Antiviral Research 102 (2014) 11–22

Contents lists available at ScienceDirect

Antiviral Research

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

Inhibition of human cytomegalovirus replication by overexpression of CREB1

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ARTICLE INFO

Article history:
Received 12 August 2013
Revised 28 October 2013
Accepted 25 November 2013
Available online 5 December 2013

Keywords:
Human cytomegalovirus
Major immediate-early genes
CREB1
NF-κB
SP1
SP3

ABSTRACT

Expression of the human cytomegalovirus (HCMV) major immediate-early (MIE) genes is regulated by a strong enhancer-containing promoter with multiple binding sites for various transcription factors, including cyclic AMP response element binding protein 1 (CREB1). Here we show that overexpression of CREB1 potently blocked MIE transcription and HCMV replication. Surprisingly, CREB1 still exhibited strong inhibition of the MIE promoter when all five CREB binding sites within the enhancer were mutated, suggesting that CREB1 regulated the MIE gene expression indirectly. Promoter deletion analysis and site-directed mutagenesis identified the region between −130 and −50 upstream of the transcription start site of the MIE gene as the "CREB1 responsive region". Mutations of SP1/3 and NF-κB binding sites within this region interrupted the inhibitory effect induced by CREB1 overexpression. Our findings suggest that overexpression of CREB1 can cause repression of HCMV replication and may contribute to the development of new anti-HCMV strategies.

1. Introduction

Human cytomegalovirus (HCMV), a member of beta-herpesvirus family, is a wide-spread human pathogen. Infection by HCMV is life-threatening for people who are immunocompromised, such as human immunodeficiency virus (HIV) infected persons, organ transplant recipients, and new born infants (Mocarski, 2002). Like all other herpesviruses, HCMV can remain latent in the host over long periods of time. HCMV replicates in various differentiated cells, such as macrophages, fibroblasts, epithelial cells, endothelial cells, and smooth muscle cells (Fish et al., 1995a,b; Ibanez et al., 1991; Sinzger et al., 1995). In contrast, the virus is maintained in a latent state and does not replicate in poorly differentiated cells such as progenitor cells of the bone marrow and monocytes of the blood (Sinclair and Sissons, 1996).

The major immediate-early (MIE) genes of HCMV play a key role in re-activation of the virus from latent to lytic phases. The MIE promoter contains a very strong enhancer between −550 and −50 relative to the transcription start site of +1 with multiple binding sites for transcription factors such as cyclic AMP response element binding protein/activating transcription factor (CREB/ATF), SP1/SP3, NF-κB/rel, AP1, NF1, YY1, retinoic acid receptor, ELK-1, and serum response factor (Meier and Stinski, 1996; Stinski and Isomura, 2008). An approximate 200-bp region between the MIE enhancer and the UL127 open reading frame (at position −741) is referred to as the unique region (UR). The UR functions as a boundary domain or an insulator, conferring repression on homologous as well as heterologous promoters in a cell-specific manner (Angulo et al., 2000; Lashmit et al., 2004; Lee et al., 2007b; Lundquist et al., 1999; Meier and Stinski, 1996; Stinski and Isomura, 2008). Three cellular homeoproteins, pancreatic-duodenal homeobox factor-1 (PDX1), special AT-rich sequence binding protein 1 (SATB1), and CCAAT displacement protein (CDP), bind to the UR (Chao et al., 2004; Lee et al., 2007a). PDX1 and CDP may function *in vivo* as repressors of HCMV MIE and UL127 transcription, respectively (Chao et al., 2004; Lee et al., 2007a).

Expression of the MIE gene can also be regulated by the cell cycle. In the G_0/G_1 compartment of the cell cycle, transcription of

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http://dx.doi.org/10.1016/j.antiviral.2013.11.012

the MIE gene is activated but transcription is subsequently suppressed when cells are in the S compartment (Fortunato et al., 2002; Salvant et al., 1998). Using specific cyclin-dependent kinase (CDK) inhibitors like flavopiridol and roscovitine (Chao et al., 2000; Chao and Price, 2001; Pumfery et al., 2006), several groups demonstrated the involvement of CDKs in controlling the transcription of the viral genes (Bresnahan et al., 1997; Kapasi and Spector, 2008; Sanchez et al., 2004; Sanchez and Spector, 2006; Zydek et al., 2010).

CREB1 is a member of the CREB/ATF protein family. The involvement of CREB1 in the regulation of viral transcription, including human T-lymphotropic virus, herpes simplex virus, and Epstein-Barr virus, has been reported (Kirby et al., 2000; Millhouse et al., 1998; Yoshimura et al., 1990). With HCMV, five putative CREB/ATF binding sites have been identified within the enhancer of the MIE genes (Lang et al., 1992; Meier and Stinski, 1996; Stinski and Isomura, 2008). *In vitro* association between CREB1 protein and the CREB/ATF binding elements has been confirmed by electrophoretic mobility shift assays (EMSAs) (Prosch et al., 2000; Rideg et al., 1994; Staak et al., 1997). The functional significance of the CREB/ATF binding sequences on the MIE gene expression has been investigated. The CREB/ATF sites in the presence of the other transcription factor binding sites of the distal and proximal enhancer did not contribute appreciably to the HCMV MIE promoter activity in infected human foreskin fibroblast (HFF) cells (Keller et al., 2003). In contrast, with viral bacterial artificial chromosome (BAC) DNA in the absence of the distal enhancer and in the presence of the SP1 sites at −75 and −55, the CREB/ATF site at −137 had a more significant effect on virus replication than the other transcription factor binding sites (NF-1, NF-κB, AP-1) (Lashmit et al., 2009). Furthermore, a recent report demonstrates that the CREB/ATF and the NF-κB sites have critical roles for desilencing virus replication in quiescently infected cell cultures (Liu et al., 2010).

