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Temporal profiling of the coding and non-coding murine cytomegalovirus transcriptomes

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ABSTRACT:

The global transcriptional programme of murine cytomegalovirus (MCMV) involving coding, non-coding and anti-sense transcription remains to date unknown. Here we report an oligonucleotide custom microarray platform capable of measuring both coding and non-coding transcription on a genome-wide scale. By profiling MCMV wild-type and immediate-early mutant strains in fibroblasts we find rapid activation of the transcriptome by 6.5 hours post infection with absolute dependency on *ie3*, but not *ie1* or *ie2* for genomic programming of viral gene expression. Evidence is also presented, showing for the first time, genome-wide non-coding and bi-directional transcription at late stages of MCMV infection.

INTRODUCTION:

Murine cytomegalovirus (MCMV) is a ubiquitous beta-herpesvirus with a 235 kilo-base pair genome transcribed in a classical cascade fashion(55). The genome sequence of MCMV has been available for some time (89), yet a systematic study of temporal gene expression during MCMV infection has been lacking. Double stranded PCR based cDNA microarrays were used once previously (105) to validate the expression of a subset of predicted MCMV (ORFs)(15) at a single time point (24 hpi). More advanced microarray technology based on oligonucleotide probes affording increased specificity to distinguish RNA polarity has not been reported. For human cytomegalovirus (HCMV) abundant antisense transcription has been observed (120) raising the possibility that aspects of the CMV life cycle are influenced or regulated by non-coding transcripts. For MCMV, small virus-encoded miRNAs (19, 20, 33) and larger double-stranded RNAs (21, 110) have been reported to be transcribed from multiple loci, however the frequency and abundance of non-coding transcripts throughout the MCMV genome has not yet been measured systematically on a genome-wide scale and at multiple stages of infection.

Here we have investigated the global transcriptional programme of MCMV by constructing a microarray capable of measuring sense and antisense transcripts. Microarrays were designed using 55-mer oligonucleotide probes in sense and antisense orientation to each of the 170 viral open reading frames (ORFS) predicted in the MCMV genome(89). 192 positive control probes were designed against stably expressed mouse genes for normalization purposes and 97 negative control probes were designed against yeast sequences with no homology to mouse or MCMV genomes (probe sequences see Supplementary Table S1). The 55mers were diluted to a concentration of 60uM and inkjet printed (Arrayjet, UK) onto amino-silane coated glass slides with each microarray consisting of six identical sub-arrays. Probes were printed in triplicate per array and have the capacity for developing a total of 18 measurements per probe

per sample to ensure high technical replication. Target RNA was extracted from infected fibroblasts using PureLink RNA Mini Kits with on-column DNase treatment (Invitrogen, CA, USA). 700ng of purified RNA for each sample was labelled for microarray analysis using the Agilent low input fluorescent linear amplification protocol (Agilent, CA, USA) with 3ug of Cy5 labelled target cRNA hybridised per sample. Hybridized microarrays were washed and subsequently scanned using the Agilent (CA, USA) scanner G2505B.

To perform a systematic analysis of genome-wide transcription in MCMV we infected NIH 3T3 fibroblasts with the parental MCMV strain at an MOI of 1 and performed DNA microarray analysis on total RNA harvested from duplicate cultures at 0.5, 6.5, 24 and 48 hpi. Individual probe signals were background subtracted, median-summarized and log base 2 transformed to form raw data points (Supplementary Table S2). Raw data was quality controlled and normalization between samples was performed based on a subset of 44 positive control probes highly correlated across the data set (Pearson r<0.90). Normalized expression data (Supplementary Table S3) was subjected to a statistically rigorous threshold detection methodology for providing on/off calls for each probe based on receiver operating characteristic (ROC)(12). From these ROC analyses, we evaluated specificity levels corresponding to a given sensitivity of 70%, 805 and 90%. At a moderate sensitivity of 70% we are able to obtain an average specificity of 93%, and this was chosen as affording an optimal balance between identifying true positives and excluding true negatives with stringency (for ROC plots see Supplementary Figures S1 and S2). Accordingly we detect 297 total probes having “on” calls and 163 probes for coding MCMV ORFs making 87.6% of the MCMV genome detectable at 48hpi (for list of genes detected see Supplementary Table S4).

To account for experimental variation, statistical testing (empirical Bayes moderated t-test) was applied between mock and infected groups to identify differential expression of only the

most highly significant MCMV ORFs. Using this more stringent approach, 119 ORFs were found to be significantly activated to a confidence level of p0.05 above mock levels at any time point (Table 1a). This included the DNA polymerase sub-unit M54 (59), known inhibitors of MHC class I surface expression m04 (gp34)(51), m06 (gp48)(90), and the Fc receptor m138(108). After a single round of replication at 24hpi, a total of 111 MCMV ORFs were detected at the high confidence level. To further validate these findings a sub-set of MCMV ORFs were subjected to qRT-PCR analysis (primer sequences see Supplementary File S1), and in agreement with the microarray results, each test-case showed ORF expression was also detectable by qRT-PCR (Figure 1a).

As previously noted for HCMV microarray analysis, there is no overt positional bias towards expression of coding ORFs based on genomic location could be linked to the patterns of gene expression observed during the infection. MCMV ORFs were annotated based on Rawlinson et al(89) and updated with details from additional publications wherever possible. MCMV ORFs from recent predictions (15, 105) were aligned against 55mer probe sequences identifying five probes overlapping with newly predicted ORFs (m107-m107.2, m16-m16.2, m22-m22.1, M34-m34.2, and M58-m58.1) which were re-annotated accordingly.

