Role of mutations identified in ORFs M56 (terminase), M70 (primase) and M98 (endonuclease) in the temperature-sensitive phenotype of murine cytomegalovirus mutant tsm5

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ABSTRACT

Twenty-six non-synonymous and synonymous mutations have been identified in the temperature-sensitive (ts) mutant (tsm5) of the K181 (Birmingham) variant of murine cytomegalovirus that is deficient in DNA synthesis, processing and packaging at the non-permissive temperature and produces undetectable levels of infectious virus in mice. Non-synonymous mutations identified in the M70 (primase), M56 (terminase) and M98 (nuclease) ORFs were introduced individually and in combination into the K181 (Perth) variant using BAC technology to examine their role in the ts phenotype. The M56 (G439R) and M98 (P324S) mutations had no evident role in the ts phenotype. However, the C890Y M70 mutation alone and in combination with the M56 and/or M98 mutations rendered the virus ts, unable to replicate in mice and highly defective in DNA synthesis. Reversion of the tyrosine mutation to cysteine or introduction of C890M (experimentally) or C890S (naturally) restored the wt phenotype.

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Introduction

Most current studies aimed at identifying ORFs involved in important aspects of murine cytomegalovirus (MCMV) biology use gene knock-outs which identify genes as essential or non-essential for virus replication and imply in vivo roles for non-essential ORFs. However, this approach gives little indication of the function of the gene product or functionally important domains. Targeted mutagenesis is a more meaningful approach but targets to be mutated require prior knowledge. The classical approach, now largely neglected, is to mutate virus using mutagens and then to identify mutations associated with the observed phenotype.

Mutant tsm5, created by mutagenesis of the murine cytomegalovirus (MCMV) K181 (Birmingham) variant by treatment with N-methyl-N′-nitro-N-nitrosoguanidine (NTG), is restricted in growth at 40 °C (Sammons and Sweet, 1989). Titres of this mutant were reduced by ~90% at 40 °C, concatameric DNA was cleaved into unit length genomes with close to wt efficiency (Sweet et al., 2007). This suggested a defect in DNA replication, processing or packaging and sequence analysis of genes involved in these processes identified three mutations: a C to T mutation leading to G439R residue change in the putative large terminase subunit (M56); a C to T mutation (P324S) in the putative alkaline nuclease (M98); and a C to T mutation (C890Y) in the putative primase component (M70) of the helicase–primase complex (Sweet et al., 2007).

In this study, these mutations have been introduced individually and in combination into the K181 (Perth) strain of MCMV using the K181 MCMV bacterial artificial chromosome (BAC) to examine their role in the ts and in vivo phenotype of the virus.

Results

Comparison of K181 (Perth) and K181 (Birmingham) variants

To examine the role of the mutations identified in tsm5 in its phenotype mutations were introduced individually and in combi-
nation into the wt K181 parental strain using BAC mutagenesis. The mutant tsms was derived from the K181 (Birmingham) variant whereas the available K181 BAC was derived from the K181 (Perth) variant. As it was possible that the two K181 variants would replicate differently it was important to compare the two. The K181 (Perth) variant was recovered from the K181 (Perth) BAC following transfection of NIH 3T3 cells and passage in MEFs to remove the BAC. As shown in Fig. 1A the two strains behaved similarly at 37 °C and 40 °C thus allowing the use of the K181 (Perth) BAC to examine the role of the tsms mutations in replication.

Production of Mt70890Y, Mt98324S and Mt56439R mutants and their revertants

These mutant viruses were produced by a two-step Red-mediated recombination in which the ORF was first replaced by an rpsL-neo cassette and then by the mutated form of the respective ORF as described in Materials and methods. A further two-step protocol was required to produce their corresponding revertants whereby the mutated ORF was replaced by the rpsL-neo cassette and this in turn was replaced by the wt ORF. Mutant viruses Mt70890Y, Mt98324S and Mt56439R and their corresponding revertant viruses Rv70C890, Rv98P324 and Rv56G439 were then derived following transfection of the respective MCMV BAC plasmid into NIH 3T3 cells and selection of isolated plaques. Viruses containing BACs were then passaged, selecting individual plaques each time, to produce viruses in which the BACs had been excised as confirmed by PCR and sequencing. To remove the BACs required between 3 and 6 passages and was facilitated by homologous recombination between 249 bp of MCMV sequence flanking both ends of the BAC cassette (Redwood et al., 2005).

