Seton Hall University
eRepository @ Seton Hall

Theses

2006

Use of Bacterial Artificial Chromosome Technology to Create Recombinant Human Cytomegalovirus to Study Their Major Immediate Early Gene Expression and Viral Replication

Yamileth Rios

Follow this and additional works at: https://scholarship.shu.edu/theses

Recommended Citation
Rios, Yamileth, "Use of Bacterial Artificial Chromosome Technology to Create Recombinant Human Cytomegalovirus to Study Their Major Immediate Early Gene Expression and Viral Replication" (2006). *Theses*. 86.
https://scholarship.shu.edu/theses/86

Use of Bacterial Artificial Chromosome technology to create recombinant human cytomegaloviruses to study their major immediate early gene expression and viral replication

Yamileth Rios
B.S., College of Saint Elizabeth, 2001

Submitted in partial fulfillment of the requirements for the Degree of Master of Science in Microbiology from the Department of Biology of Seton Hall University
January 2006

APPROVED BY:

CO-MENTOR
Dr. Hua Zhu

CO-MENTOR
Dr. Sulie L. Chang

COMMITTEE MEMBER
Dr. Heping Zhou

CHAIR PERSON
BIOLOGY DEPARTMENT
Dr. Carolyn Bentivegna

ACKNOWLEDGEMENTS

I would like to thank the following people for their continuous teaching, support and encouragement:

Dr. Hua Zhu (Co-Mentor) from the Department of Microbiology and Molecular Genetics at UMDNJ. His guidance through my research is very much appreciated.

Dr. Sulie Chang (Co-Mentor) from Seton Hall University Department of Biology for all her help and dedication to see my work completed.

Dr. James Netterwald (Laboratory Mentor) from the Department of Microbiology and Molecular Genetics at UMDNJ, for introducing me to all laboratory techniques to complete my experiments as well as his teaching and support.

Dr. Heping Zhou (Committee Member) from Seton Hall University Department of Biology, for accepting to be a part of my committee.

Weijia Wang (former member of Dr. Zhu's laboratory at UMDNJ) for her collaboration in the completion of an important experiment for this research thesis.

Shaojun Shang and Michael Nguyen (Laboratory Partners) from Dr. Zhu's laboratory at UMDNJ, for answering my questions and all their support.

Family and friends for their encouragement and all their love.

TABLE OF CONTENTS

Abstract	Page 5
Introduction	Page 7
Materials and Methods	Page 15
Results	Page 23
Discussion	Page 31
Conclusions	Page 35
Literature Cited	Page 36

LIST OF FIGURES

Figure 1	Page 23
Figure 2	Page 25
Figure 3	Page 25
Figure 4	Page 26
Figure 5	Page 26
Figure 6	Page 28
Figure 7	Page 30

ABSTRACT

Human cytomegalovirus (HCMV) infection induces the secretion of interferons- alpha, gamma and beta in the host cell that leads to the activation of interferon stimulated genes (ISGs) which serve as an antiviral defense. Two consensus elements located in the ISG promoters; the ISRE (interferon-stimulated response element) and GAS (interferon-gamma activated site) serve as HCMV response elements and are responsible for the transcriptional activation of ISGs. HCMV disrupts the cellular JAK-STAT signal transduction pathway, but induces an unidentified signal transduction pathway that leads to the activation of ISGs and it is independent of viral DNA synthesis and cytokines (Netterwald *et al*, 2005; Yang *et al* 2005). The Major Immediate Early Promoter (MIEP) of HCMV contains two GAS elements which have been shown to be important in viral replication. Base-pair (bp) deletions or mutations in the GAS elements of the MIEP of HCMV result in a slow growth phenotype at low Multiplicity of Infection (MOI), but re-insertion of the wild-type GAS sequence rescues the slow growth phenotype (Netterwald *et al*, 2005). There is a 113-bp spacer region in between the GAS elements of the MIEP and our aim was to investigate the importance of this region since it has not been assessed for its role in viral replication. To investigate this, deletions were made to create a shorter 3-bp spacer region between the GAS elements. In addition, four base-pair substitution mutations were created in the GAS elements to study their effect in HCMV life cycle. Two recombinant HCMVs, one mutant (mt) and one wild-type (WT) were constructed using Bacterial Artificial Chromosome (BAC) technology. Through growth curve analysis it was observed that the (WT) recombinant virus grew similarly to the

original AD169 (WT) strain at both high and low (MOI). The (mt) had a 10-fold growth difference compared to the AD169 (WT) virus at both high and low (MOI). Protein expression analysis through Western Blotting revealed that Immediate Early proteins (IE1 and IE2) were reduced in the mutant HCMV, but not in the recombinant (WT) virus. The data in this study demonstrates that the specific base-pair mutations created in the GAS elements of HCMV MIEP resulted in a reduced viral replication in the mutant virus. The defect is due to the reduced IE1 and IE/2 expression since these two genes have been shown to play a major role in viral replication. In addition, the WT virus was not affected by the mutation in the spacer region since its growth rate was similar to HCMV-Ad169 strain suggesting that the spacer region between the GAS elements of the HCMV MIEP might not be important in viral replication.

INTRODUCTION

The human cytomegalovirus (HCMV) is a member of the herpes virus family and is the main member of the beta-herpes virus sub-group. Beta-herpes viruses are closely related to herpes viruses in virion structure and genome organization. In addition, ORFs involved in DNA replication have shown amino acid sequence homologies comparable to mammalian herpes viruses. HCMV is also similar to other herpes viruses in its ability to cause acute and chronic infections and its ability to remain latent for life once an individual gets infected with the virus (Mocarski, 2001).

HCMV is an important pathogen in immuno-compromised individuals, especially in AIDS and transplant recipient patients and newborns as well. It is estimated that between 50-90% of adults worldwide are asymptomatic carriers of HCMV meaning that they have active and functional immune systems which causes the virus to remain latent and do not cause chronic disease (Mocarski, 2001; Sissons *et al* 2002).
A primary infection with HCMV is associated with virus replication in cells which as a consequence causes lysis of the cells and surrounding tissue. After this primary infection, the immune system usually clears the infectious virus, but the virus will remain latent for life. The latent virus can be reactivated by factors such as stress and inflammation. In immuno-competent individuals, infection with this virus does not seem to cause major problems since their immune system can clear the virus. In immuno-compromised individuals, the immune system might take longer to clear the virus and meanwhile the virus can cause major damage to cells and tissues, ultimately leading to organ failure (Mocarski, 2001).

During HCMV infection there is a continuous viral shedding in body fluids such as urine, saliva, breast milk, blood and genital secretions, which can guarantee an efficient source for transmission to other uninfected people (Mocarski, 2001). HCMV primarily infects cells of hematopoietic myeloid-lineage such as monocytes. It utilizes these types of cells which lack differentiation as targets for life time latency (Gredmark *et al* 2004). HCMV induces inhibition of macrophage differentiation by binding to the cell surface molecule CD13/aminopeptidase N which is involved in cell adhesion, cell cycle control and signal transduction. Cellular differentiation would eliminate virus upon entry since it has not started DNA replication yet, and it is important for the virus to remain unnoticed by the immune system, especially in immuno-competent hosts (Gredmark *et al* 2004). Under stress, inflammatory conditions and in immuno-compromised individuals, HCMV induces monocyte differentiation into macrophages which are permissive to viral replication. The monocytes would then transport the virus to the different organs and once they differentiate into macrophages, the virus can start its DNA replication and infect nearby cells and organs (Smith *et al*, 2004). In cell culture, HCMV infects very few cell types and human skin fibroblasts are highly permissive and susceptible to infection. Besides monocytes, macrophages and fibroblasts, HCMV also infects epithelial and endothelial cells during natural infections (Mocarski, 2001; Isomura *et al*, 2003).