As a result of the statistical cut-off (p0.05), MCMV probes for ORFs M44, M70, M75, m135, m143, m144m, m153 and m157 failed to be included, although these genes have been reported to be expressed in previous MCMV studies or are homologues of HCMV genes reported to be expressed (21, 29, 34, 79, 80, 89, 92, 110, 118). It is most likely that the specific probes for these genes exhibit false negative resultsNevertheless, in this study, we aim to purposefully avoid false positives at the sacrifice of capturing a modest level of false negatives. For this reason, we also do not detect *ie1* or *ie3* expression until 24hpi based on the statistical cut-off,

however, these genes are detectable as early as 0.5hpi and 2.5hpi using a more sensitive qRT-PCR approach (Supplementary Figure S3).

To gain further insight into the transcriptional programming of MCMV, we next sought to profile gene expression from three well-characterized MCMV mutants (5, 23, 36, 72) alongside the parental MCMV strain (111) (for schematic of strains see Figure 1b). To characterize the mutant strains before microarray analysis, we sought to; a) determine equivalent infectious doses at the genomic level by measuring genome / PFU ratio for each stock, b) ensure generating MCMVdie3 in a complementing cell line did not drastically alter infectious particle ratio, c) ensure viral growth phenotypes were consistent with those previously published (5, 36, 72), and d) ensure no viral transcription was occurring from deleted loci. On the basis of qPCR we detect equivalent numbers of MCMV genome copies per PFU for each viral strain (Figure 1c) using as a calibrator reference plasmid containing the m115 (gL) gene (nts 166387-167208 GenBank NC_004065) (for detailed account of this approach see (98)). Equivalent numbers of MCMV genomes were also found inside infected cells at 2hpi from different MCMV strains (data not shown). Multi-step growth curves confirmed viral growth phenotypes (Supplementary Figure S4) and qRT-PCR (Qiagen 1-step, Germany) confirmed transcription was not detectable from the respective deleted loci for *ie1*, *ie2* and *ie3* (data not shown). These data show the four MCMV strains are experimentally comparable for downstream gene expression analysis.

Based on previous virologic characterization of these MCMV IE mutants, we expect to observe different gene expression profiles for each MCMV strain. For example, given that removal of the *ie2* gene causes no reported phenotype (45), we anticipate few gene expression changes in the MCMVdiie2 strain relative to the parental MCMV strain. Alternatively, given *ie3* has an indispensable regulatory function and is essential for viral growth (5), we expect little or no

153 viral gene expression to be detectable from the MCMVdie3 strain. However, for the *ie1*
154 deletion mutant (MCMVdie1) it is less clear given that it has wild-type growth characteristics
155 yet the IE1 protein is well known have transcriptional regulatory activity (24, 39, 76), and is
156 further known to interact with cellular host factors (81, 104). In order to profile the gene
157 expression of each mutant strain, we infected NIH 3T3 fibroblasts in parallel at MOI of 1 and
158 harvested total RNA for microarray analysis at 0.5, 6.5 and 48 hpi, along with mock-infected
159 cells.
160
161 Figure 2 shows the comparative activation of viral transcriptomes between the four MCMV
162 strains at 48hpi and indicates the transcriptomes of MCMVdie1 and MCMVdiie2 are activated
163 with very similar profiles of viral gene expression to the parental MCMV strain over the 48-
164 hour period (Figure 2a). A similar number (within 10%) of MCMV probes were detectable
165 from MCMVdie1 (103 ORFs), MCMVdiie2 (114 ORFs) and the parental strain (113 ORFs) at
166 48hpi. The degree of similarity in expression profiles between the MCMVdie1, MCMVdiie2
167 and parental MCMV strains suggests that *ie1* and *ie2* have a redundant or negligible
168 transcriptional regulatory role in controlling downstream MCMV gene expression during
169 fibroblast infection. Hierarchical clustering (Figure 2b) further indicates few, if any,
170 differences in the global gene expression profiles of MCMVdie1 and MCMVdiie2 compared to
171 the parental MCMV strain. These results could potentially point to a redundant role for the *ie1*
172 and *ie2* genes in controlling downstream viral gene expression, or alternatively a lack of
173 sensitivity in controlling the viral genomic programme in fully permissive fibroblast cells.
174
175 In marked contrast to the *ie1* and *ie2* mutant strains, MCMVdie3 exhibited an undetectable
176 level of viral gene expression, suggesting *ie3* acts as a global trans-activator of downstream
177 MCMV gene expression as indicated by previous studies(5) To further examine the
178 transcriptional status of MCMVdie3 using a more sensitive approach, immediate early (IE) and

179 downstream MCMV genes were measured using qRT-PCR in MCMVdie3-infected cells both
180 in the presence and absence of 50µg/ml cyclohexamide (Sigma UK, C7698) at 2.5hpi. These
181 experiments confirmed IE kinetic class genes were expressed in MCMVdie3, but genes beyond
182 the IE region were not (Supplementary Figure S5).
183
184 The design of the MCMV microarray platform enables selective detection of transcripts
185 originating from both strands of the viral genome by having probes designed in sense (S) and
186 antisense (AS) orientation to each MCMV ORF. At 24hpi we detect antisense transcripts from
187 23 AS loci, five of which (m104as, M113as, m147as, m163as, m168as) have overlapping
188 ORFs on the opposite strand of the genome indicating known or predicted regions of bi-
189 directional transcription based on prior annotation(105). Three other loci were found to have
190 neighbouring but non-overlapping ORFs in their vicinity (M57as, m74as and M88as). An
191 additional 15 MCMV AS probes detected at 24hpi were found to have no overlapping or
192 nearby ORFs located on the opposite strand, indicating previously unknown non-coding
193 transcripts derived from regions outside of MCMV ORFs (m04as, m05as, m06as, m07as,
194 m09as, m13as, m41as, M47as, M50as, M87as, M102as, M115as, m119.2as, m124as, m145as).
195 At 48 hpi an additional eight antisense probes were detectable but all have overlapping or
196 nearby ORFs on the opposite strand of the genome (M48as, m69.1as, M89as, M94as, m108as,
197 m119.3as, m132as, and m144as). In total, evidence of antisense transcription was detected
198 from 35 loci over the four time points as measured by microarray analysis (Table 1 and Figure
199 3a). 26 of these loci were also found to have significant signal from their corresponding sense
200 (S) probe indicating a potential site of bi-directional transcription. A trend towards antisense
201 transcription occurring more frequently at the terminal ends of the MCMV genome is also
202 noted (Figure 3a).
203