The strategy used to confirm the correct structure of the constructed BACs is shown for the mutant Mt56439R in Fig 2. This analysis was done at each step of the procedure and for the final isolated mutant or revertant. Further confirmation that no unexpected rearrangements had taken place was obtained using restriction fragment polymorphism analysis with HpaI (Fig. 3). This pattern agreed well with the theoretical RFLP pattern. Other enzymes tested include EcoRI, AseI, and Dral (data not shown). Sequencing demonstrated that the correct mutation had been introduced (data not shown).

Replication kinetics of viruses in tissue culture

Mutants Mt56439R and Mt98324S showed the wt phenotype when examined over 9 days at 37 °C and 40 °C (Figs. 1B and C). Both viruses showed similar growth kinetics at 37 °C and yields were reduced by ~10 fold at 40 °C. No significant differences in growth kinetics were observed for the corresponding revertant viruses Rv56439R and Rv98324S (Figs. 1B and C).

In contrast, mutant Mt70890Y was temperature-sensitive (Fig. 1D). Its growth at 37 °C was similar to Mt56439R and Mt98324S and to revertant Rv70C890 but it replicated poorly at 40 °C and this replication was considerably delayed until days 8–9 when titres of 1.0–2.0 log₁₀ pfu/ml were detected. Although yields varied somewhat at 40 °C between experiments the results shown in Fig. 1D, which are the means ± SD of 3 separate experiments, clearly demonstrate that Mt70890Y is temperature-sensitive. Sequencing showed that the virus isolated at 9 days post infection was still mutant (data not shown). One of these experiments, in which titres were relatively high, was continued up to 21 days post infection (Fig. 4A); yields only reached a maximum of 2.8 log₁₀ pfu/ml and virus remained mutant (data not shown).
Production of $\text{Mt98}^{324570890Y}$ and $\text{Mt98}^{324570890Y56439R}$ mutants and their revertants

Mutant $\text{Mt70}^{890Y}$ is thus temperature-sensitive and shows similar replication kinetics to $\text{tsm}^{5}$ at 40 °C. However, different stocks of $\text{tsm}^{5}$ are mixtures of mutants and show allelic polymorphism in their $\text{M56}$, $\text{M98}$ and $\text{M70}$ mutations with either mutant nucleotide, wt nucleotide or both (Timoshenko et al., 2009). It was thus possible that other mutations were modifying the effect of the $\text{M70}$ mutation in $\text{tsm}^{5}$. To examine this, a double mutant containing the $\text{M70}$ and $\text{M98}$ mutations ($\text{Mt98}^{324570890Y}$) and a triple mutant ($\text{Mt98}^{324570890Y56439R}$) containing all 3 mutations were constructed together with their revertants. As this involved up to 6 recombination events for the triple mutant care was taken to analyse each virus for correct construction, lack of DNA rearrangement and inserted mutation at each step as described above (data not shown).

Mutant $\text{Mt70}^{890Y}$ replicated similarly to mutants $\text{Mt70}^{890Y}$ and $\text{Mt98}^{324570890Y56439R}$ and to its corresponding revertant, $\text{Rv98}^{324570890}$, at 37 °C and showed similar poor yields to mutant $\text{Mt70}^{890Y}$ at 40 °C (Fig. 4B). This experiment was repeated 3 times, each time with 3 replicates. In one of the replicates, virus titres were higher than in the other 8 replicates, reaching yields of 3.4 log$_{10}$ pfu/ml by day 9 post infection (data not shown). Interestingly, this virus had

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Fig. 2. PCR confirmation of rpsL-neo cassette insertion in the M56 gene of the MCMV BAC and its replacement with mutated M56 to produce the mutant Mt56439R BAC. (A) Schematic representation of the primer binding sites. Viral primers are shown in solid arrows, rpsL-neo primers are shown in broken arrows. (B) PCR products obtained with primer sets $a + b$ and $b' + a'$ in lanes 1 and 2 indicated the correct rpsL-neo cassette insertion into the targeted gene. No PCR products obtained with primer sets $a + c$ and $c' + a'$ in lanes 3 and 4 indicated the absence of the corresponding wt ORF in the BAC. Positive ((+)ve) and negative ((−)ve) controls for primer sets $a + c$ and $c' + a'$ are shown in lanes 5-6 and 7-8, respectively. (C) PCR products obtained with primer sets $a + c$ and $c' + a'$ in lanes 1 and 2 indicated reinsertion of viral DNA sequence in the targeted gene. No PCR products obtained with primer sets $a + b$ and $b' + a'$ in lanes 3 and 4 indicated the absence of the rpsL-neo cassette in the BAC. Positive ((+)ve) and negative ((−)ve) controls for primer sets $a + b$ and $b' + a'$ are shown in lanes 5-6 and 7-8, respectively. Molecular size markers (lanes M), in kilobases, are indicated.

