks. When just subconfluent, the cells were transfected with 5 μg of plasmid DNA in OPTI-MEM medium containing Fugene transfection reagent (Roche, Mannheim, Germany). Forty-eight hours after transfection, the cells were lysed [10 mM Tris, pH 7.5; 1% NP-40; 150 mM NaCl, 1 × Protease inhibitors (Roche)]. For immunofluorescence analyses, COS-7 cells (5 × 10⁶) were seeded onto glass coverslips and incubated overnight. The cells were then transfected with 0.5 μg of plasmid DNA in OPTI-MEM 1 medium and Fugene transfection reagent. Transfection of the expression plasmid pSV-gp24 into COS-7 cells by electroporation was carried out essentially as previously described (Loh et al., 1994). Finally, Lipofectamine Plus reagent (Invitrogen) was used for the transfection of Balb/3T3 fibroblasts with cDNA according to the manufacturer's instructions.

Anti-M73.5 monoclonal antibody production

To produce anti-M73.5 monoclonal antibody CF24 (IgG2a), C57BL/6J mice were injected two times by the intraperitoneal (i.p.) route with 5 × 10⁶ COS-7 cells transfected with the M73.5 c-myc-tagged construct together with 150 μL of MPL + TDM adjuvant (Sigma). A final boost of M73.5-c-myc COS-7-transfected cells plus MPL + TDM adjuvant was injected by the i.p. route. Three days later, splenocytes were removed and fused with X63 myeloma cells as described (Harlow and Lane, 1988) and positive clones identified by dot blot analysis.

Production of monoclonal antibodies against MCMV proteins was previously described (Loh and Qualtiere, 1988). In particular, while both MAb 6A1.21A (IgM) and the newly characterized MAb 8B6.21A (IgG3) recognized gp24 in immunoprecipitation and immunofluorescence assays, only 6A1.21A possessed neutralizing activity in the presence of complement (Loh, 1989).

Western blot analyses

Lysates from untransfected or transfected COS-7 cells were boiled in reducing or nonreducing sample buffers and then electrophoresed on NuPAGE Novex 4–12% Bis–Tris Gels (Invitrogen, Carlsbad, CA) at 200 V together with SeeBlue Plus2 Prestained standards (Invitrogen). Following electrophoresis, proteins were transferred to Trans-Blot nitrocellulose membrane (Bio-Rad, Hercules, CA) before incubation with the murine anti-c-myc MAb 9E10, or murine polyclonal anti-MCMV hyperimmune serum, or the anti-M73.5 MAb CF24, followed by alkaline phosphatase-conjugated goat anti-mouse Ig's (Biosource International, Camarillo, CA) and detection with BCIP and NBT substrates.

Immunofluorescence and confocal microscopy

Transfected or untransfected COS-7 cells on glass coverslips were fixed with 4% paraformaldehyde and either permeabilized by 0.1% Triton X100, or left unpermeabilized. Cells were incubated with the murine anti-c-myc monoclonal antibody (MAb) 9E10 or the murine polyclonal anti-MCMV hyperimmune serum, followed by incubation with AlexaFluor 488-conjugated Goat-anti-mouse Ig (Molecular Probes, Eugene, OR) and incubation with the nuclear stain Hoechst 33342. Cells were examined by
standard epifluorescence or by confocal microscopy (MRC 1000/1024 UV laser scanning confocal microscope; Bio-Rad, Hemel-Hempstead, U.K.). The program Confocal Assistant was used to generate merged images.

To complement the confocal microscopy experiments, Balb/3T3 cells grown on coverslips were processed for immunofluorescence 48 h after DNA transfection as previously described (Loh et al., 1999), with the exception that the Triton X-100 treatment was omitted when nonpermeabilized cells were required for monitoring surface expression of glycoproteins. Immunofluorescence images were captured digitally with a Zeiss Axioskop microscope with an epifluorescence attachment using the Northern Eclipse image analysis system and software (Empix Imaging, Inc.). When simultaneous visualization of two proteins under the fluorescence microscope was required, cells were first stained with rabbit and mouse antibodies specific for these proteins, and subsequently incubated with goat anti-rabbit and anti-mouse antibodies (Jackson Laboratories) conjugated to fluorescein isothiocyanate (FITC) or rhodamine, respectively. The two-color images (green and red) were superimposed and analyzed with the tools built into the public domain program Image J (available at http://rsb.info.nih.gov/ij). Final images were prepared for publication using Adobe Photoshop CS and Adobe Illustrator CS.

Radiolabeling, immunoprecipitation, and Endoglycosidase F digestion of viral glycoproteins

Mouse 3T3-L1 cells infected with the Smith strain of MCMV at a moi of 5 were radiolabeled with \([35S]\)methionine (60 Ci/mL, New England Nuclear) between 21 and 44 h post-infection in RPMI media (Invitrogen) containing one-tenth the normal amount of methionine. Similarly, COS-1 cells transfected with expression plasmids were radiolabeled with \([35S]\)methionine (60 μCi/mL) at 32 h post-transfection for 14 h. Cells were lysed at the end of the labeling period and the lysates immunoprecipitated with monoclonal antibodies as previously described (Loh and Qualtiere, 1988). When appropriate, the immunoprecipitates were digested with Endoglycosidase F (Genzyme Corp.) overnight to remove N-linked carbohydrates from the viral glycoproteins using established protocols (Loh, 1991). The immunoprecipitated proteins were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and visualized by autoradiography.

Sequencing of the gp24 glycoprotein

To obtain enough protein for sequencing, 3T3-L1 cells grown in four glass rolling bottles were infected with the Vancouver strain of MCMV. Cells were scraped off the bottle when virus production was at its peak, pelleted by low-speed centrifugation, and solubilized in 45 mL of immunoprecipitation buffer. The cell lysate was clarified by centrifugation at 13,000 rpm for 45 min in a refrigerated Beckman centrifuge and incubated with 50 μL of 6A1.21A-containing ascitic fluid or normal mouse serum overnight at 4 °C. Then gp24 and its precursor 18.4-kDa glycoprotein were precipitated by the addition of 100 μL of protein-L agarose beads (Sigma Chemical Co., Canada), electrophoresed on a 12% SDS-polyacrylamide gel, and stained with the Colloidal Blue staining kit (Invitrogen). The 18-kDa band was excised from the lane containing the 6A1.21A immunoprecipitate and sent to the Harvard University Microchemistry facility for sequence analysis. A similar area of the gel from an adjacent lane containing the normal mouse serum precipitate was also excised and analyzed for comparative purposes. The glycoprotein was digested with trypsin “in gel” and sequence analysis was performed at the Harvard facility by microcapillary reverse-phase HPLC (high performance liquid chromatography) nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Finnigan LCQ DECA quadrupole ion trap mass spectrometer. The fragmentation spectra were then correlated with known sequences using the algorithm Sequest developed at the University of Washington (Eng et al., 1994) and programs developed in the Harvard laboratory (Chittum et al., 1998).

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