In order to independently validate AS transcripts identified by microarray analysis, we generated cDNA libraries from MCMV infected fibroblasts pooled from either 4, 8 and 12 hpi (IE library), 16, 24 and 32 hpi (E library) or 40, 60, 80 and 100 hpi (L library). cDNA libraries were generated as described previously for HCMV (120). cDNA clones overlapping AS microarray regions were found at m04as, m74as, M94as (none of which have overlapping ORFs on the opposite strand of the genome) and m148as (which has the m147 ORF on the opposite strand of the genome) (for validated AS transcripts see Table 1b). We also find one large cDNA clone that overlapped three MCMV ORFs (m119.2, m119.3 and m119.4), two of which had potential AS regions identified by microarray probes (m119.2 and m119.3). Four additional AS cDNA clones were found to overlap AS regions not identified by microarray analysis (m19as, M72as, m149as and m151as). These experiments thus reveal for the first time that antisense transcription occurs frequently throughout the MCMV genome, an observation that will likely seed further studies.

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627 **FIGURE LEGENDS**

628

629 **Figure 1 - MCMV ORF detection and characterization of MCMV IE deletion mutant**
630 **strains 1a i** Microarray signals for 10 MCMV ORFs in mock and MCMV-infected cells. **1a ii**
631 qRT-PCR validation of the 10 MCMV ORFs at 24hpi. Y axis represents crossing point (Cp)
632 values from qPCR amplification curves with low Cp values indicating high transcript
633 abundance. **1b** Schematic representation of recombinant MCMV IE deletion mutants. The map
634 of the parental MCMV genome is shown at the top with structure of the *ie1*, *ie2* and *ie3*
635 transcripts below. Coding exons are shown in black with arrows indicating the direction of
636 transcription and white triangles represent deleted loci. The grey box marks the MCMV
637 enhancer ie1/ie3 promoter. The MCMVdie3 revertant strain (**1b iii**) was renamed in this study
638 to MCMVdiie2 as the HCMV MIEP is inserted between two HpaI sites spanning the
639 transcription start site of the *ie2* gene, disrupting *ie2* expression (marked with a cross) **1c** Viral
640 titres and genome particle / PFU equivalence of four MCMV strains as determined by qPCR.

641

642 **Figure 2 - Gene expression program of MCMV, MCMVdie1, MCMVdiie2 and**
643 **MCMVdie3 2a** Volcano plots compare microarray signals from mock and infected samples
644 at 48hpi using p-value (y-axis) and fold change (x-axis) comparisons derived from empirical
645 Bayes testing with two biological replicates per group. **2b** Hierarchical clustering of high
646 confidence MCMV probes with each row representing a single probe normalized to its mean
647 value across the data set to show relative expression. Yellow indicates increased expression
648 and blue indicates decreased expression relative to the mean. Genes clustered based on the
649 similarity of their expression profile across the dataset, with similar genes connected at the
650 hierarchical tree on the left. Data represents mean values from two biological replicates.

651

652 **Figure 3 - MCMV genome activation measured by microarray analysis and qRT-PCR**

653 Transcript abundance of ORFs expressed from the parental MCMV measured using

654 oligonucleotide microarrays at 0.5 hpi **(i)**, 6.5 hpi (**ii**), 24 hpi (**iii**) and 48 hpi (**iv**) in NIH 3T3

655 cells at MOI1. Histograms represent mean values from two replicate samples after background

656 (mock) subtraction. Transcripts are arranged in order from left to right according to ORF

657 names ranging from m01 up to m170 with sense probes shown in the left boxes and anti-sense