Fig. 3. Restriction fragment analysis of mutant and revertant BAC genomes. Mutant, revertant and wt K181 (Perth) BAC DNA (∼2 µg) was digested with 1 µl of HpaI restriction enzyme and resulting fragments were separated on a 0.4% agarose gel for ∼19 h at 2 V/cm. Molecular size markers (lanes M), in kilobases, are indicated.
reverted to the wt C nucleotide in the M70 ORF as early as 3 days post infection but the M98 ORF remained mutant. Continuing the experiment for the 6 replicates in the other 2 experiments for up to 21 days post infection showed that yields did not increase and the viruses remained temperature-sensitive with no reversion (Fig. 4B).

In contrast, the initial experiment with the triple mutant Mt98324S70890Y56439R showed considerably improved growth at 40 °C compared to the other mutants, reaching titres of ~4 log10 pfu/ml by 8 days post infection (Fig. 4C). This experiment was repeated with similar results (results in Fig. 4C are the means ± SD of the 2 experiments). Interestingly, sequencing showed that in all 6 replicates the virus had further mutated from the mutant UAC (ATG on complementary strand) tyrosine codon to the mutant UCC (AGG on complementary strand) serine codon in M70 and, as early as 1 day post infection at 40 °C, the virus was a mixture (Mt98324S70890Y/890S56439R) while by 9 days post infection the

Fig. 4. Growth curves of mutant viruses in tissue culture. Virus mean titres (log10 pfu/ml) from 0 to 9 or 21 days post infection (−1 day represents inoculum titre) with an MOI of 0.05 for (A) Mt70890Y (■, □) and Rv70C890 (●, ○) at 37 °C (■, ●) and 40 °C (□, ○); (B) Mt983247098P324 (■, □) and Rv98P32470C890 (●, ○) at 37 °C (■, ●) and 40 °C (□, ○); and (C) Mt983247098P32470C890 (■, □) and Rv98P32470C89098P32470 (●, ○) at 37 °C (■, ●) and 40 °C (□, ○). For (A) the mean ± SD of 3 replicates in a typical growth curve is shown, for (B) and (C) the results are the means ± SD of 2 individual experiments each with 3 replicates.

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In contrast, the initial experiment with the triple mutant Mt983247098P32470C890 showed considerably improved growth at 40 °C compared to the other mutants, reaching titres of ~4 log10 pfu/ml by 8 days post infection (Fig. 4C). This experiment was repeated with similar results (results in Fig. 4C are the means ± SD of the 2 experiments). Interestingly, sequencing showed that in all 6 replicates the virus had further mutated from the mutant UAC (ATG on complementary strand) tyrosine codon to the mutant UCC (AGG on complementary strand) serine codon in M70 and, as early as 1 day post infection at 40 °C, the virus was a mixture (Mt983247098P32470C890/56439R) while by 9 days post infection the

Fig. 5. Sequencing over nucleotide position 99,286 of the M70 gene in the stock of the serine triple mutant (Mt983247098P32470C890) virus grown at 37 °C and one of the replicates of this virus at the end of the growth curve at 37 °C and 40 °C. Total DNA was isolated from virus-infected cells, M70 gene fragment PCR amplified and sequenced. The original mutation T is replaced with the new mutation G partially at 37 °C and completely at 40 °C.
serine mutant (Mt98324S70890S56439R) only was detectable (Fig. 5). Furthermore, the virus that emerged at 37 °C was also a mixture of viruses containing the M70 serine and tyrosine mutations although the latter greatly predominated (Fig. 5). These results strongly suggest that the M70 serine mutation had occurred during production of virus working stock although this could not be detected by sequencing as the virus inoculum contained only virus with M70 tyrosine.

Two further transfections were performed with the Mt98324S70890Y56439R BAC construct which produced 3 different virus stocks. All 3 viruses contained the M70 tyrosine mutation (as determined by sequencing) and had similar growth properties to Mt70890Y and Mt98324S70890Y viruses (data not shown).

