Virology 392 (2009) 114–122

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Role of mutations identified in ORFs M56 (terminase), M70 (primase) and M98 (endonuclease) in the temperature-sensitive phenotype of murine cytomegalovirus mutant *tsm*5

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ARTICLE INFO

Article history:
Received 29 April 2009
Returned to author for revision
24 May 2009
Accepted 30 June 2009
Available online 29 July 2009

Keywords:
Primase
Terminase
Endonuclease
M70
M56
M98
Murine cytomegalovirus
Temperature-sensitive mutant
Attenuated
*tsm*5

ABSTRACT

Twenty-six non-synonymous and synonymous mutations have been identified in the temperature-sensitive (*ts*) mutant (*tsm*5) 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.

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 *tsm*5, 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 ~4 $\log_{10}$ at 40 °C compared to 33 °C or 37 °C and failed to reach detectable levels in any tissue of 1-week-old mice for up to 21 days following i.p. inoculation with 4×10^3 pfu of virus. Electron microscopy showed that *tsm*5 had a defect in DNA encapsidation and Southern hybridisation indicated that while DNA synthesis was significantly 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 *tsm*5 in its phenotype mutations were introduced individually and in combi-

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doi:10.1016/j.virol.2009.06.049

nation into the *wt* K181 parental strain using BAC mutagenesis. The mutant *tsm*5 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 *tsm*5 mutations in replication.

Production of Mt70^{890Y}, Mt98^{324S} and Mt56^{439R} 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 Mt70^{890Y}, Mt98^{324S} and Mt56^{439R} and their corresponding revertant viruses Rv70^{C890}, Rv98^{P324} and Rv56^{G439} 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 Mt56^{439R} 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 DraI (data not shown). Sequencing demonstrated that the correct mutation had been introduced (data not shown).

Replication kinetics of viruses in tissue culture

Mutants Mt56^{439R} and Mt98^{324S} 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 Rv56^{G439} and Rv98^{P324} (Figs. 1B and C).

In contrast, mutant Mt70^{890Y} was temperature-sensitive (Fig. 1D). Its growth at 37 °C was similar to Mt56^{439R} and Mt98^{324S} and to revertant Rv70^{C890} but it replicated poorly at 40 °C and this replication was considerably delayed until days 8–9 when titres of 1.0–2.0 $\log_{10}$ 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 Mt70^{890Y} 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 $\log_{10}$ pfu/ml and virus remained mutant (data not shown).

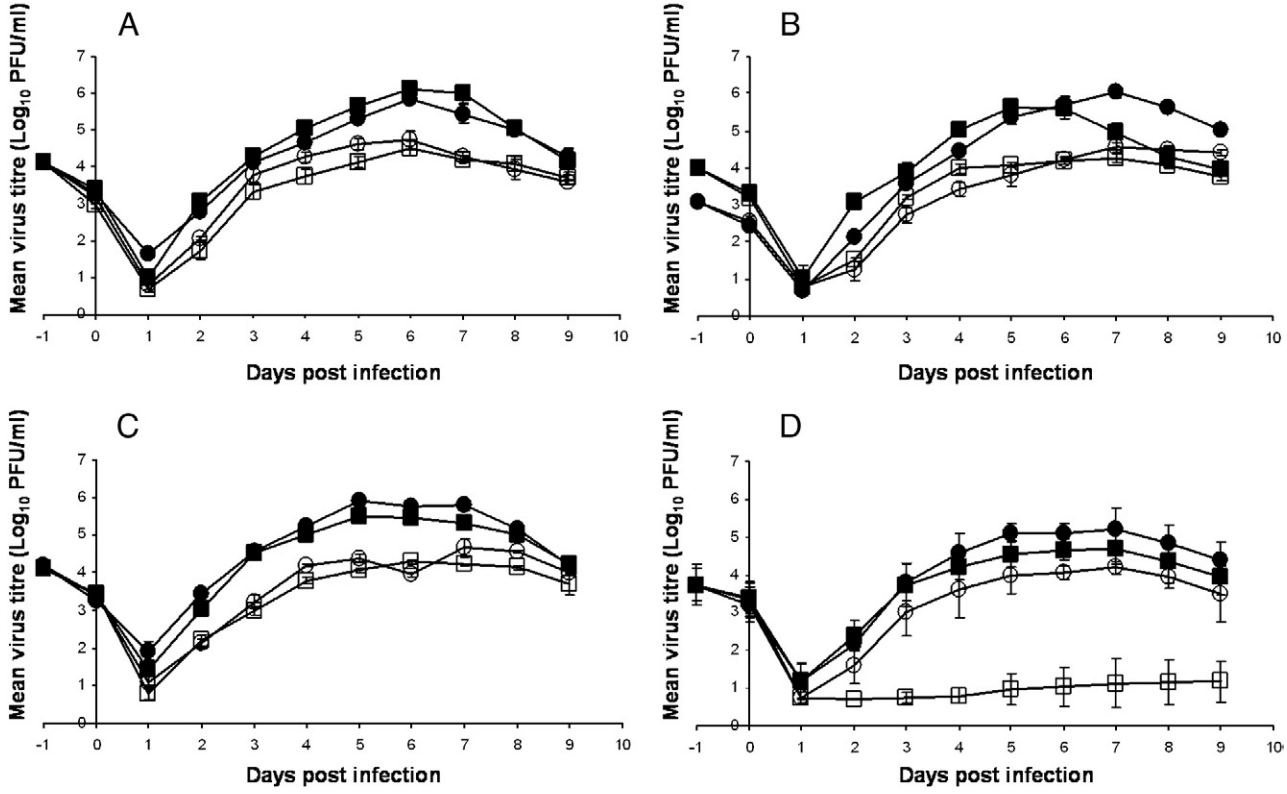

Fig. 1. Growth curves of parental viruses and single mutants in tissue culture. Virus mean titres ($\log_{10}$ pfu/ml ± SD) from 0–9 days post infection (−1 day represents inoculum titre) with an MOI of 0.05 for (A) K181 B'ham (■, □) and K181 Perth (●, ○) at 37 °C ~■, ●) and 40 °C (□, ○); (B) Mt56^{439R} (■, □) and Rv56^{G439} (●, ○) at 37 °C (■, ●) and 40 °C (□, ○); (C) Mt98^{324S} (■, □) and Rv98^{P324} (●, ○) at 37 °C (■, ●) and 40 °C (□, ○); and (D) Mt70^{890Y} (■, □) and Rv70^{C890} (●, ○) at 37 °C (■, ●) and 40 °C (□, ○). For (A)–(C) the mean ± SD of 3 replicates in a typical growth curve is shown, for (D) the results are the means ± SD of 3 individual experiments each with 3 replicates.