658 probes on the right. All raw data are available in Supplementary Material

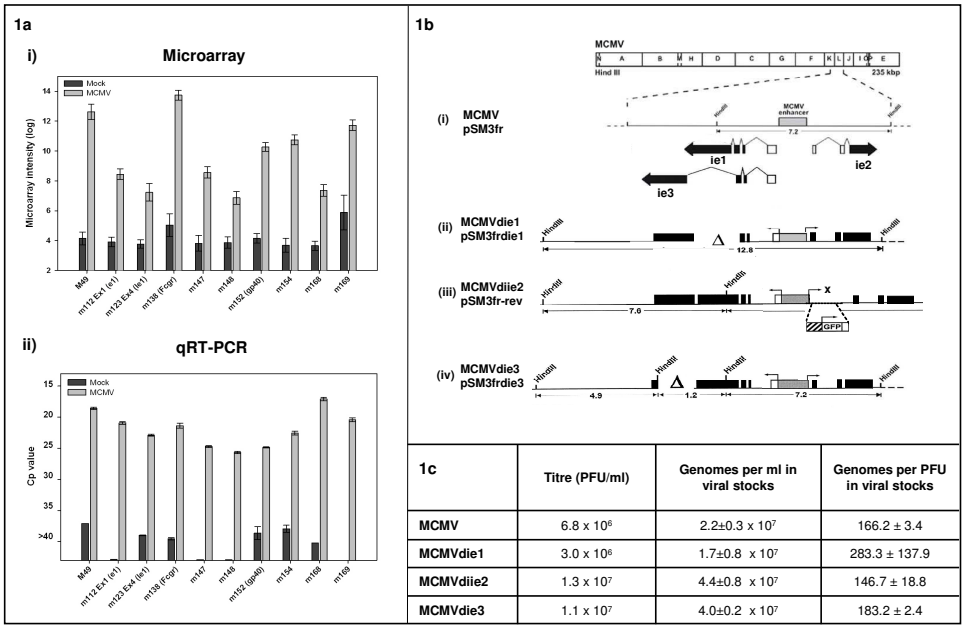
659

660 **Table 1. High confidence MCMV microarray probes**
661

662 Table 1a - Microarray probes for coding MCMV ORFs found to be significantly upregulated in
663 MCMV infected cells versus mock to a high confidence level ($p<0.05$ empirical Bayes). Probe
664 ID, ORF name, MCMV and HCMV gene name, time detected (hpi), protein type, annotation
665 and virion association (based on (49)) are shown in columns. Table 1b - Microarray probes for
666 antisense transcripts found to be significantly upregulated in MCMV infected cells versus
667 mock to a high confidence level ($p<0.05$ empirical Bayes). AS probes with significant signal
668 also found from the corresponding sense probes are marked with an "S" in the rightmost
669 column. Details of cDNA cloning validation is also noted in the annotation column where
670 applicable.

671

Figure 1 - MCMV ORF detection and characterization of MCMV IE deletion mutant strains 1a i Microarray signals for 10 MCMV ORFs in mock and MCMV-infected cells. **1a ii** qRT-PCR validation of the 10 MCMV ORFs at 24hpi. Y axis represents crossing point (Cp) values from qPCR amplification curves with low Cp values indicating high transcript abundance. **1b** Schematic representation of recombinant MCMV IE deletion mutants. The map of the parental MCMV genome is shown at the top with structure of the *ie1*, *ie2* and *ie3* transcripts below. Coding exons are shown in black with arrows indicating the direction of transcription and white triangles represent deleted loci. The grey box marks the MCMV enhancer ie1/ie3 promoter. The MCMVdie3 revertant strain (**1b iii**) was renamed in this study to MCMVdiie2 as the HCMV MIEP is inserted between two HpaI sites spanning the transcription start site of the *ie2* gene, disrupting *ie2* expression (marked with a cross) **1c** Viral titres and genome particle / PFU equivalence of four MCMV strains as determined by qPCR.



1c	Titre (PFU/ml)	Genomes per ml in viral stocks	Genomes per PFU in viral stocks
MCMV	6.8×10^6	$2.2 \pm 0.3 \times 10^7$	166.2 ± 3.4
MCMVdie1	3.0×10^6	$1.7 \pm 0.8 \times 10^7$	283.3 ± 137.9
MCMVdiie2	1.3×10^7	$4.4 \pm 0.8 \times 10^7$	146.7 ± 18.8
MCMVdie3	1.1×10^7	$4.0 \pm 0.2 \times 10^7$	183.2 ± 2.4

Figure 2 - Gene expression program of MCMV, MCMVdie1, MCMVdiie2 and MCMVdie3 **2a** Volcano plots compare microarray signals from mock and infected samples at 48hpi using p-value (y-axis) and fold change (x-axis) comparisons derived from empirical Bayes testing with two biological replicates per group. **2b** Hierarchical clustering of high confidence MCMV probes with each row representing a single probe normalized to its mean value across the data set to show relative expression. Yellow indicates increased expression and blue indicates decreased expression relative to the mean. Genes clustered based on the similarity of their expression profile across the dataset, with similar genes connected at the hierarchical tree on the left. Data represents mean values from two biological replicates.

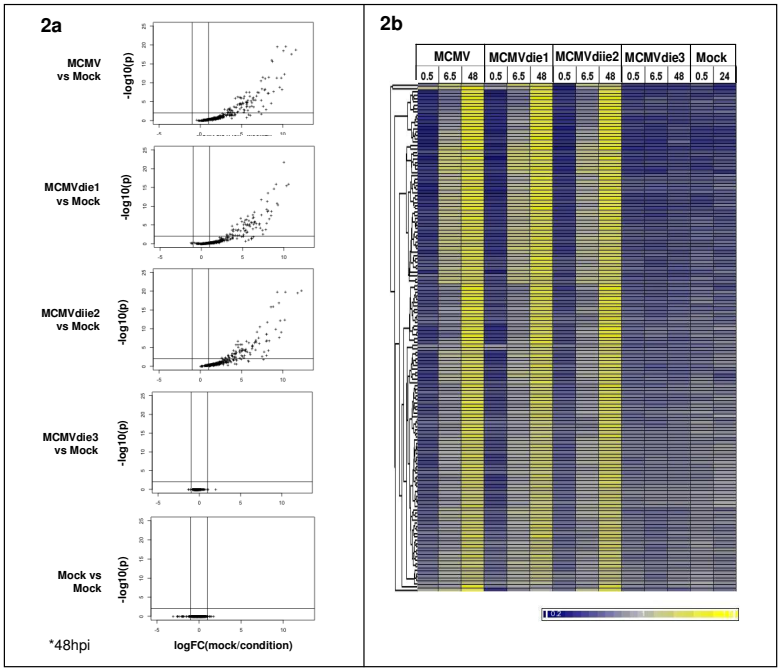


Figure 3 - MCMV genome activation measured by microarray analysis and qRT-PCR Transcript abundance of ORFs expressed from the parental MCMV measured using oligonucleotide microarrays at 0.5 hpi (**i**), 6.5 hpi (**ii**), 24 hpi (**iii**) and 48 hpi (**iv**) in NIH 3T3 cells at MOI1. Histograms represent mean values from two replicate samples after background (mock) subtraction. Transcripts are arranged in order from left to right according to ORF names ranging from m01 up to m170 with sense probes shown in the left boxes and anti-sense probes on the right. All raw data are available in Supplementary Material