HCMV is a double-stranded DNA virus which has the largest genome of all human herpes-viruses that is approximately 236 kb with about 250 potential open reading frames (ORF) (Netterwald *et al* 2004). The virion is composed of an icosahedral nucleo-

capsid with attached proteins and a large matrix and enclosed in an envelope which contains a lipid bilayer and many viral glycoproteins (Mocarski, 2001).

Human cells infected by HCMV exhibit different types of viral particles that differ from each other in their physical characteristics and ability to infect cells. Virions are the infectious particles; the noninfectious envelope particles (NIEPs) lack a DNA core, but contain viral assembly and scaffolding proteins and lastly the dense bodies, are composed of the tegument protein pp65 and lack the nucleocapsid and the DNA core. The (NIEPs) and the dense bodies are very abundant and together help form the large plaque forming units observed on infected cell cultures (Mocarski, 2001).

The nucleocapsid of HCMV contains seven proteins. Mainly they are involved in structural, scaffolding, assembly and posttranslational processes. The virion envelope is composed of glycoprotein complexes that play a significant role in viral binding and entry into its target cell. The major glycoprotein is gB which is encoded by the gene UL55 and participates in virus binding to the cell's surface, virus entry, transmission and spreading of viral particles to adjacent cells. Other glycoproteins that also participate in viral entry, but are less abundant than gB are gH, gL and gO (Mocarski, 2001). The tegument or matrix, which is between the nucleocapsid and the envelope is composed of 25 proteins with the most abundant phosphoproteins (pp) being pp150 and pp65 (Mocarski, 2001).

Although HCMV begins its DNA synthesis between 16-24 hours after infection, its life cycle is slow since it can take up to 72 hours until complete virus particles are released. Attachment of the virus to the cell is via its glycoprotein gB which binds to the

heparin sulfate cell-surface proteoglycan (Compton *et al* 1993). Virus entry into the cell involves fusion of the viral envelope with the cell membrane and is believed to be mediated by viral glycoproteins gH and gL, although the exact process is not totally understood yet. (Mocarski, 2001; Keay *et al* 1991). Once viral DNA is directed to the nucleus of the cell, viral gene expression and activation of signal transduction pathways begin. HCMV has been shown to inhibit the cell cycle and block the pro-apoptotic gene p53 activity (Fortunato *et al* 2000; Speir *et al* 1994).

Immediate early (IE), early and late viral genes are synthesized after infection. The early genes encode proteins involved in DNA replication, while the late genes encode structural proteins and are only synthesized after replication of viral DNA is completed (Fortunato *et al* 2000). There are two major IE genes in the HCMV genome: UL122 and UL123 (IE1 and IE2 respectively) which encode a number of MIE regulatory proteins (Mocarski, 2001). These two genes are the first to be expressed after infection and play a major role in the efficiency of DNA replication as well as early and late viral gene expression (Isomura *et al* 2003). HCMV containing IE1 gene deletions had significant growth defects at a low (MOI) in human fibroblasts, but by some process not yet identified this mutant grew normally at a high (MOI) (Mocarski, 2001; Gawn *et al* 2002; Greaves *et al* 1998). On the other hand the IE2 gene has been shown to be necessary for viral replication because HCMVs with IE2 deletions did not express early viral genes or show viral DNA replication (Isomura *et al* 2003; Heider *et al* 2002; Marchini *et al* 2001; White *et al* 2004).

IE1 and IE2 genes get transcribed from a region of the HCMV genome identified as major immediate early (MIE) promoter enhancer region. This enhancer lies between bp (-65 to -550) (Meier *et al* 2000), and it contains *cis*-acting elements which act to stabilize the transcription initiation by RNA polymerase II and are the sites where transcription factors can bind and activate the promoter (Isomura *et al* 2003; Steinberg *et al* 1996; Mocarski, 2001). Transcription factors such as CREB, Sp1, AP1, NF-KB bind the (MIE) promoter immediately after infection and act to activate gene expression from the promoter enhancer region by binding to their corresponding repeat sequences of different (bp) sizes (Isomura *et al* 2003). An abundant tegument protein pp71, is a potent activator of the (MIE) promoter (Homer *et al* 1999). In fact the expression of this protein occurs at nuclear domain ND10, the same site where (MIE) gene expression occurs (Ishov *et al* 2002). PP71 has binding sites for CREB and AP-1 transcription factors (Morcasky, 2001). Deletions of pp71 have resulted in slow growth phenotype only at low (MOI)s of (0.001-0.1). PP71 may be important for the activation of gene expression via the (MIE) promoter at low (MOI)s (Bresnehan *et al* 2000).

IE gene expression occurs within one hour after infection and does not depend on *de novo* protein synthesis. IE1 and IE2 can positively or negatively regulate HCMV and host cell gene expression. After early gene expression, these two genes can auto-regulate their own expression via NF-KB binding on the (MIE) promoter. In the course of the infection, IE2 represses gene expression through a cis-repression sequence (crs) at the start of the transcription site. IE2 also acts as a switch to control the expression between IE, early and late genes (Mocarski, 2001).

As an antiviral strategy, human cells induce the secretion of interferons (IFN) which act to induce the transcription of interferon-stimulated genes (ISG) or antiviral genes. The types of (IFN)s are alpha, beta and gamma. Upon binding of HCMV to the cell (signal transducers and activators of transcription: STAT1 and STAT2) get activated to initiate (IFN) alpha and beta signaling. The STATs get translocated to the nucleus where they associate with p48 (a DNA binding factor) to form a complex called interferon-stimulated gene factor 3 (ISGF3). This complex activates the promoter by binding to interferon-stimulated response element (ISRE). (IFN) gamma also forms STAT1 complexes which bind to the (IFN)-gamma activation sequence (GAS). This results in the transcription from genes that activate the major-histocompatibility complex II promoters (Fortunato *et al* 2000). The binding of (ISRE) and (GAS) elements by STAT proteins results in the transcription of (ISGs) (levy *et al* 2002).

HCMV (IE1) protein blocks the JAK-STAT pathway which activates (IFN)-alpha signaling (Miller *et al* 1999). Interestingly, HCMV also activates an unknown signal transduction pathway that leads to the expression of (ISGs) (Zhu *et al* 1998). It has been shown that cytokines such as IFN-gamma and TNF-alpha which induce (ISGs) have a positive effect on the replication cycle of HCMV (Cebulla *et al* 1999).