**DNA kinetics**

The effect of the M70 mutation on DNA synthesis was examined in virus-infected cells at 37 °C and 40 °C from 1 to 9 days post infection using real-time PCR as described in Materials and methods. The kinetics of DNA synthesis at 37 °C was essentially similar for all viruses examined (Fig. 6A) increasing ~10^4–10^5 fold by 3 days post infection and maintaining these levels up to day 9 post infection. The kinetics were similar for K181 (Perth), Rv70890Y, Rv9870890Y, and Rv9870890Y56439R viruses at 40 °C and similar to that at 37 °C (Fig. 6B). In contrast, DNA synthesis was markedly reduced in Mt70890Y, Mt98324S70890Y, and Mt98324S70890Y56439R virus-infected cells at 40 °C (~100–1000 fold) at day 1 post infection. It is possible that the delayed DNA kinetics at later time points may be due indirectly to other blocks in virus replication but this is unlikely as the revertant virus, in which only the primase mutation had been repaired, has wt DNA levels at all time points indicating no other defects in the replication cycle. Interestingly, DNA synthesis in the serine mutant Mt98324S70890Y/890S56439R virus-infected cells was similar to that of the other M70890Y containing mutants initially (day 1) but gradually increased to similar levels to K181 (Perth) and revertant viruses by day 9 (Fig. 6B). These results agree well with those for virus yields and show that the major defect in Mt70890Y is its inability to synthesise DNA at the non-permissive temperature.

**Fig. 6.** Real-time PCR quantification of MCMV DNA. MEF cells were infected with K181 Perth (●), Mt70890Y (●), Mt98324S70890Y (●), Mt98324S70890Y56439R (●), Rv70890Y (○), Rv98324S70890Y (△) or Rv98324S70890Y56439R (◊) at an MOI of 0.05 pfu per cell and incubated at 37 °C (A) and 40 °C (B). Results are the mean±SD of two to five separate experiments.

serine mutant (Mt98324S70890S56439R) only was detectable (Fig. 5). Furthermore, the virus that emerged at 37 °C was also a mixture of viruses containing the M70 serine and tyrosine mutations although the latter greatly predominated (Fig. 5). These results strongly suggest that the M70 serine mutation had occurred during production of virus working stock although this could not be detected by sequencing as the virus inoculum contained only virus with M70 tyrosine.

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Replication in immunocompetent BALB/c mice

Infectious virus was readily detected in the salivary glands of BALB/c mice inoculated with revertant viruses Rv70C890 and Rv9852470C890 or the wt K181 (Perth) variant and examined 21 days post infection (Fig. 7A). The mean viral titre for Rv70C890 was $10^{4.9}$ pfu/salivary gland; the mean viral titres for Rv9852470C890 and wt K181 (Perth) were lower at $10^{4.1}$ and $10^{4.6}$ pfu/salivary gland but these differences were not significant ($P>0.05$). In contrast, no infectious virus was detected in mice inoculated with mutants Mt70890Y or Mt9852470890Y (Fig. 7A). In agreement with the infectivity assays, the average number of viral genomes per salivary gland 21 days post infection, as estimated by real-time PCR, were similar for revertant and wt K181 (Perth) virus being $10^{6.8}$, $10^{7.3}$ and $10^{7.0}$ respectively for Rv70C890, Rv9852470C890 and wt (Fig. 7B). DNA was difficult to detect in mice infected with either mutant but the data presented in Fig. 7B indicate that at least 1 animal in each group had detectable DNA but these levels were at least 6300 fold lower for mutant Mt70890Y than for revertant Rv70C890. Thus mutants containing the M70 tyrosine mutation were also attenuated for salivary gland replication in mice.

Discussion

Twenty-six mutations have now been identified in MCMV mutant tsm5 (Sweet et al., 2007; Timoshenko et al., 2009), sixteen of which result in an amino acid change. Previous studies have demonstrated that different virus stocks of tsm5 are different mixes of mutants resulting in polymorphisms in at least 6 loci, ORFs M27, M36, M53, M56, M70 and M98 (Sweet et al., 2007; Timoshenko et al., 2009). However, the relative proportion of each mutant within the virus stock varies. Thus, the identified amino acid may be either mutant, wt or both (Timoshenko et al., 2009). One or more of these identified mutations could contribute to the tsm5 phenotype as they are in genes identified as having a role in DNA synthesis, cleavage and packaging (Pari and Anders, 1993; Homa and Brown, 1997). Three of these have been examined in the present study.

