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Genes of murine cytomegalovirus exist as a number of distinct genotypes

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Abstract

Murine cytomegaloviruses encode a number of genes which modulate polymorphic host immune responses. We suggest that these viral genes should themselves therefore exhibit sequence polymorphism. Additionally, clinical isolates of human cytomegalovirus (HCMV) have been shown to vary extensively from the common laboratory strains. Almost all research conducted on murine cytomegalovirus (MCMV) has used the laboratory strains Smith and K181, which have been extensively passaged in vitro and in vivo since isolation. Using the heteroduplex mobility assay (HMA) to determine levels of sequence variation 11 MCMV genes were examined from 26 isolates of MCMV from wild mice, as well as both laboratory strains. Both the HMA and sequencing of selected genes demonstrated that whilst certain genes (M33, mck-2, m147.5, m152) were highly conserved, others (m04, m06, M44, m138, m144, m145 and m155) contained significant sequence variation. Several of these genes (m06, m144 and m155) exist in wild MCMV strains as one of several distinct genotypes.
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Keywords: Murine cytomegalovirus; Immune evasion; Gene polymorphism; Multiple genotypes; Wild mice

Introduction

Murine cytomegalovirus (MCMV) is a double-stranded DNA virus with a genome size of approximately 230 kb. It is capable of forming life-long, asymptomatic, and persistent infections, characterised by periodic reactivation from latency (Sweet, 1999). Free-living mice may be infected with multiple strains of MCMV (Booth et al., 1993), laboratory studies having shown infection of mice with 2 strains of virus can happen either concurrently or sequentially (Gorman et al., 2006).

The genome of MCMV is co-linear with that of human cytomegalovirus (HCMV), consisting of approximately 200 open reading frames (Brocchieri et al., 2005; Rawlinson et al., 1996) with approximately 50% of genes identified in MCMV having homologues in HCMV. Targeted deletion studies of the HCMV (Towne strain) genome have shown that over 72% of all ORFs (117 ORFs from a total of 162) are dispensable for in vitro growth, and presumably function to allow virus replication in vivo (Dunn et al., 2003). Whilst some of these genes encode cell-tropism and anti-apoptopic factors, the majority appear to allow virus survival in vivo by modulating host immune responses.

The complete genome of several different strains of HCMV has been sequenced, either as viral DNA or as unrepaired BAC clones (Chee et al., 1990; Murphy et al., 2003; Dolan et al., 2004). Levels of amino acid sequence similarity between the same gene from the different isolates range from 25% to 100%. Laboratory strains of viruses that have had multiple passages may have lost a significant number of genes (Cha et al., 1996). Gene content may also vary between clinical isolates (Murphy et al., 2003).

Interestingly, sequence variation in the UL18 MHC class I homologue of HCMV results in differential avidity of binding to the ILT2 receptor, possibly indicating functional variation (Vales-Gomez et al., 2005).

As is the case with HCMV (Haberland et al., 1999), the glycoprotein gB gene of MCMV also demonstrates genetic heterogeneity (Xu et al., 1996), presumably due to selection for evasion of host immune control. The ie1 gene product in MCMV has also been shown to exist as a number of distinct genotypes (Lyons et al., 1996) among isolates isolated from wild mice.

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The heteroduplex mobility assay (HMA) is a rapid method of comparing PCR products. Two different PCR products are mixed, denatured, and allowed to re-anneal. Where strands from the separate PCR products re-anneal, regions of sequence variation cause 'bubbles' along the double stranded DNA. These imperfectly annealed regions result in a conformational change to the DNA molecule, and can be detected due to a shift in electrophoretic mobility. Variations of this methodology are widely used to analyse viruses such as HIV, TT-virus, avian hepatitis, and respiratory syncytial virus (Kuroiwa et al., 2004; Sawadogo et al., 2003; White et al., 2000), particularly as a pre-screen prior to large-scale sequencing.

We analysed genetic variation in 26 strains of MCMV using PCR and HMA, in an attempt to identify the levels of variation occurring in wild viruses. Genes in which sequence variation was evident were sequenced from multiple isolates, to determine the level and nature of this variation. We have shown that several MCMV genes exist as multiple genotypes within strains of virus isolated from free-living mice. These results demonstrate that wild strains of MCMV are different from the laboratory strains of virus and from each other, suggesting that the large body of MCMV research conducted in inbred mice using only 1 or 2 laboratory strains of MCMV may not reflect aspects MCMV infection in wild mice.

Results

PCR primers were designed, using the published Smith sequence, to amplify entire genes. These primers were designed outside of the coding sequence so as not to bias the HMA results due to included primer sequence. Should a 'normal' PCR protocol not be able to amplify specific genes from a majority of MCMV isolates, indicating sequence variation in the primer binding sites, a 'Touchdown' protocol was used. Failed PCR reactions were repeated at least twice, but failed reactions proceeded into the heteroduplex assay to serve as additional negative controls. PCR failures are assumed to be due to significant sequence variation in one or both primer binding sites.

For all genes, PCR products from all virus isolates were initially compared with the PCR product amplified from the Smith strain. If heteroduplexes were formed in this reaction, the HMA was repeated using other isolates as comparators (Table 1). For all genes, selected PCR products were completely sequenced to validate the HMA result, and to identify the location and nature of any sequence variation identified. Aligned gene sequences not presented in the results can be found in the Supplementary Data (Figs. S2A-S2D).

Genes with low-levels of variation

M33

M33 is homologous to the HCMV gene UL33, encoding a spliced 7-transmembrane-spanning protein with homology to cellular chemokine receptors (Davis-Poynter et al., 1997). It has recently been shown to functional similarities to the HCMV gene UL28, in that it signals constitutively and is a ligand for

Table 1
Summary of HMA analyses of selected genes from 26 strains of MCMV

Gene	Variation (vs. Smith)	HMA genotypes
m04	ND	NA
m06	+	>4
M33	−	NA
M44	+	3
mck-2 ex1	−	NA
mck-2 ex2	−	NA
m138	+	>2
m144	+	3
m145	+	ND
m147.5	−	NA
m152	−	NA
m155	+	2

Variation was initially determined by a capacity to form heteroduplexes when compared against the Smith strain gene. Genotypes were further determined by comparison against genes from other strains. ND = not determined. NA = not applicable.

RANTES (Melnychuk et al., 2005; Waldhoer et al., 2002). This gene was amplifiable from all but 1 of the 26 isolates tested. HMA analysis compared to the Smith gene demonstrated that 5 isolates produced heteroduplexes, however the retardation of these within the gel was not significant (Fig. S1A). Sequence analysis of selected isolates identified very few SNPs within the coding sequence, although higher levels of variation were found within the intron (Supplementary Fig. S2A). Sequences were deposited in the EMBL database under accession number AM236129.