Recent studies have shown that (ISRE) and (GAS) elements serve as HCMV response sites (VRS) to which HCMV-activated proteins bind to initiate (ISG) expression (Yang *et al* 2005). In the HCMV MIEP, two GAS elements are found to which host transcription factors bind to initiate (MIE) gene expression. These 9-bp (GAS) elements are found first between base pairs -381 to -390 and 503 to -512 in the MIEP (Meier *et al*

2000). Since HCMV activates proteins that bind to ISRE and GAS, gel shift analysis showed that mutations of DNA sequences in ISRE and GAS of the MIEP resulted in the inability of HCMV proteins to bind to activate these interferon sequences. In the GAS wild-type sequence, HCMV proteins bound indicating that the WT sequence was necessary for the interaction between HCMV proteins and GAS (Yang *et al* 2005). Mutant HCMVs which contained deletions or mutations of the GAS sequences grew significantly slowly compared to the wild-type virus (Netterwald *et al* 2005).

The GAS elements in the HCMV MIEP contain a 113-bp spacer region and the importance of this has not been investigated. In the present study, we aimed to determine the effects of mutations in the GAS elements of the HCMV MIEP and their spacer region in viral replication. To investigate this, recombinant mutant (mt) and wild-type (WT) viruses were constructed which both contained a mutated short spacer region of 3-bp. The mt virus contained four base-pair substitution mutations in the GAS elements and the WT contained the original GAS sequence. Bacterial Artificial chromosome (BAC) technology was used in this study to create the recombinant viruses. HCMV genome was cloned into a (BAC) and replicated in *Escherichia coli* (*E. coli*) (Yu *et al* 2002). Mutagenesis was performed using recombinant DNA technology. The recombinogenic DY380 strain of *E. coli* was used to create recombinant HCMVs. This strain produces a lambda prophage that harbors recombination genes: *exo, bet and gam* and that are controlled by a temperature sensitive lambda cI repressor.

The expression of the cI repressor is functional at 32°C; the activity of the recombination genes mentioned above shuts off, preventing lambda phage expression and killing of the

cell. At 42°C, the Lambda cI repressor activity is inhibited and this activates the expression of *exo, bet and gam* which then allow the cell to uptake linear DNA, thus becoming recombinogenic (Yu *et al* 2000).

Growth curve analysis data showed that the (mt) recombinant HCMV had a 10-fold growth difference compared to the HCMV-AD169 virus at both high and low (MOI). The (mt) protein expression analysis through Western Blotting revealed that immediate early proteins (IE1 and IE2) were reduced in the (mt) HCMV, but not in the recombinant (WT) virus. The data collected in this study suggests that base-pair substitution mutations in the GAS elements of the HCMV MIEP result in a slow growth phenotype as well as reduced viral IE1/IE2 expression. In addition, the WT recombinant virus constructed in this study grew similarly to HCMC AD169 original wild-type strain, and its ability to do so may be attributed to its original GAS sequence, therefore the mutated spacer region did not affect the growth or IE protein expression in this (WT) recombinant virus. We then suggest that the spacer region between the GAS elements of HCMV MIEP is not important in viral replication and further confirm the importance of the GAS elements in HCMV life cycle.

MATERIALS AND METHODS

Plasmids

P-GEMT vector (Promega, Madison, WI) was used for Thymine-Adenine (TA) cloning. Its 5' thyamine edges are able to base-pair with 3' adenine edges, thus allowing for the (TA) cloning of PCR products. A pGEM-lox-zeo (provided by J. Netterwald) and described in detail in Netterwald *et al* 2005 was created by PCR amplification of a zeocin resistance gene expression cassette (zeo^r) from a pCMV/zeo plasmid and then cloning into a pGEMT vector.

Bacterial strains

Plasmids (pGEMT and pGEMT-lox-zeo) were maintained in the *E. coli* DH5α strain. The HCMV (Ad169 BAC DNA), a gift by T. Shenk (Princeton University) was mutagenized into electroporation-competent *E. coli* DY380 cells. This HCMV-BAC DNA was maintained in *E. coli* DH10B cells. J. Netterwald provided the *E. coli* DY380 (Ad169-BAC) cells, but they needed to be made electroporation-competent to mutagenize the recombinant DNA sequences used in this study. In addition, these cells were induced to repress the lambda gene so that recombination could take place and the GAS element sequences be inserted into the viral genome. The exact procedure for mutagenesis will be described later in this section.

Making electroporation-competent cells and induction of lambda-phage recombination

The following procedure was adapted from (BioRad) with minimal changes made. *E. coli* DY380 (Ad169-BAC) cells were grown overnight (O/N) on Luria-Bertani agar plates containing kanamycin (30 ug/ml) at 30°C. An isolated fresh colony was picked and used to inoculate 20 ml of YT broth (0.8% Bacto-tryptone, 0.5% yeast extract and

0.5% NaCl; pH 7.6 and 30 ug/ml kanamycin). This liquid culture was grown overnight at 30°C with vigorous shaking. Five hundred ml of YT broth was inoculated with 5 ml of (O/N) liquid culture in a standard one litre flask and grown with vigorous shaking to an $OD_{600} = 0.6$. To induce the lambda repressor gene, the flask with the liquid culture was placed in a water bath for 15 minutes and at 42°C while shaking. The flask was immediately chilled for 30 minutes in an ice water bath. The chilled culture was then centrifuged at 4000g for 15 minutes at 0°C. The pellet was suspended in 200 ml of ice cold milliQ water and centrifuged again. All water was removed and the pellet was suspended in 100 ml of ice cold milliQ water and centrifuged as above. Twenty-five ml of 10% glycerol in milliQ water previously sterilized by autoclaving was used to suspend the next pellet. Centrifugation followed. After removing all liquid, the cells were suspended in 2 ml of 10% glycerol in milliQ water. Cell aliquots of 40 ul each were frozen on dry ice and then stored at -70°C.

Cells and viruses

Primary human foreskin fibroblasts (HFF) cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% fungisome and 1% sodium pyruvate at 37°C in humidified incubator with 7.5 % CO_2. HFF cells used for the different experiments in this study were between passages 10-20. The HCMV strain AD169 was from the American Type Culture Collection and its virulence has been attenuated for use as a live vaccine (Morcarsky, 2001). HCMV strain ($AD169_{BAC}$) was a gift by T. Shenk (Princeton University). This strain contains a gene marker for chloramphenicol resistance that allows for selection in *E. coli*. In addition, a

green fluorescence protein marker (GFP) is also present and it is excisable by the Cre gene.

Generation of point mutations in the GAS elements and partial deletion of spacer region

The two recombinant HCMVs constructed in this study (WT and mt) contained two GAS elements separated by a mutated spacer region. Base-pair substitution mutations in the GAS elements were created in the (mt) recombinant virus only. The (WT) recombinant virus contained the original GAS sequence but also a partially deleted spacer region. The original spacer region is 113-bp long and it was made into a shorter 3-bp region. Bacterial Artificial Technology (BAC) was used to construct the recombinant viruses as described previously in Lee *et al* 2001 and explained under construction of HCMV mutant and wild-type recombinant viruses in this section. The mt and WT recombinant sequences were borrowed from (J. Netterwald) and the exact procedure is described in (Netterwald *et al* 2005). It should be noted that for this project the GAS elements were not deleted; only a partial deletion of the spacer region was created by J. Netterwald. The spacer region was partially deleted by mutagenesis of HCMV-BAC. $AD169_{BAC}$ DNA was electroporated into electro-competent *E. coli* DY380 cells. Polymerase chain reaction (PCR) was used to amplify a mutagenesis cassette that contained markers for kanamycin resistance (Kan^r) and beta-galactosidase (LacZ) function from a p-GEMT plasmid (given by Dr. Shenk, Princeton University). The kan/lacZ primers used contained base-pair sequences homologous to the spacer region sequences between the GAS elements in the MIEP. The PCR product with the homologous sequences was electroporated into *E. coli* DY380 (HCMV-BAC) cells and transformants were selected

based on their ability to grow on kanamycin if homologous recombination took place and the formation of blue colonies (Netterwald *et al* 2005).