The studies to-date have focused on the DNA binding sites of the transcription factors in the HCMV MIE distal and proximal enhancer, but little has been done to investigate the biological requirement of these transcription factors in either latency or productive HCMV replication. Here we report that overexpression of CREB1 inhibits transcription from the HCMV MIE promoter. Pre-expression of CREB1 followed by infection with HCMV reduced the number and size of viral plaques, and the production of recombinant virus. Surprisingly, the inhibition of HCMV MIE transcription by CREB1 is not mediated through the CREB/ATF binding elements located within the distal or proximal enhancer. The region between −130 and −50 of the MIE promoter is necessary for CREB1-mediated repression of the MIE promoter. Further mutational analysis indicates that the "CREB1 responsive region" is at or overlapping the SP1/3 and NF-κB binding sites within this region.

2. Materials and methods

2.1. Cells, plasmids, siRNAs, and reagents

293T cells were purchased from American Type Culture Collection. HFFs were cultured as previously described (Lashmit et al., 2009). The plasmids pWT and pM5 containing the wild-type (i.e. CMV WT) and the CREB mutant (i.e. CMV 5C mt, in which all five putative CREB binding sites were mutated) promoters of HCMV MIE gene, respectively, were previously described and kindly provided by Dr. Jeffery Meier (University of Iowa) (Keller et al., 2003). To generate the firefly luciferase (Luc) reporters, the HCMV MIE promoters of the pWT and pM5 were amplified by polymerase chain reaction (PCR). The amplified PCR fragments were used to replace the promoter region of pcDNA6 (Invitrogen), followed by inserting the coding region of Luc under the HCMV promoter, to generate CMV-Luc WT and CMV-Luc 5C mt (with all five CREB sites mutated). A series of CMV-Luc reporter plasmids with deletions of the MIE promoter were generated by molecular cloning, including 600-Luc (containing the promoter sequence from −600 to +1), 300-Luc (−300 to +1), 130-Luc (−130 to +1), and 50-Luc (−50 to +1). QuikChange site-directed mutagenesis kit (Stratagene) was utilized to generate 130-Luc C5 mt, 130-Luc C5/S1 mt, 130-Luc C5/S2 mt, 130-Luc C5/S1/S2 mt, and 130-Luc C5/S1/S2/N1 mutant plasmids with the CREB (one CREB1 site located within 130-Luc, i.e. C5), SP1/3 (two SP1/3 sites in 130-Luc, i.e. S1 and S2), or NF-κB binding elements (one NF-κB site in 130-Luc, i.e. N1) mutated. The primers used for mutagenesis are listed in Table 1. The human T-lymphotropic virus (HTLV) Luc reporter plasmid (i.e. HTLV-Luc) was a kind gift from Dr. Arnold Rabson (Lin et al., 2005). The CREB1, CREB2, SP1, and SP3 complementary DNAs (cDNAs), which were purchased from Open Biosystems, were used as the templates for PCR amplification. The amplified coding sequences of CREB1, CREB2, SP1, and SP3 were subcloned into the pUB6 vector (Invitrogen) to generate pUB-CREB1, pUB-CREB2, pUB-SP1, and pUB-SP3 in which the gene expression was driven by human ubiquitin C promoter. The NF-κB expression vectors, including p50 and p65 plasmids, were kindly provided by Dr. Hirotoshi Tanaka (Ouchida et al., 2003). The siRNA sets against SP1 and SP3 were purchased from Qiagen. Each siRNA set contained four individual siRNAs.

2.2. Transient transfection, luciferase assays, cell proliferation assays, and Western blotting

Transient transfection of 293T cells was performed using FuGENE6 (Roche) transfection reagent. Neon (Invitrogen) electroporation systems were used to transfect HFF cells. For luciferase assays, cells were grown to 40–60% confluence in 96-well plates and transiently transfected with the indicated plasmids. The amount of transfected plasmids was kept constant in each reaction by adjusting with the empty vector. Luc activities were measured 48 h post-transfection by the Bright-Glo assay system (Promega) and the activities were determined using an Infinite 200 multiplate reader (Tecan). For cell proliferation assays, cells were transfected with CREB1 and green fluorescent protein (GFP) expression vectors. Transfection efficiency was determined by monitoring the expression of GFP under a fluorescence microscope and cell proliferation was measured by CellTiter-Glo Assay according to the manufacturer's instruction (Promega). The primary antibodies used in Western blotting, including antibodies against CREB1, CREB2, SP1, SP3, p50, p65, lamin B, and actin, were obtained from Santa Cruz Biotechnology and the horseradish peroxidase-conjugated secondary antibodies were purchased from Pierce. The blots were then incubated with SuperSignal West Pico Substrate (Pierce) and the chemiluminescent signal was detected using an X-ray film (Thermo Scientific). The film was then scanned and the protein bands were quantified by the GS-800 densitometer (Bio-Rad).