MCK-2

MCK-2 is the product of a spliced transcript from the open reading frames m131 and m129, and is a CC-chemokine homologue (Fleming et al., 1999). It confers increased inflammation, higher levels of viremia, and higher titers of virus in salivary glands, consistent with a role in promoting dissemination by attracting an important mononuclear leukocyte population (Saederup and Mocarski, 2002). The 2 exons of this gene were examined independently, as we hypothesised that the intron would be a major source of variation. Both exon 1 (m131) and exon 2 (m129) were amplifiable from all isolates. HMA analysis compared against the Smith gene produced no heteroduplexes at all for exon 1, and whilst 6 of the isolates produced heteroduplexes for exon 2 the degree of retardation was minor, indicating very low level sequence variation (Fig. S1B). Sequence analysis of selected isolates confirmed these results, with exon 1 being identical for all isolates and only 7 variable nucleotides within the exon 2 sequence resulting in a maximum of 4 amino acid substitutions (2 non-conservative) (Supplementary Fig. S2B). Sequences were deposited in the EMBL database under accession numbers AM236130–AM236132.

m147.5

m147.5, (or mod b7.2) is a recently discovered gene which encodes a spliced transcript (Loewendorf et al., 2004).. The product of this gene is capable of down-regulating the expression of CD86, a co-stimulatory molecule on the surface

of MCMV-infected antigen-presenting cells. Exon 1 of this spliced gene only consists of 59 nt, and it was therefore considered unsuitable for this type of PCR-based analysis. Exon 2 could be amplified from all but 2 of the MCMV isolates tested (and these 2 samples were amplified subsequently), and upon HMA comparison to the Smith gene no heteroduplexes were formed (Fig. S1C). Sequence analysis of the G4 isolate confirmed this observation with only 3 SNPs within the 402 nt coding sequence, resulting in 2 conservative amino acid substitutions (Supplementary Fig. S2C). Sequences were deposited in the EMBL database under accession number AM236133.

m152

Interestingly, the m152 gene product (gp40) has two functions. It prevents the transport of MHC class I molecules to the cell surface, causing them to be retained in the cis-Golgi (Ziegler et al., 1997). It has also been shown to act similarly to the m155 gene product in protecting infected cells against clearance by NK cells, although by down-regulating a different ligand for NKG2D, RAE-1 (Lodoen et al., 2003). m152 was amplifiable from all isolates, and upon HMA comparison with the Smith gene none of the isolates formed a heteroduplex (Fig. S1D). Sequence analysis of isolates C4A and G4 confirmed this, with only 14 variable nucleotides within the gene (Supplementary Fig. S2D), resulting in a maximum of 10 amino acid substitutions (4 between Smith and G4, 8 between Smith and C4A). Sequences were deposited in the EMBL database under accession numbers AM236134–AM236135.

Genes which vary considerably between MCMV isolates

m04

m04 encodes a protein (gp34) which binds to MHC Class I molecules without preventing expression on the cell surface (Kleijnen et al., 1997), presumably preventing antigen presentation (Kavanagh et al., 2001a, 2001b). Despite using 6 combinations of PCR primers (2 5′ to the gene and 3 3′ to the gene), m04 was only amplifiable from 5 of the 26 MCMV isolates (Smith, K181, G4, C4D and C12A) and was therefore not analysed by HMA. Sequence analysis of the m04 gene from these isolates demonstrated significant nt sequence variation (ranging from 73% to 90% similarity to Smith), particularly at the 5′ end of the gene (Fig. 1). Analysis of the predicted protein sequence of this gene identified a conserved transmembrane region and cytoplasmic tail, but a highly variable extracellular domain (amino acid identity to Smith ranging from 58% to 84%). Sequences were deposited in the EMBL database under accession numbers AM236096–AM236098.

m06

m06 encodes a protein (gp48) which interferes with the MHC class I pathway of antigen presentation. This protein binds to peptide-loaded MHC class I molecules in the ER and transports them to the lysosome for degradation, therefore preventing Class I expression at the cell surface (Bubeck et al., 2002; Reusch et al., 1999). This gene was amplifiable from all isolates tested (although isolate 16, K17A, only gave a faint PCR product). Upon HMA analysis, several isolates formed heteroduplexes against Smith, K181, G2 or W4. These data suggest that the m06 gene exists as one of a number of genotypes within the MCMV population (Fig. 2A). Sequence analysis of PCR products from selected isolates demonstrates that whilst SNPs occur at a low level throughout the gene (at 34 locations within the gene length of 1053nt), these predominantly occur in only one or two of the 11 isolates sequenced. Almost 59% of the SNPs result in an amino acid change in at least one of the isolates, although only 3 (8.8%) of these are non-conservative substitutions. Interestingly, there are 3 regions within the gene at which conserved indels (insertions or deletions) are found. These are found in different combinations in different isolates, suggesting that there may be up to eight m06 genotypes circulating (2^3 combinations of indels) (Fig. 2B). However, we have currently only identified viruses containing 5 of these possible genotypes. The distribution of these indels does not match the phylogeny of the total m06 gene sequences derived using maximum parsimony as gaps are excluded in this analysis (Fig. 2C). The distribution of indels is, however, predicted by the heteroduplex patterns. The functionally important di-leucine motif encoded towards the 3′ end of the gene is unchanged in all isolates (CTGCTC). Sequences were deposited in the EMBL database under accession numbers AM236099–AM236108.

M44

M44 (encoding pp50) is the MCMV homologue of the HCMV DNA polymerase accessory protein gene UL44 (Loh et al., 2000), and was originally chosen for analysis as it was assumed that it would be highly conserved between viruses. UL44 has sequence identity of between 98 and 99% between 6 laboratory and clinical isolates of HCMV (Murphy et al., 2003). This gene was consistently not amplifiable from 4 of the 26 MCMV strains, immediately indicating a level of sequence variation not seen in HCMV. Upon HMA (compared to the Smith gene) more than half of all isolates produced heteroduplexes with significantly retarded mobility through the gel (Fig. 3A). Sequencing of selected isolates demonstrated that the level of SNPs is low throughout the gene sequence, however all isolates contained a deletion (compared to the Smith sequence) at the 3′ end of the gene (C-terminal end of the protein). This deletion was only 3nt (a single amino acid) in isolates G4 and C4B, but was 12nt (4 amino acids) in isolate WP36A. In none of the isolates were the previously identified RGD motif or nuclear localisation signal affected by the deletions (Fig. 3B). Sequences were deposited in the EMBL database under accession numbers AM236109–AM236110.

m138

m138 encodes a protein which is a functional homologue of a cellular Fc-receptor. However, recombinant MCMVs constructed with an inactivated m138 gene are still attenuated for growth in antibody-deficient mice (Crnkovic-Mertens et al., 1998). This gene is amplifiable from all MCMV isolates, however in some isolates faint amplification suggested possible