For the base-pair substitutions made in the GAS elements of the recombinant mutant virus in this study, Netterwald et al. constructed a plasmid with a Zeo^r gene expression cassette. The primers used contained loxP sites: Zeo-loxP-F and Zeo-loxP-R. The 550 PCR product was cloned into a p-GEMT plasmid (Promega) yielding a pGEM-lox-zeo vector. A two step PCR was employed to generate a total of 4 base pair substitutions in the GAS original sequences with the use of $AD169_{BAC}$-DNA as a template for the reaction. BAC DNA technology was used to insert the GAS elements into *E. coli* DY380 by homologous recombination.

Construction of HCMV mutant and wild-type recombinant viruses

HCMV (mt and WT) recombinant sequences were each cloned separately into pGEMT vectors. The recombinant sequences were excised from the plasmid by double restriction enzyme digestion with BglII and SphI (NE BioLabs) for 2 hours at 37°C in a 50 ul reaction. The digestion product was run through gel electrophoresis and the DNA band containing the recombinant sequences of 1.2 kb was excised from the agarose gel using a gel extraction kit following the protocol from (QIAEX II, Quiagen, Inc). The (mt and WT) recombinant sequences were each inserted by ligation at the SphI and BglII sites of pGEM-lox-zeo vectors which also contained an amp^r gene. Each product was given the name of pGEM-lox-zeo-GAS-wt and pGEM-lox-zeo-GAS-mt respectively. The ligation products were plated on LB plates containing 50 ug/ml zeocin and 100 ug/ml ampicillin.

Colonies were chosen based on their ability to grow on these plates and their ability to show a fragment at 120 kb after digestion with SphI and BglII and gel electrophoresis. A PCR was done to add the homologous base pair sequences (HA and HB) that were 50-bp each to pGEM-lox-zeo-GAS-wt and pGEM-lox-zeo-GAS-mt respectively. The primers used were long GAS HA ISG primer 5' and 3' and the reaction conditions were set for 30 cycles at 94°C (5 min.), 94°C (1 min.), 60°C (1 min.), and 72°C (1 min.). The PCR products were purified following the protocol by (QIAquick for PCR purification). Two-hundred ng of each PCR product were used to electroporation-transform *E. coli* DY380 (HCMV-BAC) with GAS deletion. The purpose was to replace the kanamycin cassette that these cells contained as previously mentioned with the mutagenesis cassettes: HAGAS**(mt or wt, respectively)**lox-zeo-loxHB. Each cassette was 850 bp long. Electroporation was done using Gene Pulser II electroporator (BioRad, Hercules, CA) in a cuvette at 1.6 kV, 25 μF and 200 ohms. The transformed cells were selected based on their ability to grow on LB (12.5 μg/ml Chloramphenicol) and (50 μg/ml zeocin) agar plates. In addition, the resulting recombinants were screened on kanamycin to verify the replacement of the kan/lacZ cassette by homologous recombination. BAC DNA minipreps were made from the recombinants chosen following the protocol from (Qiagen, Inc). A PCR was done using ISG element primers 5' and 3' to check which recombinants had the 1.2 kb GAS fragment. Taq DNA polymerase from (Qiagen, Inc) was used and the reaction was set for 30 cycles at 94°C, 55°C and 72°C. *E. coli* DH10B cells were electroporation-transformed with the Ad169-BAC GAS (mutant or wild-type) DNA and a maxiprep was done following the protocol from (Nucleobond) under low

copy plasmid/BAC purification to produce a good amount of transfectable DNA. Both mutant and wild-type sequences were sent for DNA sequencing at this point. HFF cells were transfected by electroporation as mentioned above with 5 ug of the Ad169-BAC GAS (mt and wt) DNA respectively. The 1.5 μg of Cre plasmid; 1.5 μg of pp71; 3.0 μg of GFP were co-transfected as well. Plaques had to be purified as described in (Flint *et al* 2004) because there were some plaques that were fluorescent (had GFP) in them and further contact could had contaminated the non-GFP plaques. In the contaminated plaques the zeocin cassette was not removed by Cre so they appeared fluorescent green. HFF cells were infected at MOI = 0.1 with the purified viral plaques to make large stocks of the viruses. Titers were determined by the plaque assay.

The removal of the 550 bp zeocin cassette was verified by PCR. The primers that can amplify this were Zeo-lox-P 5' and 3'. The presence of the GAS sequences was also confirmed by PCR using ISG element primers.

Growth curve analysis

A multi-step growth curve analysis was performed to determine the effect of mutations in the recombinant viruses on viral growth. HFF cells were grown to confluence on six-well culture plates and infected at low and high MOIs (0.1 and 5 respectively) with the recombinant viruses. For the low MOI, samples were collected every other day at the same time and for the high MOI, they were collected every day as indicated in the results section. For collection the cells were scraped from the plate with a cell lifter and the medium collected was frozen immediately on dry ice and then stored at -80°C. HFF cells

were infected with each of the samples collected and the titers were determined by the plaque assay. A growth curve was generated by plotting titer results vs. time.

Western Blotting

HFF cells were infected at low and high MOIs (0.1 and 1.0) with the recombinant viruses and the original HCMV-Ad169 wild-type. Samples were collected by scraping cells with a cell lifter at 8, 12, 24 and 48 hours. For the high MOI, samples for all three viruses just mentioned were collected only at 8 hours. After collection, the samples were centrifuged in a top speed micro-centrifuge and the pellets were washed with 1 ml PBS twice. The pellets were frozen immediately in dry ice and stored at -80°C. The monoclonal antibodies used were IE1/2 (mAB810 from Chemicon, Temecula, CA) and actin as an internal control (clone 80 from Boehringer, Mannheim, Germany). A 10% SDS-polyacrylamide gel was made; 10 ul of cell lysates were loaded onto the corresponding wells and electrophoresis was performed in 1X buffer containing 25 mM of Tris; 0.1% SDS and 200 mM of glycine. Protein transfer was allowed overnight at 4°C in 1X transfer buffer (same as 1X buffer, but with 20% methanol). The following morning, Ponceuas S (Sigma, Inc) was used to stain the membrane for 20 minutes and at room temperature. Blocking of the membrane with TBST buffer (10 mM Tris-HCl pH 8.0; 150 mM NaCl; 0.05% Tween 20) and 10 % milk for 30 min. was done to eliminate non specific binding. All fluid was removed and 1st antibody IE1/2 (mAB810 from Chemicon, Temecula, CA) was diluted (1:100) in TBST and 1% milk) and added. After one hour incubation at room temperature, the membrane was washed with TBST three times at 10 minutes each. Anti-mouse antibody (IgG-HRP) was used as a 2nd antibody

and was incubated with the membrane for one hour at room temp. The membrane was washed 3times at 20 min. each with TBST. Membrane developing was done for one min. with the Western Lightning ECL reagent (Perkin-Elmer, MA). Exposing to x-ray film (Denville Scientific, NJ) allowed the visualization of the antigen-antibody binding.