The M56 ORF encodes the putative terminase subunit which, in the herpes simplex type 1 (HSV-1) UL28 homologue, associates with the putative ATPase subunit of the terminase (UL15; MCMV M89) and is involved in inserting viral DNA into virion capsids and cleavage of DNA concatemers into unit length genomes (Tengelsen et al., 1993; Bogner et al., 1998). The tsm5 G439R mutation does not fall within the region containing the putative ATP-binding pocket (Scholz et al., 2003), shown to be essential for terminase activity (Wang and Mccoy, 2008), or any other known functional domains of the terminase. It is located within the variable “spacer” region that is poorly conserved even among cytomegaloviruses, indeed deletion of 13 of the 68 amino acids in HSV-1 UL28 had no effect on virus growth or DNA packaging (Abbotts et al., 2000). In confirmation of this, mutant Mts5G439R had a wt replication phenotype in MEF cells at both 37 °C and 40 °C (Fig. 1B).

Surprisingly, Mt9852470M56Y mutant virus also exhibited a wt phenotype (Fig. 1C). The wt proline 324 is highly conserved in motif IV of the seven herpesvirus alkaline nuclease motifs defined by Goldstein and Weller (1998). The nuclease activity of the HSV-1 UL12 protein (M98 homologue) has an important function in resolving branches formed from intra- and inter-molecular recombination of large branched head-to-tail concatemers formed during DNA replication (Reuven et al., 2003). Clearly proline 324 is not an essential residue for this function.

In contrast, the C890Y mutation in M70 has a very important role in the tsm5 phenotype as viruses containing this mutation (Mt70890Y, Mt9852470890Y and Mt9852470890Y5E4188) all replicated poorly at the non-permissive temperature and reversion of tyrosine 890 to the wt cysteine, serine or methionine restored the wt phenotype. The HCMV UL70 homologue, together with UL102 and UL105, are predicted to encode proteins that comprise the helicase–primase complex essential for DNA replication (McMahon and Anders, 2002). Like the HSV-1 primase, HCMV pUL70 contains highly conserved putative DxD and zinc finger motifs (Woon et al., 2008). Recently, other motifs have also been recognised in UL70 including a putative protein disulphide isomerase Cxxc motif, a CAMP-dependent protein kinase phosphorylation site, 20 casein kinase II phosphorylation sites, 10 protein kinase C phosphorylation sites and 2 tyrosine kinase phosphorylation sites (Woon et al., 2008). None of these encompass residue 890.

Without a structure it is difficult to know how the C890Y mutation affects primase function. The cys→his→cys zinc finger motif is conserved in all herpesviruses and is involved in DNA binding and helicase activity as well as primase activity as mutations within this motif of the HSV-1 homologue of M70/UL70 affects these functions (Chen et al., 2005). The C890 residue is conserved in all cytomelaviruses and is located 16 amino acids upstream of the zinc finger suggesting that it too may affect all 3 activities. The replacement of the cysteine with a tyrosine may have introduced a tyrosine kinase phosphorylation site and the phosphorylated tyrosine could markedly affect primase conformation and stability. However, the ScanProsite software from the Expert Protein Analysis System (ExPASy) proteomics server did not identify Y890 as a predicted tyrosine kinase phosphorylation site. Both cysteine and tyrosine provide chemically active side chains for interaction with other amino acids and the large side chain of tyrosine could sterically destabilise the primase protein structure. Replacement of cysteine with serine, a much smaller amino acid with a chemically active side chain restored wt activity. However, replacement of cysteine with methionine, a relatively inactive amino acid, also restored wt activity (data not shown) suggesting that the ability of amino acid residue 890 to interact chemically with other amino acids in the structure is not important.

The polymorphism in tsm5 was unexpected. It is possible that the polymorphism resulted from reversion but this is unlikely as different stocks of tsm5 varied in their polymorphic loci and wt virus did not emerge on passage. A more likely explanation is the production of multicapsid virions. During the production of tsm5 precautions were taken to try and eliminate multicapsid virions, which are produced in tissue culture (Hudson et al., 1976; Chong and Mims, 1981; Weiland et al., 1986) and in vivo (Reddehase et al., 1985), by plaque picking, sonication and filtration (Sammons and Sweet, 1989). However, it now seems likely that this was not achieved. Multicapsid virions consist of up to 15 capsids or virions surrounded by a single membrane (Chong and Mims, 1981) and up to 40% of virions may be multicapsids (Kurz et al., 1997). Furthermore, recent studies suggest that 80 to 500 MCMV genomes may be required to form a single plaque (Kurz et al., 1997; Heider et al., 2002) and, owing to the defective nature of mutant virus, up to 300 times more virions may be required for one pfu of mutant virus (Heider et al., 2002). Functional trans-complementation between mutant and wt MCMV viruses following co-infection of the same cell has been shown in vivo (Cicin-Sain et al., 2005) and such a mechanism may maintain the tsm5 polymorphism. Thus it may be very difficult to plaque purify single cloned mutants from such a heterogeneous mix. An inability to plaque purify a pure clone was shown for HCMV isolate TB40/E (Dolan et al., 2004; Sinzger et al., 2008). Subsequent cloning of TB40/E as a BAC was achieved. Multicapsid virions consist of up to 15 capsids or virions and, owing to the defective nature of mutant virus, up to 300 times more virions may be required for one pfu of mutant virus (Heider et al., 2002). Functional trans-complementation between mutant and wt MCMV viruses following co-infection of the same cell has been shown in vivo (Cicin-Sain et al., 2005) and such a mechanism may maintain the tsm5 polymorphism. Thus it may be very difficult to plaque purify single cloned mutants from such a heterogeneous mix. An inability to plaque purify a pure clone was shown for HCMV isolate TB40/E (Dolan et al., 2004; Sinzger et al., 2008). Subsequent cloning of TB40/E as a BAC was necessary to produce a genetically pure clone for phenotypic analysis (Sinzger et al., 2008). This method could be applied to separate genetically different variants of tsm5 for subsequent analysis of their phenotypes.