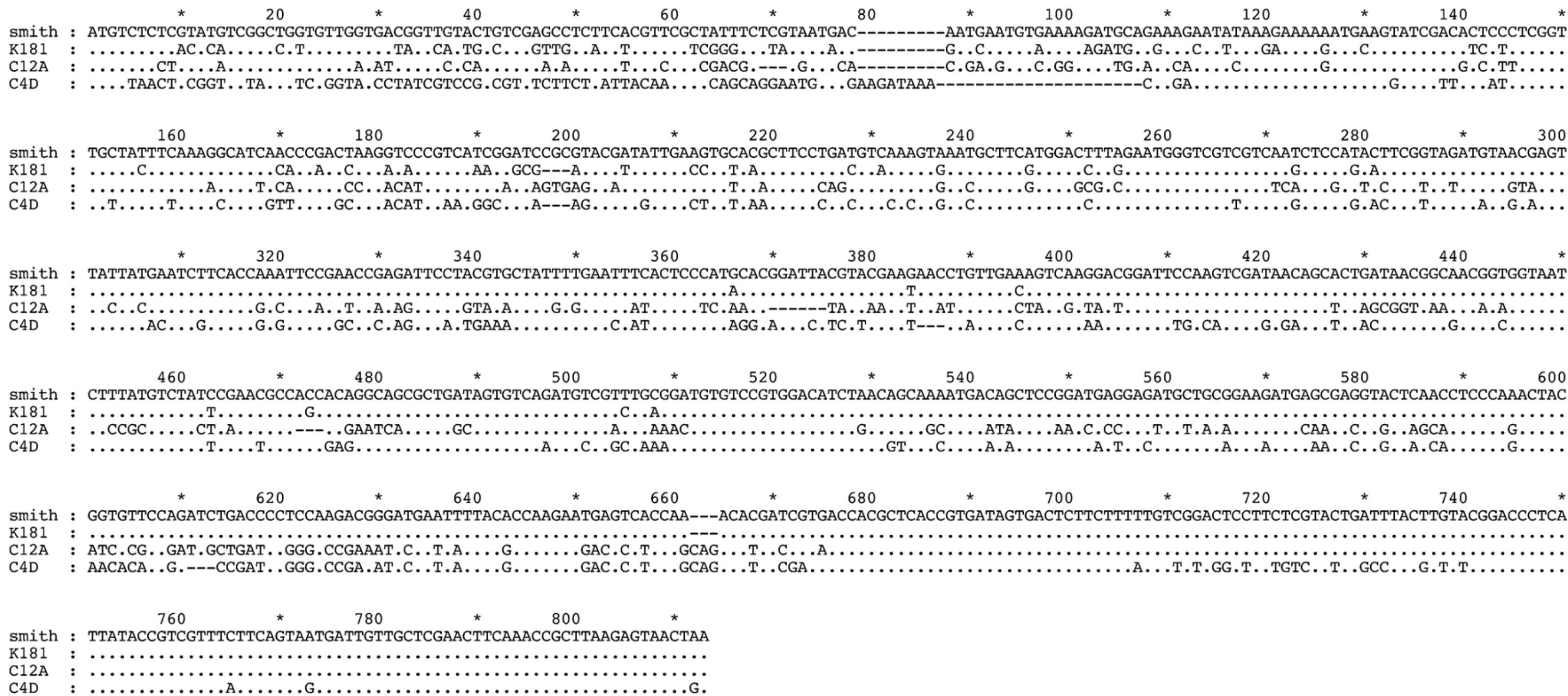

Fig. 1. Alignment of m04 sequences from isolates C12A, C4D, K181, and the published Smith strain. ‘Dot’ indicates sequence identical to Smith, ‘Dash’ indicates gap introduced into alignment.

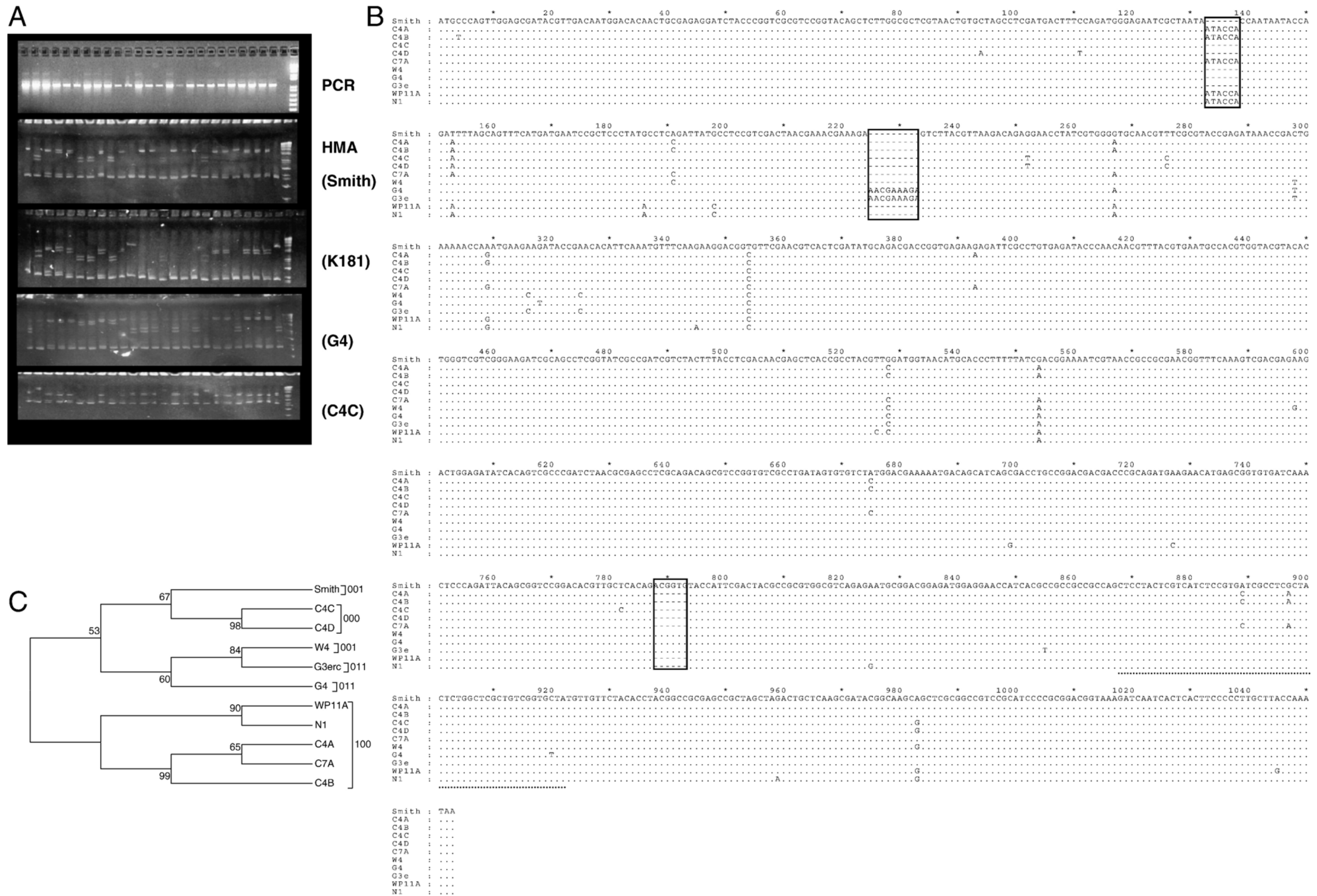

Fig. 2. Analysis of m06 gene variation. (A) HMA comparisons to the m06 genes from Smith, K181, G4 and C4C. Strains analysed and loaded in the order described in Table 3. (B) Alignments of m06 gene sequences from 11 MCMV strains. Underlined is the sequence encoding the predicted transmembrane domain of the protein, and boxed is the sequence of the di-leucine motif vital for targeting of MHC Class I to the lysosome. Indel loci are shaded. 'Dot' indicates sequence identical to Smith, 'Dash' indicates gap introduced into alignment. (C) Phylogenetic analysis of m06 gene sequences, with patterns of insertions/deletions at the 3 loci identified by 1 or 0 as appropriate.