RESULTS

Construction of HCMV wild-type and mutant recombinant viruses

Part 1: Cloning

The 120-bp fragment containing the recombinant (WT and mt) HCMV sequences were each cloned individually into a pGEM-T vector by TA cloning. Two ug of each pGEMT (GAS mt and WT) DNA were individually doubled digested with restriction enzymes: Sphl and BglII. Gel electrophoresis was run and the DNA bands showing at 1.2 kb were excised from the gel. Each recombinant GAS (WT and mt) sequence were each inserted at the SphI and BglII sites of pGEM-lox-zeo vectors. Homologous base pair sequences (HA and HB) were added to pGEM-T-lox-zeo-GAS-wt and pGEM-lox-zeo-GAS-mt respectively by PCR. (Figure 1).

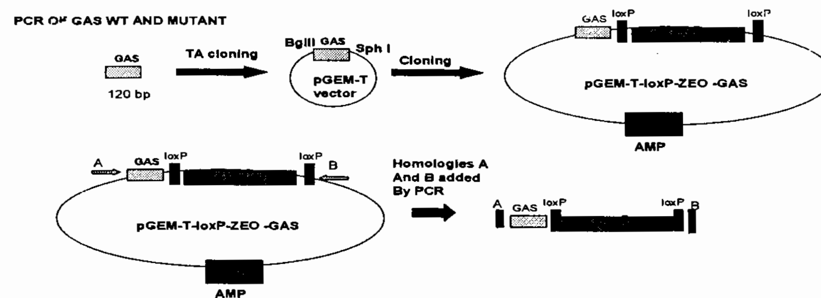


Figure 1. Cloning of (WT and mt) recombinant sequences to pGEM-T and pGEMT-loxP-Zeo vectors. The final PCR product is about 850-bp and will serve as the mutagenesis cassette to be inserted by homologous recombination into *E. coli* DY380 (HCMV-BAC) exhibiting deletion of the GAS elements and their spacer region.

Part 2: Mutagenesis

E. coli DY380 (HCMV-BAC) with GAS deletion were electroporation-transformed with two-hundred ng of each PCR product to replace the kanamycin cassette with the mutagenesis cassettes: HAGAS**(mt or WT, respectively)**lox-zeo-loxHB. A total of two recombinants for each wt and mt mutagenesis were selected based on their ability to grow on zeocin. BAC DNA minipreps were made from the recombinants selected which were zeo^r and kan^s. *E. coli* DH10B cells were electroporation-transformed with the Ad169-BAC GAS (mt and WT) DNA and a maxiprep was done to produce a good amount of transfectable DNA (Figure 2). HFF cells were transfected by electroporation with 5 ug of the Ad169-BAC GAS (mt and WT) DNA respectively. The efficiency of transfection was determined by the presence of GFP. About 10% HFF cells expressed the GFP phenotype (data not shown). At a week post-transfection, some viral plaques were observed in the mt and WT transfected cells. At two weeks post-tansfection, non-GFP plaques were purified as previously described (Flint *et al* 2004) to avoid contamination with the GFP expressing plaques. These were contaminants because the co-transfection with Cre should have removed the GFP by then. By this time, the recombinant WT virus showed many large plaques and lysis of the cells while the mutant showed only very small plaques and no visible lysis. This observation suggests that the mutant has a slow growth phenotype when compared to the recombinant WT virus. (Figure 3). The titers of both viruses were determined by the plaque assay.

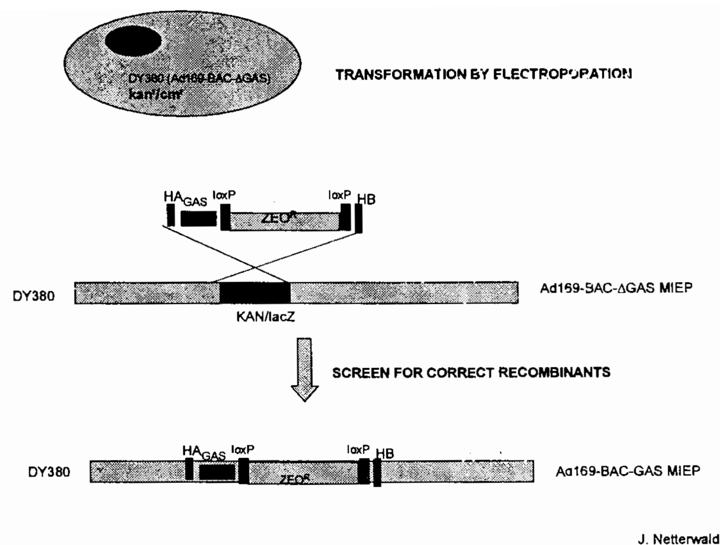
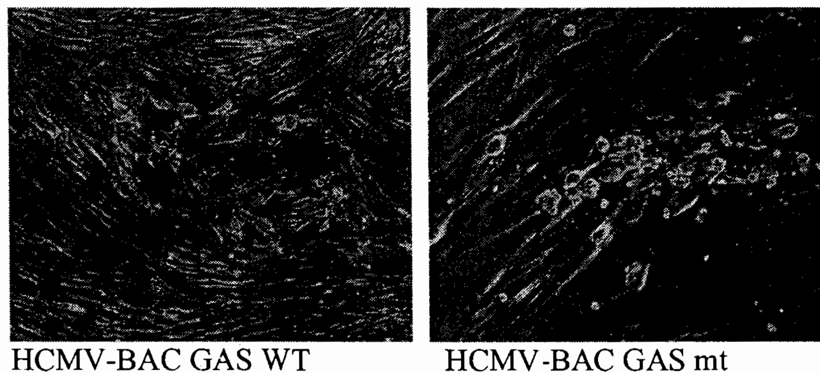


Figure 2. BAC technology. A mutagenesis cassette harboring a zeocin resistance marker and HCMV recombinant WT and mt GAS sequences was used for target replacement of a kan^r/lacZ cassette in *E. coli* DY380 (HCMV-BAC).



HCMV-BAC GAS WT HCMV-BAC GAS mt

Figure 3. Photomicrographs were taken at 10 days post-transfection. HCMV-BAC GAS WT shows large viral plaques and lysis of the cells while HCMV-BAC GAS mt shows small plaques and no lysis.

Recombinant wild-type and mutant sequences were confirmed by PCR and DNA sequencing analysis

The presence of the mutagenesis cassette containing the 1.2 kb GAS recombinant WT and mt sequences was confirmed DNA sequencing analysis and by PCR. DNA sequencing was done on the recombinant BAC DNAs prior to transfecting HFF cells. (Figure 4). The PCR was done using ISG element primers 5' and 3'. (Figure 5).