2.3. Transcription and plaque assays

For the viral transcription assay, HFFs in 6-well plates were transfected by the calcium phosphate precipitation method (Graham and van der Eb, 1973) with 3 μg of one of the expression plasmids pUB6 (empty vector), pUB-CREB1, or pUB-CREB2 and 3 μg of recombinant virus BAC DNA per well. The viral BAC DNAs contained either the wild type MIE enhancer, the proximal MIE enhancer (i.e. dl-223/-694), or the MIE enhancer with the five putative CREB/ATF sites mutated (i.e. 5C mt). All viral BAC DNAs expressing viral MIE RNA, choramphenicol acetyl transferase (CAT), or GFP used in this study have been described previously (Keller et al., 2003; Lashmit et al., 2009). Cells were transfected with viral DNA

Table 1
The sequences of forward (F) and reverse (R) primers utilized to generate 130-Luc C5 mt (C5 mt), 130-Luc C5/S1 mt (C5/S1 mt), 130-Luc C5/S2 mt (C5/S2 mt), and 130-Luc C5/S1/S2 mt (C5/S1/S2 mt) plasmids by site-directed mutagenesis.

Construct	Template	Primer sequences
C5 mt	130-Luc	F: GTAACAACTCCGCCCCATCCCCGCAAATGGGCGGTAGGC R: GCCTACCGCCCATTTGCGGGGATGGGGCGGAGTTGTTAC
C5/S1 mt	C5 mt	F: CCAAAATGTCGTAACAACTCAAAACCATCCCCGCAAATGGGCGG R: CCGCCCATTTGCGGGGATGGTTTTGAGTTGTTACGACATTTTGG
C5/S2 mt	C5 mt	F: GCCCCATCCCCGCAAATGAAAAGTAGGCGTGTACGGTGGG R: CCCACCGTACACGCCTACTTTTCATTTGCGGGGATGGTTT
C5/S1/S2 mt	C5/S1 mt	F: CAAAACCATCCCCGCAAATGAAAAGTAGGCGTGTACGGTGGGAG R: CTCCCACCGTACACGCCTACTTTTCATTTGCGGGGATGGTTTTG
C5/S1/S2/N1 mt	C5/S1/S2 mt	F: GTTTTGGCACCAAAATCAACGCCACTTTCCAAAATGTCGTAAC R: GTTACGACATTTTGGAAAGTGGCGTTGATTTTGGTGCCAAAAC

to avoid activation of the MIE promoter by virion-associated proteins such as pp71 and gB (Liu and Stinski, 1992; Yurochko et al., 1997). Cells were harvested at 14 days post-transfection and assayed for MIE RNA or CAT as described previously (Lashmit et al., 2009). The relative amounts of MIE RNAs were set to 1.00 for the plasmid pUB relative to 18S cellular RNA and 24 h viral DNA input. CAT assays were as described previously (Lashmit et al., 2009). For the viral plaque assay, HFF cells were transfected with the expression plasmids by the Neon (Invitrogen) electroporation system. Transfection efficiency was determined as described above. Two days after transfection, the cells were infected with 0.1 PFU/cell of recombinant HCMV expressing GFP. The expression of GFP was driven by an SV40 promoter in the BAC backbone. After infection, the cells were over-laid with media containing agar for plaque assay as previously described (Lashmit et al., 2009). The data were photographed as green fluorescent foci (20× magnification) or enumerated as plaques at 10 days after viral infection. The mean corrected total cell fluorescence (MCTCF) was determined using ImageJ software (Burgess et al., 2010).

2.4. EMSA

EMSA was performed as described previously with some modifications (Chao et al., 2004; Lee et al., 2007a). Briefly, the biotin-labeled oligonucleotides, which covered the region between −130 and −50 [including, C5 WT (5′-CAACTCCGCCCCATTGACGCAAATGG GCGGTAGGC-3′), C5 mt (with the C5 site mutated, 5′-CAACTCCGC CCCATCCCCGCAAATGGGCGGTAGGC-3′), and N1 WT (5′-AAAATC AACGGGACTTTCCAAAATGT-3′], were used as DNA probes. The oligonucleotides with the CREB consensus binding sequence (5′-GAGATTGCCTGACGTCAGAGAGCTAG-3′) were used as a control. Nuclear extracts were prepared from 293T cells transfected with a pUB empty vector and a pUB-CREB1 expression plasmid, respectively. The biotin-labeled DNA probe was incubated with nuclear extracts at room temperature for 30 min, in the absence or presence of the 10× excess unlabeled DNA probe (i.e. specific competitor) or an anti-CREB1 antibody. DNA and DNA–protein complexes were resolved on 6% non-denaturing polyacrylamide gels (Invitrogen) in 0.5 × TBE running buffer (Invitrogen) at 4 °C for 60 min. Following electrophoresis, the gels were transferred to a nylon membrane (GE healthcare), which was then cross-linked using a UV Stratalinker (Strategene). The membranes were incubated with horseradish peroxidase labeled streptavidin (Biolegend) and subsequently exposed to an X-ray film.