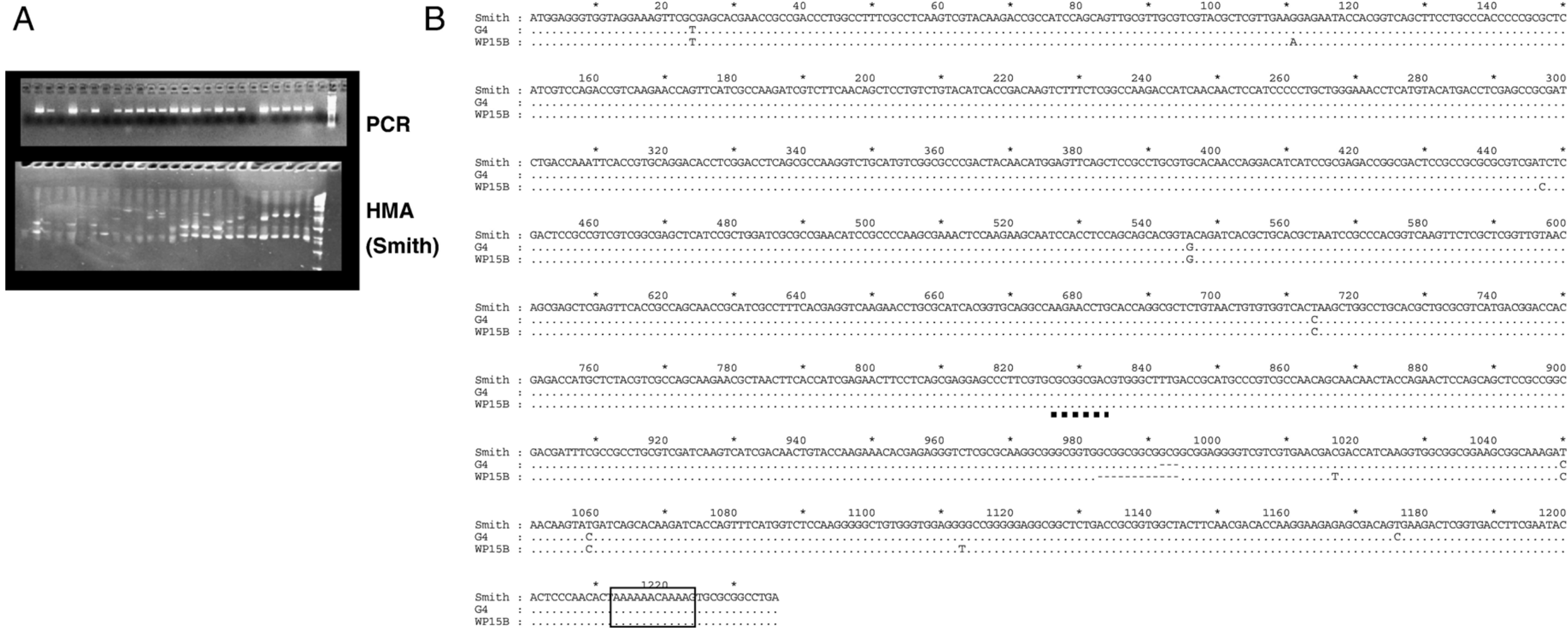

Fig. 3. Analysis of M44 gene variation. (A) HMA comparisons to the M44 gene from Smith. Strains analysed and loaded in the order described in Table 3. (B) Alignments of M44 gene sequences from 3 MCMV strains. Boxed sequence encodes the nuclear localisation signal, and underlined sequence encodes the RGD motif. ‘Dot’ indicates sequence identical to Smith, ‘Dash’ indicates gap introduced into alignment.

sequence variation in the primer binding sites. When analysed by HMA against the Smith gene, almost all isolates produced heteroduplexes with significantly retarded mobility within the gel (Fig. 4A). Sequence analysis confirmed the variability of the m138 gene with MCMV isolates. Variable nucleotides are distributed throughout the m138 gene sequence at up to 89 sites (5.2% of the gene length), 49 of which result in an amino acid substitution in at least one isolate. Of these possible substitutions, 10 are non-conservative (Fig. 4B). There are also several small indels towards the 3′ end of the gene, which do not disrupt the reading frame. Sequences were deposited in the EMBL database under accession numbers AM236111–AM236113.

m144

m144 encodes a homologue of an MHC class I molecule, similar to that encoded by the HCMV gene UL18. Like UL18, m144 is capable of binding β-2 microglobulin, but unlike UL18 it is unable to bind endogenous peptides (Chapman and Bjorkman, 1998) and it has been suggested that m144 mimics cellular MHC class I molecules and acts as a decoy for NK cells by engaging inhibitory NK-cell receptors (Cretney et al., 1999; Farrell et al., 1997; Kubota et al., 1999). The m144 gene was amplifiable from all but one isolate of MCMV. HMA analysis with comparison to the Smith, C12A and G2 genes suggested that there are 3 genotypes of m144 within populations of MCMV, with one genotype being rare (Fig. 5A). Sequence analysis of the m144 gene from several isolates shows that whilst variable nucleotides are distributed throughout the gene they are more frequent in the first 600nt of the gene sequence (Fig. 5B). Overall sequence identity to the Smith isolate ranges from 94% to 99%. Of the 93 possible variant nucleotides, 35 result in amino acid substitutions (10 of which are non-conservative). There are also 2 conserved indels, one of 3nt and one of 6nt, which appear to correlate with the genotypes identified by HMA and with the phylogeny derived using maximum parsimony (Fig. 5C). Sites of amino acid variation for G4 and WP15B (compared to the Smith sequence) were mapped onto the recently published crystal structure of the m144 protein (Natarajan et al., 2006). Both isolates contained variation within the α3 domain, with WP15B also varying within the α2 domain. Both isolates also contained variation within the region of protein for which no electron density could be observed, which is the suggested ligand binding site (Fig. S3). Sequences were deposited in the EMBL database under accession numbers AM236114–AM236121.

m145

Along with m152 and m155, m145 encodes a protein which down-regulates the cell surface expression of an NKG2D ligand. In this case, m145 expression results in down-regulation of the stress-induced ligand MULT-1 (Krmpotic et al., 2005). The m145 gene was not amplifiable from 5 of the 26 isolates, suggesting sequence variation within the primer binding regions. HMA analysis compared to the Smith gene showed that almost all viruses produced heteroduplexes, although the gels were consistently unclear (Fig. 6A). Sequence analysis of the m145 gene from 3 isolates identified a high level of sequence variation, although once again SNPs are predominantly found in only one of the sequenced isolates. Each isolate was found to contain one indel of 3 nt, again suggesting the existence of multiple m145 genotypes (Fig. 6B). Sequences were deposited in the EMBL database under accession numbers AM236122–AM236124.

m155

The m155 gene product in MCMV down-regulates the level of H60 on the surface of infected cells, thereby preventing H60 interaction with NKG2D and NK-cell clearance of infected cells (Lodoen et al., 2004). The m155 gene was amplifiable from all but 2 of the MCMV isolates (one of these was amplified subsequently). HMA analysis compared to the Smith and G2 genes indicates that there are 2 genotypes of m155 within MCMV populations, but one is comparatively rare (Fig. 7A). Sequence analysis of the m155 gene from selected isolates shows that isolates are either highly conserved compared to the Smith sequence, or like isolate G3e are significantly different. Between Smith and G3e there are 71 nt changes, 39 of which result in amino acid changes (25 of which are non-conservative). There is also a conserved indel of 3nt (one amino acid) when comparing the 2 genotypes (Fig. 7B). Sequences were deposited in the EMBL database under accession numbers AM236126–AM236128.

m157

The m157 gene product from the Smith strain of MCMV has been shown to bind the NK-cell activating receptor Ly49H in C57BL/6 mice (Smith et al., 2002). However this gene has previously been shown to be highly variable in wild isolates of MCMV , and therefore was not analysed in this research. This sequence variation has also been shown to result in functional variation, as the m157 gene product from several of these isolates has been shown to be unable to bind to Ly49H (Voigt et al., 2003).