Ad169-BAC GAS WT	**Ad169- BAC GAS mt**
TTACGGTAAACGTTACGGTAAA	TTAGGGTTAACGTTAGGGTTAA

Figure 4. Ad169-BAC GAS WT contains the WT consensus sequence of the GAS elements but a partially deleted spacer region (3-bp long) between them. Ad169- BAC GAS mt contains 4 base pair changes in the GAS elements and the same mutated spacer region as the WT.

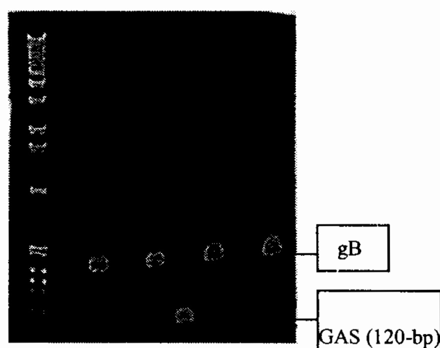


Figure 5. A PCR using recombinant BAC WT and mt DNA was done with ISG element primers which can amplify the GAS fragments to confirm their presence and that of the spacer region after mutagenesis. For both WT and mt, one of the transformants showed a band at 1.2 kb indicating the presence of the GAS elements. (WT data not shown). Glycoprotein (gB) was used as a negative control. Although the positive control (recombinant HCMV with GAS elements) failed to show a band, DNA sequencing confirmed the presence of the recombinant sequences.

Determination of recombinant HCMVs growth kinetics through growth curve analysis

The growth kinetics of both WT and mt HCMV recombinant viruses was determined by a multiple step growth curve. HFF were infected with the recombinant HCMVs at MOI

0.1 and 5 and samples were collected every other day for the low MOI and everyday for the high MOI over about a 2-week period. The titer for each sample collected was determined by the plaque assay. In figure 6A, the growth curve at low MOI 0.1 shows that both original Ad169 HCMV and the (WT) recombinant virus grow at a similar rate, while the (mt) recombinant virus has a peak titer that is about 10-fold lower than the WT viruses. This finding agrees with the observations by J. Netterwald which showed that mutations in the GAS elements cause a slow growth phenotype in recombinant HCMV viruses at low MOI. Apparently the mutations done to create a short spacer region in this study, did not have an effect in viral growth at low MOI since the recombinant WT virus grew similarly to the Ad169 original strain of HCMV. In figure 6B, the growth curve at MOI 5 shows that the recombinant WT grew similarly to the Ad169-HCMV, further suggesting that mutations in the spacer region between the GAS elements may not have a significant effect on viral growth. However, the (mt) recombinant HCMV again showed a peak titer about 10-fold lower than the WT viruses suggesting that the combination of mutations in the GAS elements and spacer region may have affected virus growth at high MOI. This finding disagrees with the data observed by J. Netterwald which demonstrated that at high MOI, (mt) recombinant HCMV containing mutations in the GAS elements had a growth rate similar to the Ad169-HCMV. (Figure 6)

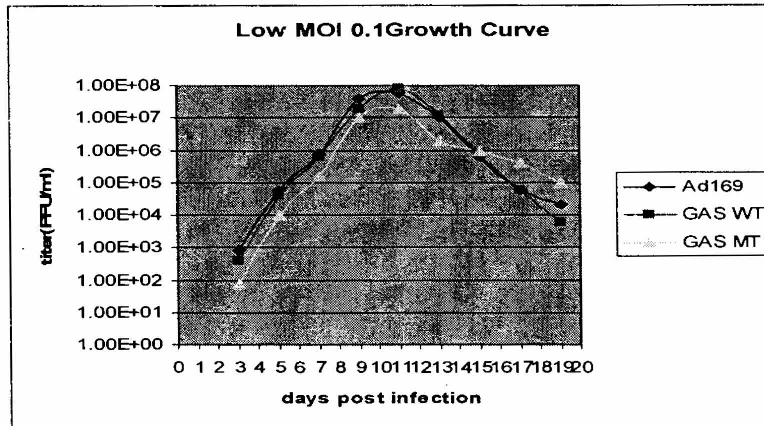


Figure 6. A. HFF cells were infected with Ad169-HCMV, recombinant WT and mt HCMVs At low MOI 0.1. Cells and viruses were harvested at 3, 5, 7, 9, 11, 13, 15, 17, and 19 days post infection. The infectious titer of each sample was determined by the plaque assay and a growth curve was constructed by plotting individual titer values against time. Ad169-HCMV and the recombinant WT virus showed a similar growth rate. The recombinant mt virus which contained mutations in the GAS elements and the spacer region showed a peak titer that was about 10-fold lower than the recombinant WT HCMV and the Ad169-HCMV.

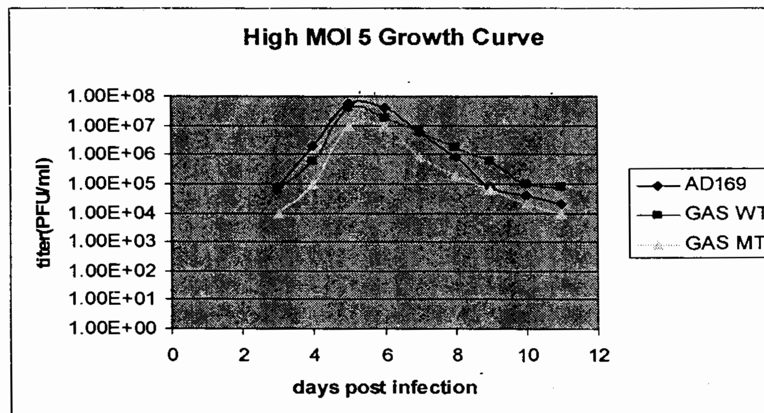


Figure 6. B. HFF cells were infected with Ad169-HCMV, recombinant WT and mt HCMVs At high MOI 5. Cells and viruses were harvested everyday for 11 days post infection. The growth curve at high MOI 5 shows that the recombinant mt HCMV once again had a peak titer about 10-fold lower than the WT viruses. The recombinant WT HCMV showed a similar growth pattern to Ad169-HCMV.

Effects of mutations in recombinant HCMVs on MIEP IE1/2 gene expression by Western Blotting

The MIEP controls the expression of IE1 and IE2 proteins which are essential in viral replication. The MIEP contains two GAS elements and previous studies have shown that mutations in the GAS elements result in a slow growth phenotype at MOI 0.1. In the present study the same slow growth phenotype was observed and our aim was to determine the effect of mutations in the GAS elements and spacer region of the recombinant viruses on MIE gene expression. The expression of IE1 and IE2 proteins was compared between the (WT) recombinant and (mt) HCMVs constructed in this study through Western Blotting. HFF cells were infected with the (mt and WT) recombinant viruses at low and high MOIs (0.1 and 1). Samples were collected at 8, 12, 24 and 48 hours. IE1/2 monoclonal antibodies were used along with actin as an internal control. Lower levels of IE1 and IE2 protein expression was observed for the (m) recombinant HCMV when compared to the WT recombinant virus at high and low MOIs. This result suggests that the mutations in the GAS elements might be the cause for the low IE1/2 expression in the mt virus since the WT showed higher expression of the proteins. The (WT) recombinant HCMV contained the mutation of the spacer region but its GAS elements were intact further demonstrating that mutations in the spacer region may not have a significant effect on MIEP IE1/2 expression. (Figure 7).