The above data demonstrates that the M70 tyrosine mutation is the first mutation identified in tsm5 and that this mutation also attenuates replication in vivo. However, previous studies with the chimaeric virus Smith/tsm5DGKi, constructed from overlapping cosmids and containing region M56-m144 from tsm5 and its 5′ and 3′ ends from the Smith strain of MCMV (Sweet et al., 2007),
replicated well in mice and was not as temperature-sensitive as tsm5. This contradicts present data as the chimeraic virus is expected to contain the M70 mutation. However, as tsm5 is polymorphic the chimeraic virus was sequenced over known mutations to examine the possibility that the M70 mutation may not be present. Sequencing confirmed mutations in M56 and M98 ORFs but revealed that M70 was wt. Thus, its ability to replicate in mice may be due to the wt M70 gene and not to the Smith component as previously suggested (Sweet et al., 2007). Interestingly, many of the other tsm5 mutations are also absent in Smith/tsx5SGIK suggesting that the M70 may not be the only mutation contributing to the tsm5 temperature-sensitive phenotype. This emphasises the difficulty of determining the contribution of specific mutations to a phenotype when multiple mutations are present and constitute only single nucleotide changes. This is made more difficult when they are subject to change in different virus stocks.

A final puzzle is why the M70^ts0507 and Mts8^ts2457^ts0907 mutant viruses were attenuated for salivary gland replication in mice. This is unlikely to be due to their temperature-sensitivity at 40 °C as mice have a normal body temperature of 37 °C. It is possible that the mutation produces a generally “stressed” virus, which is reflected by decreased replication at elevated temperature in vitro and by the in vivo environment of mice.

In conclusion, a single nucleotide change resulting in a cysteine to tyrosine amino acid change in the M70 primase of MCMV renders the virus temperature-sensitive and attenuated for salivary gland replication in mice. Whether a similar change in the conserved cysteine of HCMV produces a similar phenotype is currently under investigation.

Materials and methods

Cells and viruses

The K181 (Birmingham) variant of MCMV and mutant tsm5 have been described previously as has the method of production of primary mouse embryo fibroblasts (MEFs) and virus stocks (Sammons and Sweet, 1989; Sweet et al., 2007). The K181 (Perth) variant of MCMV was kindly provided by Dr. Alec Redwood, Perth, Australia as a BAC containing the K181 (Perth) viral genome. Viral titres were quantified in MEF cells by standard plaque assays.

PCR amplification

The standard PCR reaction was carried out with 300 ng of DNA in a 50 μl PCR reaction using 2× ReddyMix™ PCR master mix (Abgene). An initial denaturing step of 2 min at 94 °C was followed by 30 amplification cycles of 94 °C for 45 s, 50–70 °C (depending on primer melting temperature) for 45 s and 72 °C for up to 3 min depending on the length of the expected PCR product. The PCR reaction was completed with an extra extension time of 7 min at 72 °C before cooling down to 4 °C. PCR products used in mutagenesis were amplified with Extensor Hi-Fidelity PCR Master Mix (Abgene). An initial denaturing step of 3 min at 94 °C was followed by 10 amplification cycles of 94 °C for 45 s, 60 °C for 30 s and 72 °C for 1 min, then 19 amplification cycles of 94 °C for 45 s, 60 °C for 30 s and 72 °C for up to 3 min depending on the length of expected PCR product and final extension step of 7 min at 72 °C before cooling down to 4 °C.