Evolutionary analysis of MCMV genes

The level of sequence variation within isolates of MCMV was determined by sequencing of genes from selected isolates. As most of the genes analysed in this study are involved in modulating the host immune response to the virus, we were interested in determining if genetic selection was occurring within MCMV populations. For genes which exhibited high levels of variation (m138, m144, m145 and m144), the level of selection was determined by calculating the frequencies of synonymous and non-synonymous mutations within each gene, using a moving window analysis on pairwise comparisons of all available sequences (Figs. 4D, 5D, 6D and 7D, respectively). If a gene existed in clearly delineated genotypes, only one sequence from each genotype was used in analysis. In none of the genes did we find regions where the rate of non-synonymous mutations was significantly greater than the rate of synonymous mutations (i.e., dN/dS > 1), indicating that positive selection is not acting on these genes. In fact, apart from m138, all the genes contained windows where dS/dN > 5, indicating strong purifying selection.

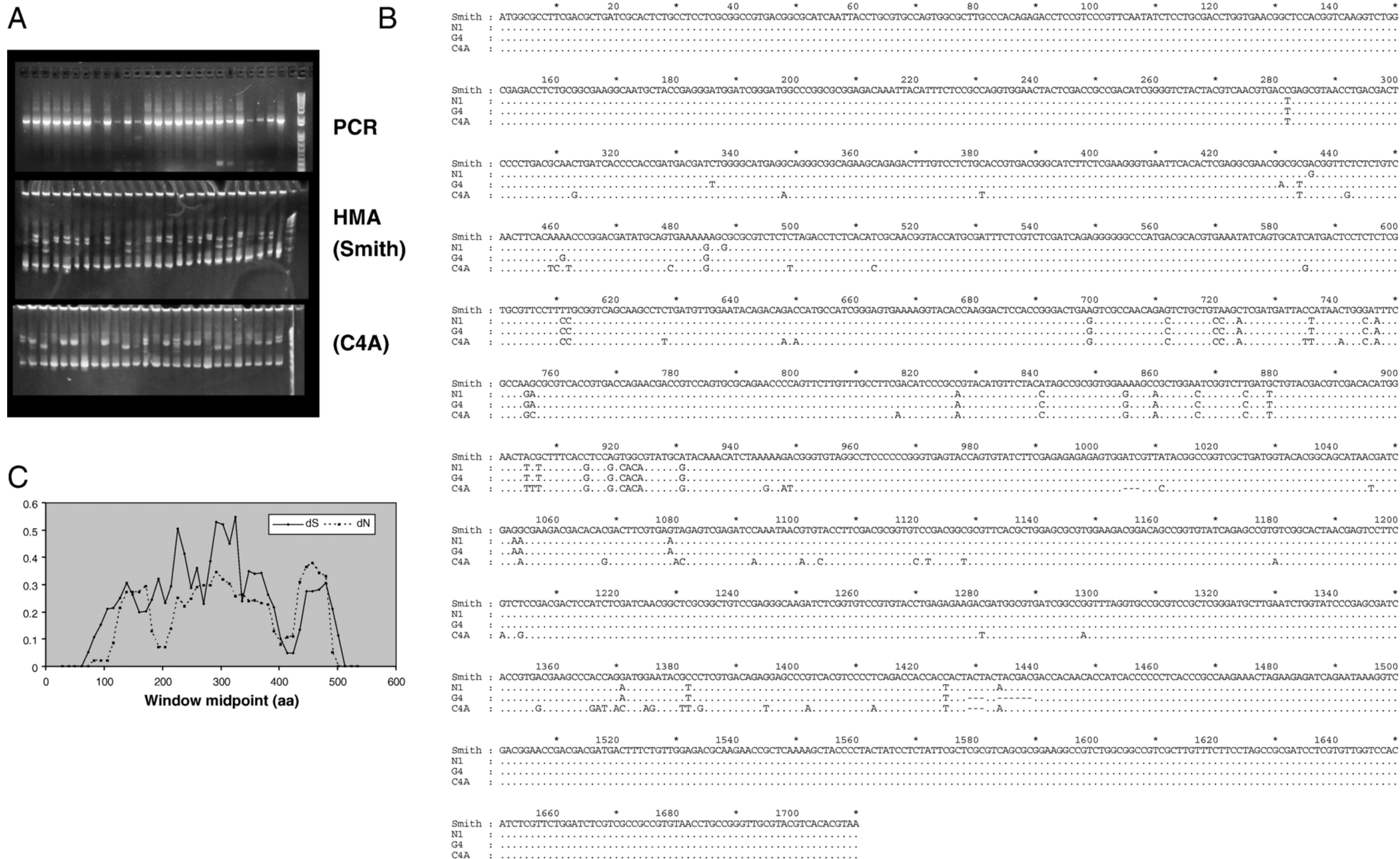

Fig. 4. Analysis of m138 gene variation. (A) HMA comparisons to the m138 genes from Smith, and C4A. Strains analysed and loaded in the order described in Table 3. (B) Alignments of m138 gene sequences from 4 MCMV strains. 'Dot' indicates sequence identical to Smith, 'Dash' indicates gap introduced into alignment. (C) Selection within m138 sequences as analysed by the rate of synonymous (dS) and non-synonymous (dN) mutations.

Fig. 5. Analysis of m144 gene variation. (A) HMA comparisons to the m144 genes from Smith, C12A, and G2. Strains analysed and loaded in the order described in Table 3. (B) Alignments of m144 gene sequences from 9 MCMV strains. 'Dot' indicates sequence identical to Smith, 'Dash' indicates gap introduced into alignment. Shaded regions indicate conserved indels. (C) Phylogenetic analysis of m144 gene sequences, with patterns of insertion/deletions identified by 1 or 0 as appropriate. (D) with m144 sequences as analysed by the rate of synonymous (dS) and non-synonymous (dN) mutations.