2.5. Microarray analysis

Total RNAs were first isolated from pUB and pUB-CREB1 transfected 293T cells using the QIAGEN RNeasy RNA isolation kit. Cells transfected with an empty vector (i.e. pUB) were used as a control. Ten μg of total RNAs was used to synthesize double-stranded cDNAs using the GeneChip Expression 3′ Amplification One-cycle cDNA synthesis kit (Affymetrix, US). The cDNA was then used as a template for synthesizing biotin-labeled complementary RNA (cRNA). The cRNAs were then purified and fragmented before hybridizing onto an Affymetrix GeneChip Human Genome U133 Plus 2.0 array, which comprises of ~39,000 of the best characterized human genes. The samples were hybridized onto the arrays at 45 °C for 16 h. The arrays were then washed and stained with Affymetrix Fluidic Station 450 before scanning using Affymetrix GeneChip Scanner 3000. The data were then extracted via GeneChip Operating Software (GCOS). MAS5.0 normalization was performed on the raw data and the data was filtered for reproducibility at *p*-value of 0.05.

2.6. Quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR)

Total RNAs of the 293T cells transfected with the CREB1 expression plasmid were isolated using RNeasy mini kit (Qiagen). One μg of the total RNAs was converted into cDNA using ImProm™-II Reverse Transcription System (Promega). Specific cDNAs were amplified using SYBR Green PCR Master Mix (Applied Biosystems). The primer pairs used in the QRT-PCR include: SP1 (5′- TCATACCAGGTGCAAACCAA-3′ and 5′-TGAGAGCTGGGAGTCAAGGT-3′), SP3 (5′-TCCAGTCAGCAGATGGTCAG-3′ and 5′-TTGGGTTTGACCAGGAAAAG-3′), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5′-AACAGCCTCAAGATCATCAGC-3′ and 5′-GGATGATGTT CTGGAGGACC-3′). GAPDH was used as a control to normalize the cDNA inputs as described previously (Livak and Schmittgen, 2001). Amplification and detection of the cDNAs were performed using ABI Prism 7000 Thermal-Cycler (Applied Biosystems).

2.7. NF-κB activity assay

293T cells were transfected with the indicated expression vectors for 2 days prior harvesting. The NF-κB activity of the transfected cells was measured using TransAm™ NF-κB Family Transcription Factor Assay Kit according to the manufacturer's instruction (Active Motif).

3. Results

3.1. Overexpression of CREB1 negatively regulates the HCMV MIE promoter

Five putative binding sites for CREB/ATF family proteins were identified in the enhancer of the HCMV MIE genes (Fig. 1A, denoted