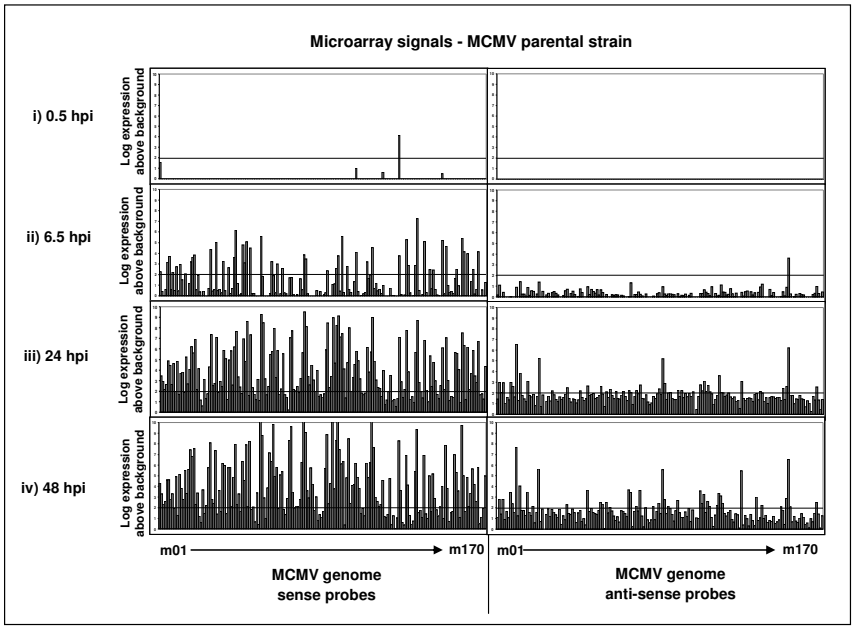


Table 1a – High confidence sense probes

Unique Probe ID	ORF	Strand	MCMV Name	HCMV name	Time on	Protein type	Annotation	Virion assoc
vMC132	m128 Ex3	C	Ie2	US22 (GF2)	0.5hpi	immediate early	The spliced m128 (ie2) gene has sequence similarity to members of the US22 family of HCMV (77)	
vIE1rem	m123 Ex4	C	ie1	IE1	6.5hpi	immediate early	ie1 exon 4, with mRNA terminating at base 179544(53-55); total length of Ex2 plus Ex3 plus Ex4 is 595 aa, total MW is 66.7 kDa	
vMC003	m03				6.5hpi	Glycoprotein m02	Sequence variation and early transcriptional kinetics found in wild-derived MCMV isolates; m03 encoded protein could be found on cell surface (28)	
vMC004	m04		gp34		6.5hpi	Glycoprotein m02	The m04 gene product (gp34) forms a complex with MHC I (51) which reaches the cell surface (46) and is required for Ly49P recognition of infected cells (57). m04/gp34 also antagonizes the effect of m152 (84)	
vMC006	m06		gp48		6.5hpi	Glycoprotein m02	m06 gene product (gp48) downmodulates the levels of MHC class I proteins on infected cells(50) and binds to the ternary complex of assembled MHC class I with antigenic peptide in the ER, redirecting this complex to the lysosome for degradation; unbound gp48 is destroyed by the proteasome (18)	
vMC008	m08				6.5hpi	Glycoprotein m02	Glycoprotein family m02	
vMC010	m10				6.5hpi	Glycoprotein m02	Glycoprotein family m02	
vMC016	m16.2				6.5hpi	Glycoprotein m02	Glycoprotein family m02 -probe overlaps with newly predicted ORF m16.2 (105)	
vMC017	m17	C			6.5hpi	Membrane glycoprotein		
vMC018	m18	C			6.5hpi	Early	Highly antigenic early gene (44)	VAP
vMC027	m25.1	C		UL23 (GF2)	6.5hpi	US22 family homolog	Mutant showed no obvious growth phenotype (75)	
vMC030	M28	C		UL28	6.5hpi			
vMC037	m34.2			UL34	6.5hpi		Re-annotations of the MCMV genome have identified three putative M34 overlapping ORFs (m33.1, m34.1, and m34.2). This microarray probe overlaps with newly predicted ORF m34.2 (105) A M34 mutant virus which interrupted all three m34 ORFs had attenuated replication both in tissue culture and in SCID mice (10)	
vMC040	M36 Ex2	C		UL36 Exon 2	6.5hpi	US22 family homolog	Possible transcriptional regulator (89) and implicated in blocking apoptosis via inhibiton of caspase-8 (FLICE) activation(100). Growth of M36 mutant was attenuated in vitro and in vivo(27). Human CMV vICA/pUL36 protects cells from extrinsic apoptosis induced via the death receptors in TNFR1, Fas/CD95 or Trail.	
vMC041	M37	C		UL37	6.5hpi	Glycoprotein	Glycoprotein and viral mitochondrial inhibitor of apoptosis (vMIA). M37 mutant is severely attenuated in growth and virulence in vivo(63). Homolog of HCMV UL37 that inhibits mitochondrial megapore activation in a manner similar to members of the anti-apoptotic Bcl family(37)]. May also be a transcriptional regulator (58)	
vMC045	m41	C			6.5hpi		Putative anti-apoptotic function (79)	
vMC046	m42	C			6.5hpi	Putative glycoprotein		
vMC047	M43	C		UL43	6.5hpi	US22 family homolog	Anti-apoptotic(79), immunoregulatory gene that modulates T helper cell response (99). Found to be not essential for viral growth in vitro and in vivo and dispensable for virulence in killing SCID mice (117)	VAP
vMC049	M45	C		UL45 (RRL)	6.5hpi	Ribonucleotide reductase	Anti- apoptotic (17) homologue of the large subunit of ribonucleotide reductase (85). Blocks NFkB activation as a result of its inhibitory effect on receptor-interacting protein kinase 1 (RIP1) signalling (71, 109)	VAP
vMC055	m48.2	C			6.5hpi	Capsid	Smallest capsid protein (14)	VAP
vMC061	M54	C	DNApol	UL54 (DNApol)	6.5hpi	DNA polymerase	DNA polymerase delta subtype (59)	VAP
vMC064	M57	C		UL57 (MDBP)	6.5hpi	DNA binding	Major ssDNA binding protein (3, 4)	VAP
vMC079	M80			UL80 (AP)	6.5hpi	Assembly protein, protease	Assembly protein and protease (11, 69) which conserves the domain structure and cleavage sites present in HCMV UL80.	VAP
vMC080	M82	C	pp71	UL82 (pp71)	6.5hpi	Upper matrix phosphoprotein	Encodes a structural protein unique to the beta-herpesvirus group, aka pp71 - same family as M83 (91)	VAP
vMC092	M94			UL94	6.5hpi	Virion-associated	Virion-associated, tegument/second evelopment protein (112)	VAP
vMC098	M99			UL99 (pp28)	6.5hpi	Phosphoprotein	Encodes small structural phosphoprotein unique to the beta-herpesvirus group shown to be around 16 kDa in size and to be associated with the virion (30, 78)	VAP
vMC100	M102			UL102 (HP)	6.5hpi	Helicase-primiase	Part of the helicase-primase complex of three proteins (M70, M102, and M105) (70, 97)	VAP
vMC103	M105			UL105 (Hel)	6.5hpi	DNA helicase	DNA helicase - part of the helicase-primase complex of three proteins (M70, M102, and M105) (70, 97)	VAP
vMC114	M118	C		UL118	6.5hpi		Possible alternate splice to m119 as for HCMV UL118 (62, 88)	
vMC117	m119.2	C	sgg1		6.5hpi		Exon 2 of M133 (sgg1) (61, 73)	