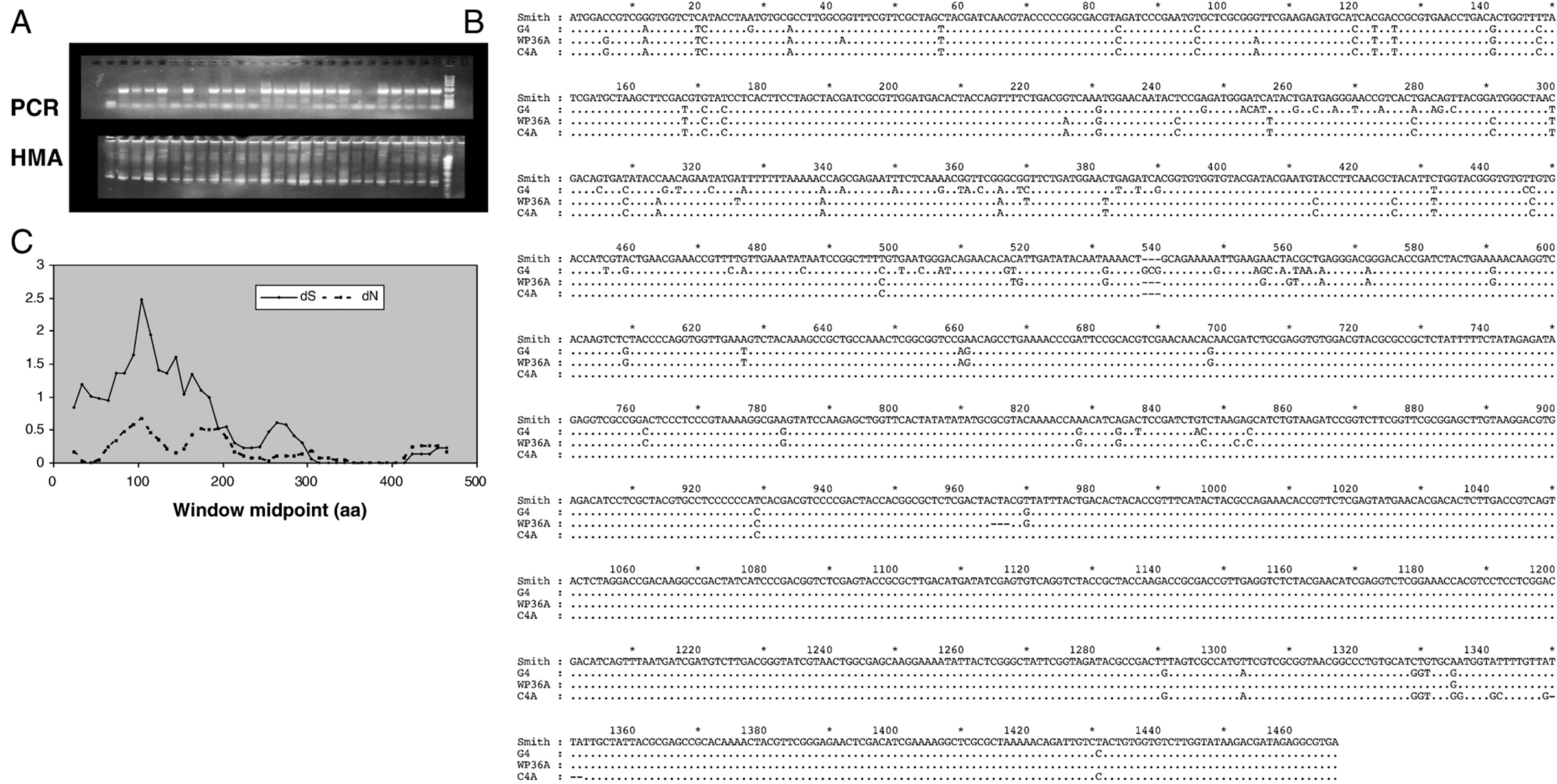

Fig. 6. Analysis of m145 gene variation. (A) HMA comparisons to the m145 gene from Smith. Strains analysed and loaded in the order described in Table 3. (B) Alignments of m145 gene sequences from 4 MCMV strains. 'Dot' indicates sequence identical to Smith, 'Dash' indicates gap introduced into alignment. (C) Selection within m145 sequences as analysed by the rate of synonymous (dS) and non-synonymous (dN)

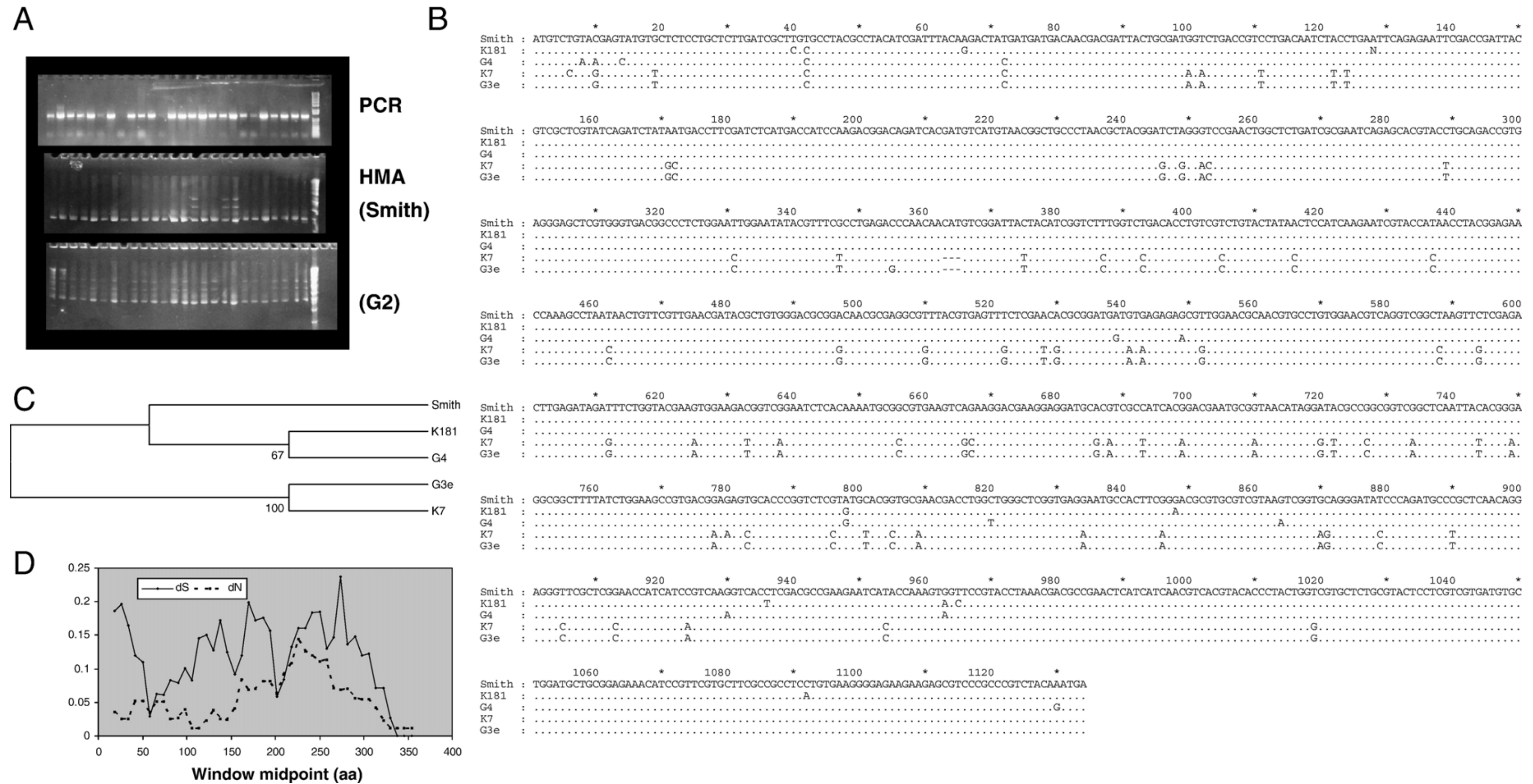

Fig. 7. Analysis of m155 gene variation. (A) HMA comparisons to the m155 genes from Smith and G2 . Strains analysed and loaded in the order described in Table 3. (B) Alignments of m155 gene sequences and phylogenetic analysis of m155 gene sequences. 'Dot' indicates sequence identical to Smith, 'Dash' indicates gap introduced into alignment. (C) Phylogenetic analysis of m155 gene sequences. (D) Selection within m155 sequences as analysed by the rate of synonymous (dS) and non-synonymous (dN) mutations.