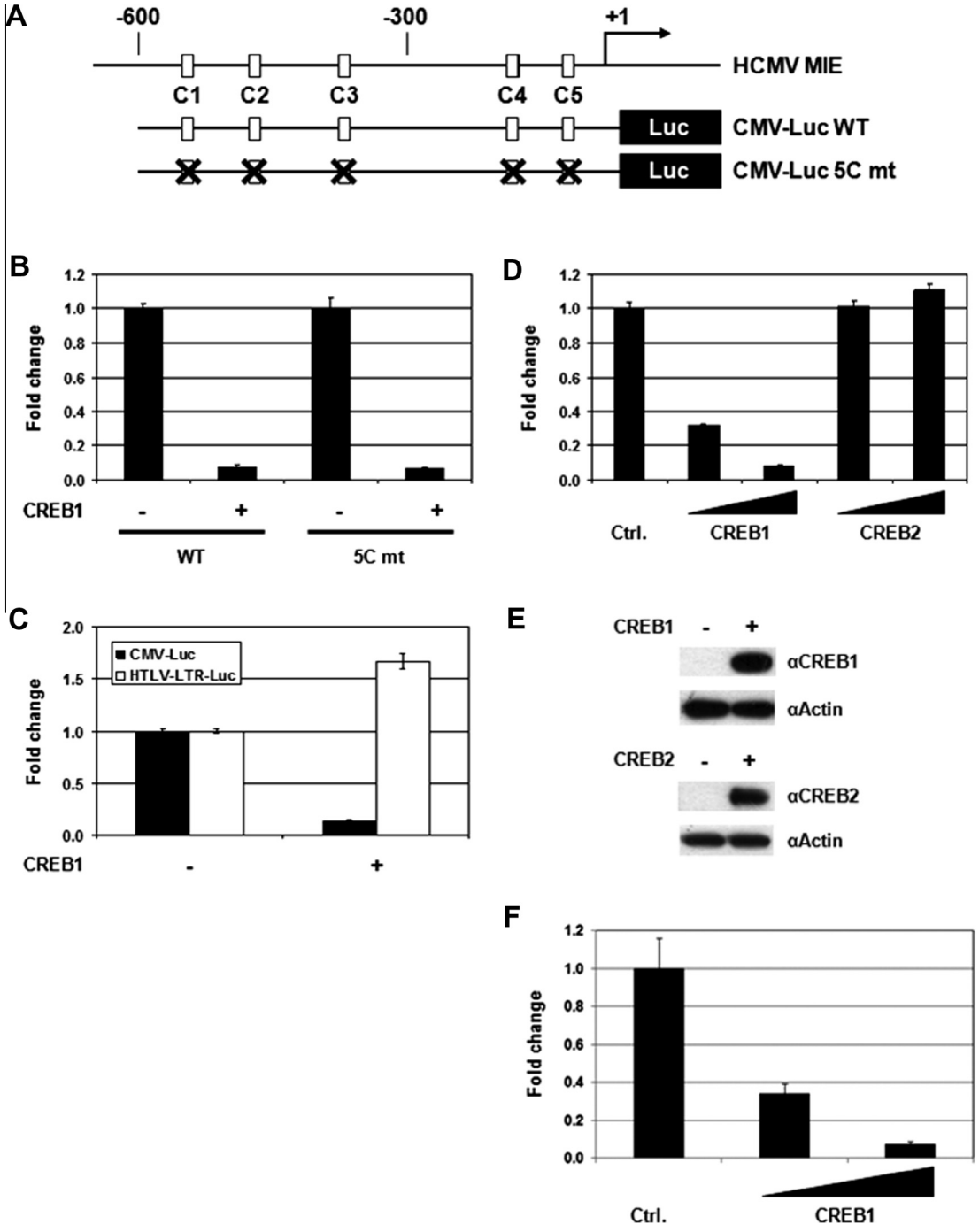

Fig. 1. Overexpression of CREB1 inhibits the HCMV MIE promoter. (A) Diagram of the HCMV MIE promoter, and the luciferase reporters containing the wild-type promoter (i.e. CMV-Luc WT) or the mutant promoter with all five CREB binding sites mutated (i.e. CMV-Luc 5C mt). The five putative CREB binding sites are labeled as C1–5. (B) Effects of CREB1 overexpression on the WT and 5C mt HCMV MIE promoters. 293T cells were cultured in a 96-well plate and co-transfected with an indicated CMV-Luc reporter and an expression vector (i.e. empty vector or CREB1 plasmid). Luc activity was relative to cells with the empty vector, which was set at 1.00. (C) Effects of CREB1 on the HCMV MIE and HTLV promoters. Cells were co-transfected with a Luc reporter (i.e. CMV-Luc or HTLV-Luc) and an indicated expression vector (i.e. empty vector or CREB1 plasmid). Luc activity was measured 2 days post-transfection. (D) Effects of CREB1 and CREB2 overexpression on the WT HCMV MIE promoter. Cells were transiently transfected with the WT CMV-Luc reporter and an indicated expression vector (i.e. empty vector, CREB1, or CREB2 plasmids). The CREB1 and CREB2 expression vectors were titrated at tenfold increment (i.e. 0.03 and 0.3 μg). (E) Cell lysates prepared from the CREB1- and CREB2-transfected cells were analyzed by Western blotting. Cells transfected with an empty vector were used as a control. Actin was utilized as a loading control for the blots.

as C1–5) (Meier and Stinski, 1996). To determine the role of CREB1 in regulation of the MIE gene expression, 293T cells were co-transfected with a CREB1 expression plasmid (i.e. pUB-CREB1) and a wild-type (WT) CMV-Luc reporter. Cells transfected with an empty vector and a CMV-Luc reporter were used as a control. When CREB1 was overexpressed, >90% inhibition of Luc expression, driven by the MIE promoter, was detected (Fig. 1B). Since five CREB binding elements are present within the MIE enhancer, it is possible that CREB1 may inhibit the MIE promoter through the direct association with the five binding sites. We generated the CMV-Luc 5C mt reporter in which all five CREB binding sites were mutated to disrupt the interaction between CREB1 and the enhancer.

Unexpectedly, ectopic expression of CREB1 still showed >90% inhibition on the mutant MIE enhancer-containing promoter (Fig. 1B). We next determined the effect of overexpression of CREB1 on a different viral promoter. There was a 67% increase in HTLV promoter activity in the CREB1-overexpressing cells. These data indicate that overexpression of CREB1 potently and selectively inhibited the HCMV MIE promoter, but activated the HTLV promoter (Fig. 1C). The impact of CREB2, another member of CREB/ATF family proteins, on the HCMV MIE promoter was also investigated. Overexpression of CREB2 showed little to no effect on the HCMV MIE promoter (Fig. 1D). Western blot analysis confirmed that both CREB1 and CREB2 expression vectors successfully increased the levels of CREB1 and CREB2 proteins in the transfected cells (Fig. 1E). The impact of CREB1 overexpression on the MIE promoter in the HCMV permissive cell, HFF, was also examined and a similar inhibitory effect on the promoter was detected (Fig. 1F).

We further investigated the effect of CREB1 overexpression on cell viability. 293T cells were transiently transfected with the indicated amounts of a pUB-CREB1 expression vector along with a pCMV-GFP plasmid as the internal control to illustrate the transfection efficiency. Overexpression of CREB1 did not cause any significant influence on cell viability (Supplementary Fig. 1A), while the transfection efficiency was estimated around 70–80% (Supplementary Fig. 1B). Expression from plasmid pCMV-GFP were observed as decreases in fluorescene in cells co-transfected with the CREB1 expression vector (Supplementary Fig. 1B). It was expected as the expression of GFP was driven by the HCMV MIE promoter.