vMC136	m132 Ex2	C			6.5hpi			
vMC141	m137	C			6.5hpi	Putative glycoprotein		
vMC142	m138	C	Fcgr	fcr1	6.5hpi	Glycoprotein	Encodes the 88 kDa Fc receptor glycoprotein (107). Fc receptor specific (m138) deletion mutants show unexpected alterations in virulence and are attenuated in normal and immunosuppressed adult mice (31). m138 down-modulates the NKG2D ligands MULT-1, H60 (65) and RAE-1 epsilon (8)	
vMC146	m142	C		US26 (GF2)	6.5hpi	US22 family homolog	Required to block PKR-mediated shutdown of celular protein synthesis and associated antiviral response (21, 110)	
vMC151	m147	C			6.5hpi	Possible membrane spanning protein	Spliced gene m147.5 selectively targets CD86 expression on APCs (68)	VAP
vMC156	m152	C	gp40		6.5hpi	Glycoprotein	The m152 gene product (gp40) is a member of MGP family that downmodulates MHC class I proteins on infected cells(50), disrupts export of MHC class I complexes from pre-Golgi compartments to the Golgi via a luminal domain(122), modulates NK cell (inhibition), antigen presentation, and modulates T cell response (40) Viruses lacking gp40 show increased susceptibility to CTL killing in vivo (43)	
vMC158	m154	C			6.5hpi	Glycoprotein	Threonine-serine-rich glycoprotein of MGP family m145, some homology to EHV1 g(X) (106)	
vMC164	m160	C			6.5hpi	Putative membrane glycoprotein		
vMC167	m163	C			6.5hpi	Putative membrane glycoprotein		VAP
vMC168	m164	C	gp36.5		6.5hpi	Putative membrane glycoprotein	MCMV viral carrier protein gp36.5(64), the m164-derived peptide 257-AGPPRYSRI-265 is presented by the MHC-I molecule D(d) (45)	
vMC170	m166	C			6.5hpi	Putative membrane glycoprotein	Required for MCMV virulence in killing SCID mice and for optimal viral growth in vivo (121)	
vMC173	m169	C			6.5hpi			
vIE3rem	M122 Ex5	C	ie3	UL122 (IE2)	24hpi	immediate early	ie3 exon 5, with mRNA terminating at base 177817(76); total length of Ex2 plus Ex3 plus and Ex5 is 611 aa, MW is 68.1 kDa	
vMC011	m11				24hpi	Glycoprotein m02		
vMC013	m13				24hpi	Glycoprotein m02		
vMC015	m15				24hpi	Glycoprotein m02		
vMC020	m20	C			24hpi		Reported irregularities in coding potential have suggested possible sequence errors in the 3'-proximal end of m20 (49)	
vMC023	M23	C		UL23 (GF2) P	24hpi		US22 family homolog	
vMC026	M25			UL25 (GF1)	24hpi	Tegument	UL25 family homologue (26, 32). Encodes a component of the MCMV tegument (116)	VAP
vMC028	M26	C		UL26	24hpi			
vMC029	M27	C		UL27	24hpi		Plays important role in MCMV growth and virulence (2) and inhibits gamma interferon signalling (56) via selective binding and downregulation of Stat2(123)	
vMC032	m29.1	C			24hpi			
vMC033	m30				24hpi			
vMC034	M31			UL31	24hpi		Reported irregularities in coding potential have suggested possible sequence errors in the 3'-proximal end (49)	
vMC035	M32	C		UL32 (pp150)	24hpi	Phosphoprotein (tegument)	Encodes large phosphoprotein homologous to HCMV tegument protein UL32 (pp150) (47, 48)	VAP
vMC038	M35			UL35 (GF1)	24hpi	Virion-associated	Virulence factor and HCMV UL25 family homologue(26, 32). Insertional mutant at M35 is defective in growth in vivo (103)	VAP
vMC039	M36 Ex1			UL36 Exon 1	24hpi		US22 family homolog	
vMC042	M38	C		UL38	24hpi			
vMC050	m45.1	C			24hpi			
vMC053	M48			UL48 (Teg)	24hpi	Tegument	Large tegument protein (101)	VAP
vMC056	M49	C		UL49	24hpi			