Both m06 and m144 exist as a number of distinct genotypes, and therefore aligned gene sequences were examined for evidence of recombination. Using several different tests (RDP, BOOTSCAN, and GENECONV), 2 regions of the m144 gene (from approximately nt 120 to nt 246, and from approximately nt 650 to nt 950) were identified as possible sites for recombination, with $P < 0.05$.

Discussion

We have analysed the level of sequence variation in selected genes of 26 isolates of MCMV, including both of the commonly used laboratory isolates. Several of these genes were shown to be highly variable or to exist as a number of distinct genotypes, leading to speculation of functional variation in naturally occurring MCMV infections and the applicability of the laboratory strains to studies of 'wild strains' of virus.

How representative are laboratory strains of MCMV of circulating viruses?

Serial passage has altered the laboratory strains of HCMV such that they are considerably different, both genetically and in their growth characteristics, from strains of HCMV isolated from patients (Murphy et al., 2003). Almost all laboratory research on murine cytomegalovirus utilises either the Smith or K181 strains of the virus. These strains have been serially passaged in vivo and in vitro for, in the case of the Smith virus, more than 50 years (Smith, 1956). For this reason, it can no longer be assumed that the laboratory strains of MCMV are representative of viruses circulating within wild mouse populations; indeed, it may be that the Smith virus found in one laboratory may differ from the Smith strain found elsewhere (Hudson et al., 1988; Osborn, 1982). Whilst we have not looked for the large-scale genomic rearrangements found in HCMV, sequence analysis of selected genes from 2 strains of MCMV shows significant levels of variation from the published Smith sequence and from each other (summarised in Table 2). Due to this level of variation, it is possible that 'wild' isolates of MCMV may prove to be more reliable models of clinical HCMV infection. Interestingly, the presence of different m06 genotypes in K181 and Smith suggests that K181 is not simply a variant of the Smith virus, but a different isolate.

MCMV genes exist as a number of distinct genotypes

Several HCMV genes have been shown to exist as a number of genotypes. These include those encoding surface glycoproteins gB and gN (Rossini et al., 2005; Steininger et al., 2005) which are presumably under selective pressure, but also genes such as the chemokine homologues UL74, UL144, UL146 and UL147 (Arav-Boger et al., 2006; Stanton et al., 2005) which have immune modulatory functions. Whilst a particular UL144 genotype was recently correlated with congenital HCMV disease (Arav-Boger et al., 2002), no such correlation was found with individual genotypes of UL146 or UL147, and there was strong evidence of purifying selection within genotypes of these genes (Arav-Boger et al., 2006). At least 3 of the genes examined in this study exist as distinct genotypes in MCMV populations (m06 has at least 5 genotypes, m144 has at least 3 genotypes, and m155 has 2 distinct genotypes) and these genotypes are also evolving under purifying selection.

Is there functional variation between the same gene from different isolates?

The m157 gene has been shown to be highly variable, with broadly 3 genotypes identified in 29 strains of MCMV (Voigt et al., 2003). It has been shown that the m157 gene product binds the Ly49I inhibitory NK-cell receptor (Arase et al., 2002). Interestingly the Smith strain (and those with identical m157 sequences) and 2 other virus strains with high m157 sequence

Table 2
Comparison of selected gene sequences from wild isolates G4 and C4A the published Smith isolate sequence

Gene	Comparison Smith to:				Comparison G4 to C4A	
	G4		C4A			
	nt (% identity)	aa (% identity/% similarity)	nt (% identity)	aa (% identity/% similarity)	nt (% identity)	aa (% identity/% similarity)
m04	96.9	91.0/93.2	NA	NA	–	–
m06	98.5	98.0/98.6	97.8	97.4/99.1	97.1	96.6/97.7
M33	ND	ND	97.8	99.5/100	–	–
M44	99.3	99.8/99.8	ND	ND	–	–
mck-2 (ex1)	100	100/100	100	100/100	100	100/100
mck-2 (ex2)	99.5	98.5/99.5	99.5	98.5/99.5	100	100/100
m138	97.1	95.1/97.5	94.7	91.2/95.8	96.2	94.4/97.2
m144	97.9	97.1/98.4	97.2	96.3/98.7	97.2	95.6/97.7
m145	93.0	92/95.9	96.4	96/97.9	93.6	92/96.3
m147.5 (ex2)	99.3	98.5/99.2	ND	ND	–	–
m152	99.5	98.9/99.5	98.9	97.9/99.2	99.1	97.9/99.2
m155	98.8	97.9/97.9	ND	ND	–	–

NA—Gene not amplifiable from this isolate. ND—Sequence not determined.

homology to Smith (N5 has 84.6% similarity and G1F has 86.7% similarity) are able to bind an NK-cell activating receptor Ly49 H, which allows effective control of MCMV infection in Ly49H$^+$ mice (Voigt et al., 2003). It has also been suggested that m157 may be able to bind other Ly49 receptors. As the Ly49 gene locus has been shown to be highly variable in wild mice, with the complement of Ly49 genes varying between individuals, then this may be the reason for existence of multiple m157 genotypes within viral populations.

The effects of sequence variation within HCMV populations has been demonstrated recently. Variation within the UL18 gene of clinical isolates of HCMV has been shown to change the binding avidity of the UL18 protein to the ILT2 receptor, with reduced binding affinity correlating to a reduced ability to inhibit ILT-2 expressing NK-cells in vitro (Vales-Gomez et al., 2005).

Interestingly, it is generally the viral genes which interact with polymorphic components of the murine immune response (m04 and m06 with MHC class I, m145 and m157 with NK-cell receptors) which are the most variable. The exception to this rule is the m152 gene which is highly conserved, however variation within this gene is probably limited as it serves 2 functions. The existence of a rare genotype of m155 is also of interest, suggesting either a correspondingly rare genotype of H60 (or an intermediate molecule), or a possible second function for m155. H60 polymorphism has been demonstrated at the level of expression (Malarkannan et al., 1998), with sequence variation in the C57BL/6 H60 also being suggested if not explicitly stated (Diefenbach et al., 2000). However it should be noted that in contrast with MCK-2, the CC chemokine homologue found in MCMV, the CXC chemokine homologues found in HCMV, UL146 and UL147, have been shown to be highly variable (Arav-Boger et al., 2006).