3.2. Overexpression of CREB1 inhibits transcription of the MIE genes and HCMV plaques

Since expression of the HCMV MIE genes is critical for viral replication, the effect of CREB1 overexpression on MIE gene expression from the viral genome was determined. HFFs were co-transfected with a CREB1 expression vector and either WT or 5C mt HCMV BAC DNAs (Fig. 2A). The 5C mt HCMV BAC DNA was described previously (Keller et al., 2003; Lashmit et al., 2009). The 5C mt BAC DNA has all five putative CREB sites mutated in the distal and proximal enhancer (Keller et al., 2003). The cells were analyzed for MIE RNA by QRT-PCR at 14 days post-transfection as described previously (Lashmit et al., 2009). To determine the effects of CREB1 overexpression on the MIE transcription, the levels of MIE RNA expression for wild type and 5C mt BAC DNAs in the cells co-transfected with the empty vector were normalized to one for each respective viral BAC DNA (Fig. 2B and Supplementary Table 1). Overexpression of CREB1 blocked >98% of the MIE gene transcription (Fig. 2B). A control set of assays were also performed by overexpressing CREB2. CREB2 did not repress MIE transcription with WT and 5C mt recombinant HCMV BAC DNAs (Fig. 2B). To determine whether the region between −694 and −223 was responsible for the repression by CREB1 overexpression, we used the dl-223/-694 BAC DNA which contains a deletion between −223 and −694 nucleotides upstream of the MIE genes and expresses CAT (Fig. 2A) (Lashmit et al., 2009). Cells transfected with an empty vector were used as control. Overexpression of CREB1 repressed CAT expression >96% (Fig. 2C). In contrast, no effect on CAT expression from dl-223/-694 BAC DNA was observed in the CREB2-expressing cells (Fig. 2C).

To determine the effects of CREB1 on HCMV infection, HFFs were transiently transfected with a CREB1 expression plasmid. Cells transfected with an empty vector were used as a control. The transfection efficiency was determined to be ∼50% (Supplementary Fig. 2). Two days after transfection, the GFP expressing recombinant HCMV was added to the culture at 0.1 PFU/cell and the cells were overlaid with medium containing agar. The enumeration of GFP-expressing plaques was described in the Section 2. Compared to the control, smaller and fewer GFP-expressing HCMV plaques were observed in the CREB1-expressing cells (Fig. 3A). Overexpression of CREB1 led to a 4-fold decrease in the number of the GFP-expressing plaques (Fig. 3B) and an ∼4-fold reduction in the total cell fluorescence (Fig. 3C), demonstrating that CREB1 could function as a negative regulator of HCMV transcription and infection.

3.3. The region located between −130 and −50 of the MIE promoter are involved in the transcriptional inhibition of the MIE gene by CREB1

Mutagenesis analysis indicated that the five CREB/ATF binding sites were not involved in the inhibition of HCMV by CREB1 overexpression (Figs. 1 and 2), suggesting that CREB1 might indirectly repress the HCMV MIE transcription without directly binding to the MIE enhancer-containing promoter. Still, we believed that the "CREB1 responsive region" must be present in the MIE promoter to mediate the inhibition by CREB1. In search for this specific DNA region, we constructed the mutant CMV-Luc reporters, including 600-Luc, 300-Luc, 130-Luc, and 50-Luc, which contained a series of deletions of the MIE enhancer-containing promoter (Fig. 4A). Overexpression of CREB1 showed strong inhibition on 600-Luc, 300-Luc, and 130-Luc, while the inhibition by CREB1 was released when 50-Luc was used as the reporter (Fig. 4B). These results indicated that the putative "CREB1 responsive region" is located between −130 and −50 position of the MIE enhancer-containing promoter. These data agreed with the previous result which suggested that the "CREB1 responsive region" was not present between −694 and −223 (Fig. 2C).

Between -130 and -50, there are one CREB, two SP1/3, and one NF-κB binding elements (Isomura et al., 2005; Meier and Stinski, 1996). A series of 130-Luc mutant plasmids were generated by site-directed mutagenesis to destroy these four binding sites and the impact of the CREB1-mediated inhibition was examined (Fig. 4C). Overexpression of CREB1 significantly inhibited the Luc expression of 130-Luc WT as expected (Fig. 4D). When the CREB site within the region was mutated, CREB1 overexpression still exhibited strong inhibition on the promoter (Fig. 4D, C5). We continued to mutate the two putative SP1/3 sites within the region. Mutation in a single SP1/3 site partially alleviated the inhibition caused by CREB1 (Fig. 4D, C5/S1 and C5/S2), while such transcriptional inhibition was significantly reduced when both S1 and S2 sites were mutated (by ∼6-fold; Fig. 4D, C5/S1/S2). Overexpression of CREB1 failed to show any inhibitory effects on Luc expression of 130-Luc when the NF-κB binding site was further mutated (Fig. 4D, C5/S1/S2/N5). Taken together, the region between −130 and −50 was identified as the "CREB1 responsive region", while the positions at or overlapping the SP1/3 and NF-κB binding sites within the region were important for the CREB1-dependent inhibition.

3.4. CREB1 binds to the CREB binding site located between −130 and −50 of the MIE promoter

We further investigated the DNA–protein interaction between CREB1 and the "CREB1 responsive region" by EMSA. Two oligonucleotides (i.e. C5 WT and N1 WT) which covered the "CREB1 responsive region" and one C5 mt oligonucleotide (with the C5 CREB binding site mutated) were used as DNA probes (Fig. 5A). Nuclear extracts were prepared from the 293T cells transfected with an empty or a CREB1 expression vector, and the expression of CREB1 in the extracts was confirmed by Western blot (Fig. 5B). A biotin-labeled oligonucleotide containing the consensus CREB binding element, TGACGTCA, was used as a positive control for the assays. A DNA–protein band was detected using the CREB1 nuclear extract (Fig. 5C, indicated by an arrow). Formation of this