Sequencing

PCR products, purified using the QIAquick PCR Purification kit (Qiagen), were sequenced using the Big Dye™ terminator ready reaction cycle sequencing kit (Applied Biosystems) and an ABI 3700 machine. The entire M56, M70 and M98 gene sequences were assembled by comparison to the K181 (Perth) consensus sequences (Redwood et al., 2005) using the NCBI BLAST network service translated using Alltrans and the whole protein sequence for each gene aligned using CLUSTALW.

Plasmids

Plasmids pRpsL-neo and pKD46 were obtained from Gene Bridges, Dresden, Germany.

Quantitative real-time PCR

Total genomic DNA from virus-infected MEF cells or homogenised salivary glands of virus-infected BALB/c mice was prepared using the DNeasy blood and tissue kit (Qiagen), according to the manufacturer’s instructions. All real-time PCRs were performed in an ABI Prism 7000 Sequencing Detection System (Applied Biosystems) in 96-well clear plates (Applied Biosystems). Primers and probes were designed using the TaqMan primer and probe design software and synthesised by Eurogentec. The target MCMV gene was m29.1 and forward and reverse primers and probes were as follows (5′→3′): forward, GAAATGCACACGGAAAAAAGC; reverse, ATCTATGATGTTGCGGTCTTCT; probe, FAM-TACCAGGTTTATGGCCATCGGC-BHQ1. Similarly, the forward and reverse primers and the probe for the mouse GAPDH gene used as a control were (5′→3′): forward, AAGA-GAGCCCTATCCTCAACTC; reverse, TAGGCCCTCCTGTATTGATGG; probe, Yacima Yellow-TCTCCCTCAAAATTCATGCCAC-BHQ1. The PCR reactions were performed in a 20-μl volume containing 10 μl of qPCR Mastermix (Eurogentec), 450 nM of each primer, 187.5 nM of the probe and 3–5 μl of the DNA sample. For thermal cycling, conditions consisted of two initial steps of 2 min at 50 °C and 10 min at 95 °C, followed by 44 amplification cycles (15 s at 95 °C, 1 min at 60 °C). Threshold Cycle (Ct) was determined as a cycle number at which the fluorescence generated in the reaction crosses the threshold set within the logarithmic phase of each PCR. For relative quantification of MCMV genomes in virus-infected MEF cells the Ct for the control gene was subtracted from the Ct for the gene of interest and the relative amount of that gene was calculated as 2 – ΔCt. For absolute quantification of MCMV genomes in salivary glands of virus-infected BALB/c mice, 10-fold serial dilutions containing 10^6 to 0 copies of K181 (Birmingham) virion DNA per assay were tested in triplicate and used to construct the standard curve by plotting the average Ct values against the logarithm of MCMV genome copy number, followed by least-squares linear regression analysis. MCMV genome copy number per known amount of DNA used in the PCR reaction was calculated using the equation obtained from the regression analysis. Data were further processed to determine the number of MCMV genomes per salivary gland. The limit of detection of this assay was determined as 400 MCMV genomes per PCR reaction or 10^5 MCMV genomes per salivary gland.

BAC mutagenesis

A two-step Red-mediated homologous recombination was performed to introduce mutations into the M56, M70 and M98 genes and to revert them to wt. Selection of recombinants is facilitated using an rpsL-neo cassette, the rpsl gene conferring streptomycin sensitivity and the neo gene kanamycin resistance. Escherichia coli DH10B (which has a mutation in its rpsl gene rendering it streptomycin resistant) containing the K181 (Perth) MCMV bacterial artificial chromosome (BAC) (chloramphenicol resistant) (Redwood et al., 2005) was transformed with plasmid pKD46, which expresses λ phage recombinases redA, redD and gam (Datsenko and Wanner, 2000), and grown at 30 °C to OD^600 of 0.3 on LB medium containing 12 μg ml^-1 chloramphenicol and 50 μg ml^-1 carbenicillin at which point the expression of Red recombinases was induced by adding l-arabinose to a final concentration of 0.3%. The cultures were incubated at 37 °C for 1 h with shaking and were then harvested at 13,000 × g for 30 s.
The cells were prepared for electroporation by washing 3 times in ice-cold 10% glycerol and used immediately.