The recent crystal structure of the m144 gene product from the Smith virus (Natarajan et al., 2006) included one region for which no electron density was determined and therefore for which no structure was predicted. However, it was suggested that this region of apparent structural flexibility between the 2 α-helices could be the ligand binding site. As our data shows, this site corresponds to a highly variable region of the m144 sequence, possibly suggesting that m144 binds a variable ligand.

Multiple infection and gene variation

Four of the isolates used in this study, C4A, B, C and D, were plaque purified viruses isolated from the same mouse, which were identified as being distinct isolates due to their RFLP patterns (Gorman et al., 2006). These isolates between them had 2 different m06 genotypes (A + B have the same genotype, as do C + D), 3 different M44 genotypes (B + D have the same genotype), 2 different m138 genotypes (A + D have the same genotype, as do B + C), and 3 different m145 genotypes (A and C have the same genotype). All 4 isolates have different m144 genotypes, and identical m155 genotypes. This pattern of multiple viral genotypes within the same animal suggests that these viruses are not simply variants of a progenitor virus which have adapted to their particular host, but are distinct viruses which co-exist within the host. We have recently shown that at least 34% of free living mice are infected with at least 2 viruses of differing ie1 genotypes (Gorman et al., 2006) and our current research suggests that this is also true for multiple m06 and m144 genotypes (unpublished data). This phenomenon, combined with possible functional variation of virus genotypes, may indicate that multiple cytomegaloviruses are capable of complementing each other within a host, enhancing survival of the entire virus population.

Materials and methods

Cells and viruses

Strains of MCMV were as described in Table 3. All viruses had been previously subjected to 3 rounds of plaque purification on murine embryo fibroblasts (MEFs). Isolates C4A, B, C, and D were all isolated from the same mouse, as were isolates WP11A and B, and isolates WP36A, B and C.

DNA extraction

Viruses were inoculated onto MEFs in 6-well trays. Upon visible cytopathic effect (CPE), medium was removed from cells and 4 M guanidine hydrochloride added to each well. DNA was extracted by 3× extractions with phenol:chloroform followed by ethanol precipitation, and resuspended in TE buffer.

Table 3
Isolates of MCMV used in this work

Number	Virus Isolate	Mouse Trapped	Reference
1	K181^{PERTH}		
2	G4	Geraldton, WA	Booth et al. (1993)
3	N1	Nannup, WA	Booth et al. (1993)
4	C4A	Canberra, ACT	Gorman et al. (2006)
5	C4B	Canberra, ACT	Gorman et al. (2006)
6	C4C	Canberra, ACT	Gorman et al. (2006)
7	C4D	Canberra, ACT	Gorman et al. (2006)
8	C7A	Canberra, ACT	Gorman et al. (2006)
9	C12A	Canberra, ACT	Gorman et al. (2006)
10	W3	Walpeup, VIC	Booth et al. (1993)
11	W4	Walpeup, VIC	Booth et al. (1993)
12	W5	Walpeup, VIC	Booth et al. (1993)
13	K4	Kerguelen Island	Booth et al. (1993)
14	K6	Kerguelen Island	Booth et al. (1993)
15	K7	Kerguelen Island	Booth et al. (1993)
16	K17A	Kerguelen Island	Booth et al. (1993)
17	G1A	Geraldton, WA	Booth et al. (1993)
18	G2	Geraldton, WA	Booth et al. (1993)
19	G3E	Geraldton, WA	Booth et al. (1993)
20	WP11A	Walpeup, VIC	unpublished
21	WP11B	Walpeup, VIC	unpublished
22	WP15B	Walpeup, VIC	unpublished
23	WP36A	Walpeup, VIC	unpublished
24	WP36B	Walpeup, VIC	unpublished
25	WP36C	Walpeup, VIC	unpublished
26	SMITH		

Table 4
PCR primers used to amplify viral genes

Gene	Primers (5′–3′)
m04	Forward: gagcgacggatggtacaag Reverse: ccggaaaatggtttactcaa
m06	Forward: atggacaatgaagccaatct Reverse: aggtactaaaacagtttcca
M33	Forward: gacgaccggctcgagatg Reverse: gccgcgctgcgatcactg
M44	Forward: cagaataaggcattcagg Reverse: tacatctccgcgggaatg
mck-2 exon 2 (m129)	Forward: aggacacgaggtatgtcat Reverse: tgtgtatatagatctcgg
mck-2 exon 1(m131)	Forward: cataggcagtaacgacac Reverse: caggtgagtgttttgatctg
m138	Forward: cgatgacttgagttacgt Reverse: ggcagagccgaggcgatg
m144	Forward: agttacgcggccctcttttaca Reverse: catgaatgtgagatgtcgc
m145	Forward: atacagagattcggacagtca Reverse: ggtgtttcaactccgcga
m147.5 exon2	Forward: caacatcacacaagtttattg Reverse: atgttaattttcacatacaga
m152	Forward: gccggtcgcacgaacatca Reverse: cgatcgctagcctgtaca
m155	Forward: gggggggaccggggtgatcattt Reverse: ttacagactttcgtcgaaaatg

PCR

PCR primers were designed to amplify several genes from the MCMV genome, based on the published Smith virus sequence (Table 4). Primers were designed to start and finish outside the gene open reading frame, so as not to bias the HMA results. PCR annealing temperatures were determined by amplification of Smith virus DNA. Genes which were not amplifiable using this (or a slightly lower) annealing temperature were PCR amplified using a touchdown protocol as follows: Initial denaturation (95 °C for 2 min) followed by touchdown protocol (15 cycles of 95 °C for 30 s, X °C for 60 s, 72 °C for 120 s, where X decreases from 63 °C by 1 °C per cycle) and a normal PCR protocol (25 cycles of 95 °C for 30 s, 48 °C for 60 s, 72 °C for 120 s).

Each reaction contained 5 nmol each dNTP and 10 pmol each primer. PCR products (2 μl) were analysed on a 1% agarose gel.

Heteroduplex mobility assay

PCR products were diluted 1:3 before use in the HMA. For each virus, 1 μl diluted PCR product was mixed with 1 μl of a ‘standard’ PCR product (from whichever virus the samples were being compared against) in a 10-μl volume containing 10 mM Tris–HCL pH 7.8, 100 mM NaCl, 2 mM EDTA. Samples were heated to 95 °C for 5 min, and then slow cooled to 68 °C at a rate of 1 °C per minute. Samples were rapidly cooled to 4 °C and stored on ice prior to loading on a gel. Samples were mixed with loading buffer and loaded onto a 6% polyacrylamide:bis (19:1) gel containing 15% urea. The gel was electrophoresed at 300v for 3 h, and stained with ethidium bromide for 15 min prior to visualisation.

Sequencing and data analysis

PCR products were purified and sequenced by Macrogen Inc (Seoul, Korea). Sequences were assembled and analysed using the Vector NTi suite of programmes (Invitrogen Inc.). Multiple sequence alignments were performed using CLUSTALW and subsequently edited manually. Aligned sequences were compared using MatGat (Campanella et al., 2003). Levels of synonymous and nonsynonymous mutations were calculated on aligned coding sequences using CRANN, with a moving window of 10% of the gene length shifted by 20% of the window length (Creevey and McInerney, 2003). The computer program RDP-V2, implementing the RDP, BOOTSCAN, and GENECONV, was used to analyse sequences for evidence of recombination (Martin et al., 2005).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2006.04.031.

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