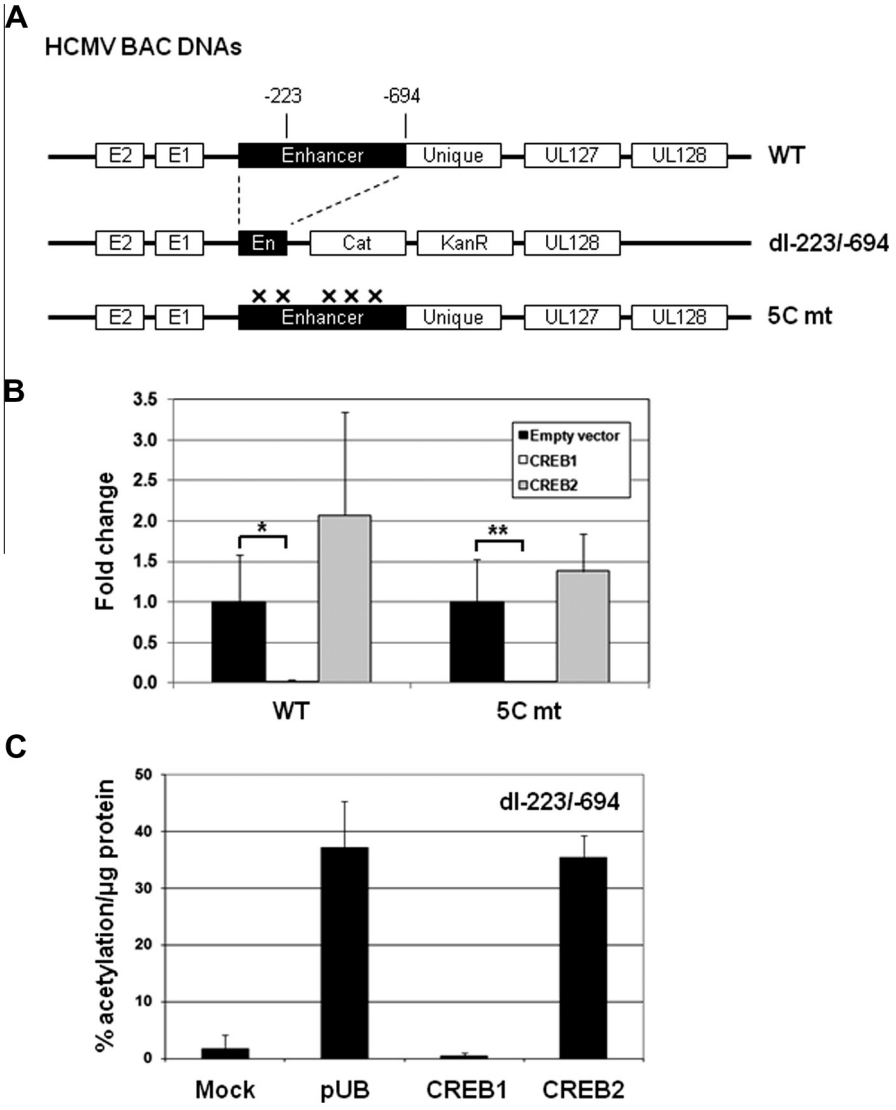

Fig. 2. Overexpression of CREB1 inhibits the transcription of the HCMV MIE gene. (A) Diagram of the WT, dl-223/-694, and 5C mt HCMV BAC DNAs. (B) HFFs were co-transfected with an indicated HCMV BAC DNA and an expression plasmid (i.e. empty vector, CREB1, or CREB2 plasmids). Transcription of the MIE genes was determined by measuring the MIE mRNAs with QRT-PCR. The "Fold change" was calculated as "MIE RNA relative to 18S RNA, relative to 24-h DNA input (gB/18S)" as described previously (Lashmit et al., 2009). The calculation is described in Supplementary Table 1 in detail. MIE RNA levels were relative to cells with the empty vector, which was set at 1.00. Data represent the average and standard deviation of three repeats (*$p < 0.06$ and **$p < 0.05$). (C) HFF cells were transfected with the dl-694/-223 BAC DNA and an indicated plasmid (i.e. empty vector pUB, CREB1, or CREB2 plasmids). Cells that were not transfected with any BAC DNAs or plasmids were used as a control (i.e. Mock). CAT activity was measured at 14 days post-transfection.

DNA–protein complex was disrupted when incubated with an unlabeled specific competitor (i.e. the unlabeled CREB oligonucleotide) or an anti-CREB1 antibody, confirming the interaction between CREB1 protein and the DNA (Fig. 6C). CREB1 was found to interact with the C5 WT oligonucleotide, while such a DNA–CREB1 complex failed to form when the C5 CREB binding site was mutated (Fig. 6C, C5 mt). No interaction between CREB1 and the NF-κB containing oligonucleotide was detected (Fig. 6C, N1 WT). Collectively, the C5 site was the only CREB1 binding site present between −130 and −50 of the HCMV MIE promoter.