vMC057	M50	C		UL50	24hpi	Nuclear export	Conserved herpesviruses proteins that form a complex essential for egress of nucleocapsids from the nucleus (94) M50/p35 inserts into the inner nuclear membrane and is aggregated by M53/p38 to form the capsid docking site (82)	
vMC059	M52			UL52	24hpi			
vMC060	M53			UL53	24hpi	Nuclear export	Conserved herpesviruses proteins that form a complex essential for egress of nucleocapsids from the nucleus (94) M50/p35 recruits the cellular protein kinase C for phosphorylation and dissolution of the nuclear lamina (82)	
vMC062	M55	C	gB	UL55 (gB)	24hpi	Glycoprotein	Glycoprotein B (29, 87)	VAP
vMC063	M56	C		UL56 (NM)	24hpi	Tegument	Tegument protein and homologue of HCMV terminase subunit and HSV ICP18.5 (13, 38)	VAP
vMC067	M69	C		UL69	24hpi	Tegument	Tegument protein similar to HCMV transactivator UL69 (113) which in HCMV induces a G1 block (42)	VAP
vMC071	M72	C		UL72 (dUTPase)	24hpi	Putative dUTPase	Homology to HCMV UL72 - a putative dUTPase enzyme required for nucleotide metabolism, replication, and/or repair (86)	VAP
vMC072	M73			UL73	24hpi			
vMC077	M78			UL78	24hpi	G protein-coupled receptor	G protein-coupled receptor (GCR) homologue, same family as M33(38). Has subcellular trafficking properties(96). Mutants in M78 exhibit reduced replication in cultured cells as well as severe attenuation in the infected host (83)	
vMC078	M79	C		UL79	24hpi			
vMC081	M83	C		UL83 (pp65)	24hpi	Virion-associated	Homologue of HCMV virion-associated factor with IFN repressor function (pp65) (1, 16, 74) M83 mutant has attenuated viral growth and virulence in SCID mice (119)	VAP
vMC083	M85	C		UL85	24hpi	Capsid	Homologue of HCMV minor capsid protein (9)	VAP
vMC084	M86	C		UL86 (MCP)	24hpi	Capsid	Homologue of HCMV major capsid protein (25)	VAP
vMC086	M88			UL88	24hpi	Virion-associated	Homologue of HCMV virion protein (9)	VAP
vMC091	M93			UL93	24hpi			
vMC093	M89 Ex1	C		UL89	24hpi			
vMC094	M95			UL95	24hpi			
vMC095	M96			UL96	24hpi			
vMC096	M97			UL97 (PK)	24hpi	Phosphotransferase	Homologue of HCMV UL97 phosphotransferase gene whose product phosphorylates ganciclovir in HCMV infected cells (67, 102)	VAP
vMC097	M98			UL98 (DNase)	24hpi	Exonuclease	Alkaline exonuclease (DNase) gene (89)	VAP
vMC099	M100	C	gM	UL100 (gM)	24hpi	Glycoprotein	Glycoprotein M with seven hydrophobic stretches that are potential membrane-spanning regions (66, 93)	VAP
vMC101	M103	C		UL103	24hpi			
vMC104	m106	C			24hpi			
vMC105	m107				24hpi			
vMC106	m108	C			24hpi			
vMC107	M112 Ex1		e1	UL112	24hpi	Early	Exon 1 of e1(22); total e1 length is 330 aa, MW is 36.4 kDa. IE3 and the early M112/113 gene products colocalize and coimmunoprecipitate (104). Neuron-specific activation of the e1 promoter observed in transgenic mice (7)	
vMC109	M114	C		UL114 (UNG)	24hpi	Glycosylase	Uracil DNA glycosylase enzyme homolog (114) found in herpesviruses required for nucleotide metabolism, replication, and/or repair	
vMC110	M115	C	gL	UL115 (gL)	24hpi	Glycoprotein	M115 (gL) (52)that contains five potential glycosylation sites, has significant amino acid similarity to gL homologs in HCMV and HHV-6 and has previously been shown to be glycosylated in virions (118)	
vMC115	m119				24hpi			
vMC116	m119.1				24hpi	Potential glycoprotein		
vMC119	m119.4				24hpi		predicted MCMV homologue of HCMV spliced ORF (89)	