A linear DNA fragment (1.3 kb) containing a streptomycin sensitive and kanamycin resistant rpsL-neo cassette flanked at the 5′ end with 52 nt of the M56 ORF (nt 86,484 to 86,535; based on K181 (Perth) genomic sequence, GenBank database accession number AM886412), the M70 ORF (98,989 to 99,040) or the M98 ORF (142,813 to 142,864) and at the 3′ end with 51 nt of the M56 ORF (nt 87,035 to 87,085), the M70 ORF (99,540 to 99,590) or the M98 ORF (143,365 to 143,415) was amplified by PCR from the rpsL-neo plasmid template using Extenfor Hi-Fidelity PCR master mix (Abgene) and the following primers: M56F (5′-CCGCTTGGGAGAAGACCGCTCATTATTTCTGGAGACA-3′) and ETM56F (5′-CCGCTTGGGAGAAGACCGCTCATTATTTCTGGAGACA-3′) and ETM56R (5′-GGGAA-GACCCGAGCGGTGGATGTTCGAGATAGTGG-3′) and ETM70R (5′-GGGAA-GACCCGAGCGGTGGATGTTCGAGATAGTGG-3′) and ETM98R (5′-GGGAA-GACCCGAGCGGTGGATGTTCGAGATAGTGG-3′).

rpsL-neo cassette was replaced by linear DNA fragments carrying a tms5-specific nucleotide substitution in the M56 (C to T at base position 86,783), M70 (C to T at position 99,286) or M98 (C to T at base position 143,114) gene was amplified by PCR from tms5 or the chimaeric Smith/tms5GIK (Sweet et al., 2007) viral DNA template using Extenfor Hi-Fidelity PCR master mix (Abgene) and the following primers: M56F (5′-GTGTCGCTCCAGATGGT-3′) and M56R (5′-CTGTTTCTCATGCATAG-3′) and M70F (5′-GTCCGGACATGTGCCTCGCC-3′) and M70R (5′-GGCCCGATCTCCTCTCATGAC-3′) and M98F (5′-GAGAGGACCCGTCA-GAGTG-3′) and M98R (5′-TCAGAAGAACTCGTCAAGAAGGCG-3′). PCR products were digested with DpnI (NEB) to remove any residual template DNA.

PCR fragments (100–200 ng in 1–2 μl of water) were added to the cells. Electroporation was performed using a 1 mm gap ice-cold cuvette and a Bio-Rad Gene Pulser set to 25 μF, 2.3 kV with pulse controller set at 200 Ω. LB medium (1 ml) was added immediately after electroporation. The cells were incubated at 30 °C for 2–3 h with shaking, spread on plates containing 12 μg ml−1 chloramphenicol, 20 μg ml−1 kanamycin and 50 μg ml−1 carbenicillin and grown at 30 °C overnight.

To identify recombinants, colonies were restreaked in parallel on plates containing 12 μg ml−1 chloramphenicol plus 20 μg ml−1 kanamycin and 50 μg ml−1 streptomycin. Recombinants were selected based on their chloramphenicol and kanamycin resistance and streptomycin sensitivity and integration of the rpsL-neo cassette confirmed by PCR.

A linear DNA fragment carrying a tms5-specific nucleotide substitution in the M56 (C to T at base position 86,783), M70 (C to T at base position 99,286) or M98 (C to T at base position 143,114) gene was amplified by PCR from tms5 or the chimaeric Smith/tms5GIK (Sweet et al., 2007) viral DNA template using Extenfor Hi-Fidelity PCR master mix (Abgene) and the following primers: M56F (5′-GTGTCGCTCCAGATGGT-3′) and M56R (5′-CTGTTTCTCATGCATAG-3′) and M70F (5′-GTCCGGACATGTGCCTCGCC-3′) and M70R (5′-GGCCCGATCTCCTCTCATGAC-3′) and M98F (5′-GAGAGGACCCGTCA-GAGTG-3′) and M98R (5′-TCAGAAGAACTCGTCAAGAAGGCG-3′). PCR products were used to replace the rpsL-neo cassette as described above. Recombinants were replica plated as above and suitable colonies selected based on their chloramphenicol and streptomycin resistance and kanamycin sensitivity. Recombinant BAC plasmid DNA, isolated using the NucleoBond® BAC kit (Macherey-Nagel), was PCR-screened and sequenced to confirm the desired rpsL-neo cassette insertion.

Viruses were harvested, homogenised in growth medium, centrifuged and supernatants titrated for virus.

Viruses in animal models

Specific pathogen-free 3-week-old immunocompetent BALC/c mice (Charles River, UK) were inoculated intraperitoneally with 105 pfu of virus. Infected animals were sacrificed at 21 days p.i., salivary glands harvested, homogenised in growth medium, centrifuged and supernatants titrated for virus.

References


Statistical analysis

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