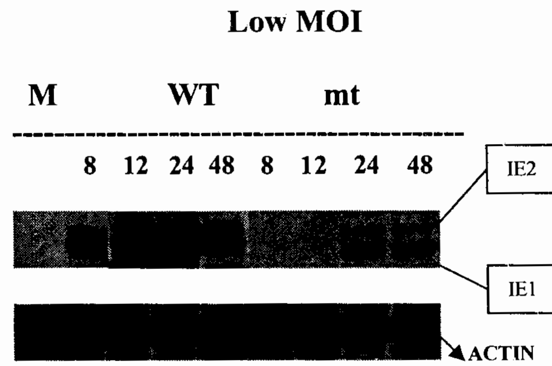


Figure 7. A. HFF cells were infected with recombinant HCMV-BAC GAS WT and HCMV-BAC GAS mt at MOI 0.1. Cells were harvested at 8, 12, 24 and 48 hours post infection (hpi) and the expression of IE1 and IE2 proteins was measured by Western blotting using mAb810 which reacts with IE1 and IE2. The mock (M) does not show any binding of the mAb810. The recombinant WT virus shows strong bands at 12, 24 and 48 (hpi) while the mt shows no binding of mAb810 at 8 and 12 hpi and reduced binding at 24 and 48 hpi compared to the recombinant WT virus.

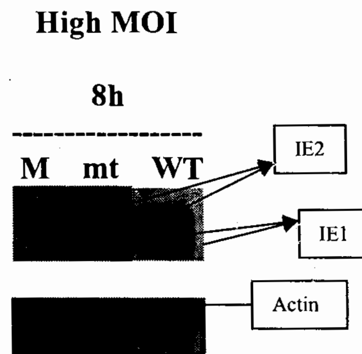


Figure 7. B. HFF cells were infected with recombinant HCMV-BAC GAS WT and HCMV-BAC GAS mt at MOI 1. Cells were harvested only at 8 hpi and IE1 and IE2 expression was measured. The mock (M) shows no reactivity with the mAb810 while the mutant shows reduced levels of IE1 and IE2 expression when compared to the recombinant WT virus at high MOI.

DISCUSSION

BAC technology was utilized in this study to create and purify recombinant HCMVs containing defined mutations of the GAS elements and their spacer region located in the MIEP. The HCMV was maintained as a BAC clone in *E. coli* cells making it easier for efficient mutagenesis to take place by homologous recombination. In this study a kan/lacZ cassette harboring a deletion of the GAS elements and their spacer region was successfully replaced with a zeo^r gene expression cassette containing the recombinant GAS and spacer region sequences. The BAC technology allowed for the rapid and efficient recovery of recombinant viruses facilitating the analysis to define the effects of the mutations created in HCMV.

HCMV gene expression gets initiated from the MIE region that is expressed prior to viral transcription. The MIEP produces the two major IE genes in the HCMV genome: IE1 and IE2 which encode a number of MIE regulatory proteins and are the first to be expressed after infection and play a major role in the efficiency of DNA replication (Marchini et al 2001; Isomura *et al* 2003; Mocarski, 2001). Studies have shown that deletions to the HCMV MIEP can significantly reduce viral replication at low MOI (Meier et al 2000). Two GAS-like elements are found in the MIEP and a recent study by Netterwald *et al.* demonstrated that deletions and mutations of the GAS elements in HCMV resulted in a slow growth phenotype, but the defect was only observed at low MOI, whereas at high MOI the virus grew similar to its WT strain. In the present study, bp-substitution mutations and a partial deletion of the spacer region between the GAS elements were created resulting in a slow growth phenotype at low and high MOIs as

observed in growth curve analysis. With this observation we suggest that important transcriptional binding sites are located within the mutated regions causing a decrease in the activation of transcription from the MIEP. In order to determine the biological cause of the growth defect observed in the recombinant mt virus constructed in this study, we tested the expression of IE1 and IE2 in the recombinant viruses by Western Blotting since these two genes are also transcribed from the MIEP. Our results indicated that IE1 and IE2 expression was reduced in the mt virus when compared to the WT at both high and low MOIs. At low MOI, the WT showed strong bands of IE1/IE2 as soon as 8 hpi while the mt showed reduced IE1/IE2 expression only after 24 hpi. At high MOI and at 8 hpi a clear reduction of IE1/2 expression by the mt virus was observed when compared to the WT virus.

HCMV initiates a signal transduction pathway in the cell upon its binding to cell surface receptors which results in the induction of interferons and later the transcription of ISGs. STATS get activated first as a response to the virus and the complex formed binds to ISRE and GAS elements which activate the transcription of ISGs (levy *et al* 2002). Interestingly enough, studies have shown that cytokines such as IFN-gamma and TNF-alpha synthesized during the signal transduction pathway that HCMV initiates have a positive effect in HCMV replication (Cebulla *et al* 1999). The HCMV MIEP has two GAS like elements which participate in MIE gene expression since their mutations and deletions not only caused a slow growth phenotype in the mt virus at low MOI, but also reduces the expression of IE1 and IE2 genes which are involved in viral replication. This study suggested that the HCMV-initiated signal transduction pathway not only stimulates

ISGs, but also stimulates IE gene expression through the GAS elements in the MIEP (Netterwald *et al* 2005).

In this study, we also investigated the importance of the spacer region between the GAS elements in the MIEP. This 113-bp region is a large insertion between the 9-bp GAS sequences (TTACGGTAA). The spacer region was deleted to 3-bases (ACG) to separate the GAS elements. However it was not completely deleted because a gel shift analysis using a luciferase reporter plasmid (pELu-GAS) demonstrated that the combination of base-pair mutations in the GAS elements and partial deletion of their spacer region resulted in the inability of HCMV proteins to bind to GAS sequences (Netterwald *et al* 2005). The data collected in the present study suggests that the base-pair mutations in the GAS elements of the recombinant mt HCMV constructed in this study are responsible for its slow growth phenotype and the reduced IE protein expression observed. The WT recombinant virus containing the original GAS sequence and mutated spacer region had a similar growth rate to the HCMC AD169 original WT strain; therefore the deletion in the spacer region did not affect its growth. IE1 and IE2 protein expression in the WT virus was significantly higher than that of the mt virus, demonstrating that the replication of this virus was not affected by the deletion in the spacer region and that this region is not essential for HCMV life cycle as long as the GAS elements are intact. A recent study by Netterwald et al. demonstrated that deletion of the GAS elements and their spacer region caused a significant growth defect. The present study demonstrates that the GAS elements and not their spacer region are important in HCMV replication.

A possibility exists that the close proximity of the GAS elements in the WT recombinant virus created by deleting most of their spacer region may have induced a synergistic effect. This means that the two GAS elements next to one another might have given a stronger promoter activation that compensated for any growth defects induced by the deletion of the spacer region. Further investigations need to be done to determine if there are any important transcription binding sites in the spacer region of the MIEP GAS elements to help prove the statement about the synergistic effect just mentioned.