vMC120	m119.5				24hpi			
vMC121	m120	C			24hpi			
vMC123	M122 Ex5	C	ie3	UL122 (IE2)	24hpi	immediate early	ie3 exon 5, with mRNA terminating at base 177817(76); total length of Ex2 plus Ex3 plus and Ex5 is 611 aa, MW is 68.1 kDa	
vMC127	m124				24hpi		ORFs m124, m124.1, and m125 located within the enhancer region are nonessential for MCMV growth in vitro (6)	
vMC137	m132 Ex1	C	sgg1		24hpi		Exon 2 of M133 (sgg1) (61, 73)	
vMC143	m139	C		US22 (GF2)	24hpi		US22 hamily homolog	
vMC149	m145				24hpi	Glycoprotein	Downmodulates expression of the cellular MULT-1 (60) and is a member of membrane glycoprotein (MGP) family m145	
vMC152	m148				24hpi			
vMC159	m155				24hpi	Glycoprotein	Member of membrane glycoproteins (MGP) family m145 that modulates NK cell (inhibition) and T cell response, impedes a NKG2D ligand (H60) (41, 79)	
vMC163	m159	C			24hpi	Putative membrane glycoprotein		
vMC165	m161	C			24hpi	Putative glycoprotein	Putative membrane glycoprotein	
vMC172	m168				24hpi			
vMC174	m170	C			24hpi			
vMC005	m05				48hpi	Glycoprotein m02		
vMC012	m12				48hpi	Glycoprotein m02		
vMC014	m14				48hpi	Glycoprotein m02		
vMC043	m39	C			48hpi			
vMC066	m59				48hpi		potential ORF lcoated wtihin the origin of replication (89)	
vMC073	m74	C		UL74 P	48hpi	Putative glycoprotein	Functional homolog of HCMV gO and has key role in determining the entry pathway of MCMV (95)	VAP
vMC108	M113			UL113 P	48hpi		Potential alternative splice to M112 Ex1 (e1) (22) as found for HCMV UL112/UL113 (115)	
vMC118	m119.3				48hpi		predicted MCMV homologue of HCMV spliced ORF	
vMC133	m129	C			48hpi		m131/129 is a chemokine homolog and determinant of viral pathogenicity (35) and may modulate cytokine signalling (79)	

Table 1b – High confidence antisense probes

Unique Prot	**ORF**	**Strand**	**MCMV h**	**HCMV name**	Time on	**Protein type**	**Annotation**	**Sense p**
vMC336	m148as	-	-	-	6.5hpi	Antisense transcript	Validated with AS cDNA clone L151 position 208012-207467 (5' to 3') overlapping m148 (AS) and m149 (S) clone length 545nt	S
vMC357	m169as	-	-	-	6.5hpi	Antisense transcript		S
vMC175	m05as	-	-	-	24hpi	Antisense transcript		S
vMC180	m163as	-	-	-	24hpi	Antisense transcript		S
vMC188	m04as	-	-	-	24hpi	Antisense transcript	Validated with AS cDNA clone IE150 position 4043-3943 (5' to 3') overlapping m04 (AS), clone length 384nt	S
vMC189	m05as	-	-	-	24hpi	Antisense transcript		S
vMC190	m06as	-	-	-	24hpi	Antisense transcript		S

vMC191	m07as	-	-	-	24hpi	Antisense transcript		-
vMC193	m09as	-	-	-	24hpi	Antisense transcript		-
vMC197	m13as	-	-	-	24hpi	Antisense transcript		S
vMC229	m41as	-	-	-	24hpi	Antisense transcript		S
vMC236	M47as	-	-	-	24hpi	Antisense transcript		-
vMC241	M50as	-	-	-	24hpi	Antisense transcript		S
vMC248	M57as	-	-	-	24hpi	Antisense transcript		S
vMC257	m74as	-	-	-	24hpi	Antisense transcript	Validated with AS cDNA clone L147 position 104825-105449 (5' to 3') overlapping m74 (AS), clone length 624nt	S
vMC269	M87as	-	-	-	24hpi	Antisense transcript		-
vMC270	M88as	-	-	-	24hpi	Antisense transcript		S
vMC283	M102as	-	-	-	24hpi	Antisense transcript		S
vMC285	M104as	-	-	-	24hpi	Antisense transcript		-
vMC291	M113as	-	-	-	24hpi	Antisense transcript		S
vMC293	M115as	-	-	-	24hpi	Antisense transcript		S
vMC299	m119.2as	-	-	-	24hpi	Antisense transcript		S
vMC311	m124as	-	-	-	24hpi	Antisense transcript		S
vMC333	m145as	-	-	-	24hpi	Antisense transcript		S
vMC335	m147as	-	-	-	24hpi	Antisense transcript		-
vMC351	m163as	-	-	-	24hpi	Antisense transcript		S
vMC356	m168as	-	-	-	24hpi	Antisense transcript		S
vMC239	M48as	-	-	-	48hpi	Antisense transcript		S
vMC251	m69.1as	-	-	-	48hpi	Antisense transcript		-
vMC272	M89 Ex2as	-	-	-	48hpi	Antisense transcript		-
vMC277	M94as	-	-	-	48hpi	Antisense transcript	Validated with AS cDNA clone L164 position 137299-137096 (5' to 3') overlapping m94 (AS), clone length 203nt	S
vMC289	m108as	-	-	-	48hpi	Antisense transcript		S
vMC300	m119.3as	-	-	-	48hpi	Antisense transcript		S
vMC319	m132 Ex2as	-	-	-	48hpi	Antisense transcript		S
vMC332	m144	-	-	-	48hpi	Antisense transcript		