CONCLUSIONS

The main purpose of this study was to determine the effects of mutations in the GAS elements and spacer region of HCMV in viral MIE gene expression and replication. To accomplish this, BAC technology was utilized to create recombinant HCMV harboring the desired mutations.

We conclude from this study that the GAS elements located in the HCMV MIEP are important for viral replication. The specific base-pair substitutions made in the mt recombinant virus caused a growth defect at both low and high MOI. The defect was due to the reduced MIE (IE1 and IE2) expression. This finding further suggests that HCMV induces an unknown signal transduction pathway to activate gene expression from its MIEP via the GAS elements.

The recombinant WT virus which had the original sequence of the GAS elements, but a mutated 3-bp spacer region grew similarly to HCMC AD169 original WT strain and its IE1 and IE2 expression was not affected neither at low or high MOI. This data suggests that the spacer region located between the GAS elements in HCMV MIEP may not be important in HCMV life cycle as long as the GAS elements are intact.

LITERATURE CITED

Bresnahan, W.A. and Shenk, T.E. UL82 virion protein activates expression of immediate-early viral genes in human cytomegalovirus-infected cells. 2000. Proceedings of the National Academy of Sciences USA 97: 14506-14511.

Cebulla, C.M., Miller, D.M., and Sedmak, D.D. Viral inhibition of interferon signal transduction. 1999. Intervirology 42: 325-330.

Compton, T., Nowlin, D.M., and Cooper, N.R. Initiation of Cytomegalovirus infection requires initial interaction with cell surface heparan sulfate. 1993. Virology 193: 834-841.

Fortunato, E.A., McElroy, A.K, Sanchez, V., and Spector, D. Exploitation of cellular signaling and regulatory pathways by human cytomegalovirus. 2000. Trends in Microbiology 8: 111-119.

Gawn, J.M. and Greaves, R.F. Absence of IE1 p72 protein function during low-multiplicity infection by Human Cytomegalovirus results in a broad block to viral delayed-early gene expression. 2002. Journal of Virology 76: 4441-4455.

Greaves, R.F. and Mocarski, E.S. Defective growth correlates with reduced accumulation of a viral DNA replication protein after low-multiplicity infection by a human cytomegalovirus ie1 mutant. 1998. Journal of Virology 72: 366-379.

Gredmark, S., Britt, W.B., Xie, X., Lindbom, L., and Soderberg-Naucler, C. Human Cytomegalovirus induces inhibition of macrophage differentiation by binding to human aminopeptidase N/CD13. 2004. Journal of Immunology 173(8): 4897-907.

Heider, J.A., Bresnahan, W.A., and Shenk, T.E. Construction of a rationally designed human cytomegalovirus variant encoding a temperature sensitive immediate-early 2 protein. 2002. Proceedings of the National Academy of Sciences USA 99: 3141-3146.

Homer, E.G., Rinaldi, A., Nicholl, M.J., and Preston, C.M., Activation of herpesvirus gene expression by the human cytomegalovirus protein pp71. 1999. Journal of Virology 73: 8512-8518.

Ishov, A.M., Vladimirova, O.V., and Maul, G.G. Daxx-mediated accumulation of human cytomegalovirus tegument protein at ND10 facilitates initiation of viral infection at these nuclear domains. 2002. Journal of Virology 76: 7705-7712.

Isomura, H., and Stinski, M.F. The human cytomegalovirus major immediate-early enhancer determines the efficiency of immediate-early gene transcription and viral replication in permissive cells at low multiplicity of infection. 2003. Journal of Virology 77: 3602-3614.

Keay, S., and Balwin, B.R. Anti-idiotype antibodies that mimic gp86 of human cytomegalovirus inhibit viral fusion but not attachment. 1991. Journal of Virology 65: 5124-5128.

Levy, D.E., and Darnell, J.E., Jr. STATS: Transcriptional control and biological impact. 2002. National Review of Molecular Cell Biology 3: 651-662.

Marchini, A., Liu, H., and Zhu, H. Human cytomegalovirus with IE-2 (UL122) deleted fails to express early lytic genes. 2001. Journal of Virology 75: 1870-1878.

Meier, J.L., and Pruessner, J.A. The human cytomegalovirus major immediate-early distal enhancer region is required for efficient viral replication and immediate-early gene expression. 2000. Journal of Virology 74: 1602-1613.

Miller, D.M., Zhang, Y., Rahill, B.M., Waldman, W.J., and Sedmak, D.D. Human cytomegalovirus inhibits IFN-alpha-stimulated antiviral and immunoregulatory responses by blocking multiple levels of IFN-alpha signal transduction. 1999. Journal of Immunology 162: 6107-6113.

Mocarski, E.S. 2001. Cytomegaloviruses and their replication, p. 2629-2673. *In* D.M. Knipe et al. (ed.), fields virology, 4th ed., vol. 2. Lippincott Williams &Wilkins, Philadelphia, Pa.

Netterwald, J.R., Jones, T.R., Britt, W.J., Yang, S.J., McCrone, I.P., and Zhu, H. Post attachment events associated with viral entry are necessary for induction of interferon-stimulated genes by human cytomegalovirus. 2004. Journal of Virology 78: 6688-6691.

Netterwald, J.R., Yang, S.J., Wang, W., Ghanny, S., Cody, M., Soteropoulos, P., Tian, B., Dunn, W., Liu, F., and Zhu, H. Two gamma interferon-activated site-like elements in the human cytomegalovirus major immediate-early promoter/enhancer are important for viral replication. 2005. Journal of Virology 79: 5035-5046.

Sissons, J.G.P., Bain, M., Wills, M.R., and Sinclair, J.H., Latency and reactivation of human cytomegalovirus . 2002. Journal of Infection 44: 73-77.

Smith, M.S., Bentz, G.L., Alexander, J.S., and Yurochko, A.D. Human cytomegalovirus induces monocyte differentiation and migration as a strategy for dissemination and persistence. 2004. Journal of Virology 78: 4444-4453.

Speir, E., Modali, R., Huang, E.S., Leon, M.B., Shawl, F., Finkel, T., and Epstein, S.E. Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. 1994. Science 265: 391-394.

Steinberg, R.M. The human cytomegalovirus major immediate-early gene. 1996. Intervirology 39: 343-349.

White, E. A., Clark, C.L., Sanchez, V., and Spector, D.H. Small internal deletions in the human cytomegalovirus IE2 gene result in nonviable recombinant viruses with differential defects in viral gene expression. 2004. Journal of Virology 78: 1817-1830.

Yang, S., Netterwald, J., Wang, W., and Zhu, H. Characterization of the elements and proteins responsible for interferon-stimulated gene induction by human cytomegalovirus. 2005. Journal of Virology 79: 5027-5034.

Yu, D., Smith, G.A., Enquist, L.W., and Shenk, T. Construction of a self-excisable bacterial artificial chromosome containing the human cytomegalovirus genome and mutagenesis of the diploid TRL/IRL13 gene. 2002. Journal of Virology 76: 2316-2328.

Zhu, H., Cong, J.P., Mamtora, G., Gingeras, T., and Shenk, T. Cellular gene expression altered by human cytomegalovirus: global monitoring with oligonucleotide arrays. 1998. Proceedings of the National Academy of Sciences USA 95: 14470-14475.