3.5. Cellular transcription factors affected by CREB1 overexpression

We continued to investigate the inhibition of HCMV MIE genes by CREB1. CREB1 is a transcription factor and therefore, overexpression of CREB1 should significantly affect the gene expression profile of the host cells. We hypothesize that a specific set of cellular genes regulated by CREB1 may be required for the inhibition of the MIE promoter. To identify these genes, microarray analysis was carried out using the RNAs isolated from the CREB1-overexpressing 293T cells. As shown in Supplementary Table 2, the expression of thousands of cellular genes was affected by CREB1 overexpression. It is likely that most of them are non-specific targets. Therefore, we only focused on the transcription factors having the binding elements within the MIE enhancer region. Based on these criteria, several candidate genes were selected from the microarray analysis (Supplementary Table 3). It was noted that SP1, SP3, and NF-κB p50 precursor, p105, were among the list (Supplementary Table 3). Since the binding sites for SP1/3 and NF-κB were found between −130 and −50, the requirement of SP1/3 and NF-κB for CREB1-mediated inhibition was further investigated.

3.6. SP1, SP3, and NF-κB proteins are not involved in the CREB1-mediated inhibition of the HCMV MIE expression

Significant decreases in the mRNA levels of SP1 and SP3 genes were detected in the CREB1 overexpressing 293T cells by microarray analysis (Supplementary Table 2). To confirm the effects of

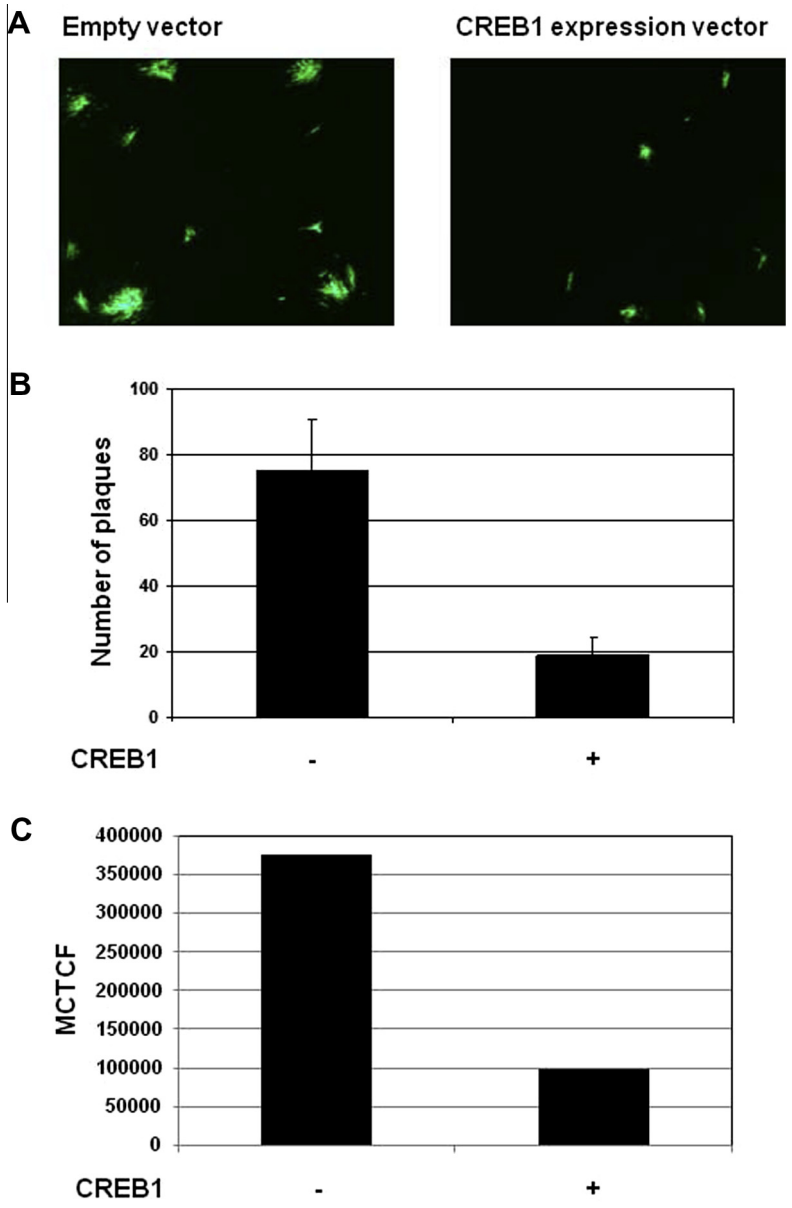

Fig. 3. Overexpression of CREB1 blocks infection by recombinant HCMVs. HFFs were transfected with pUB6 (empty vector) or pUB-CREB1, infected with GFP-expressing recombinant HCMV at a multiplicity of 0.1 PFU/cell, and overlaid with medium containing agar as described in the Section 2. (A) GFP-HCMV foci at 10 days post-infection (d p.i.). (B) GFP-HCMV plaques at 10 d p.i. (C) Mean corrected total cell fluorescence (MCTCF) was determined as described in Section 2.

CREB1 overexpression on SP1 and SP3, QRT-PCR and Western blot were carried out. Analyses of QRT-PCR indicated about 40% and 20% decreases in the SP1 and SP3 mRNAs respectively when CREB1 was overexpressed (data not shown). Western blot analysis showed that CREB1 overexpression caused a 2.8-fold decrease in SP1 protein level, but no effect on the SP3 protein level (Supplementary Fig. 3). In addition, overexpression of CREB1 showed no influence on the protein levels of two subunits of NF-κB, p50 and p65 (Supplementary Fig. 3), even though a slight increase in the mRNA level of p50 precursor (i.e. p105) was observed in microarray data (Supplementary Table 3).

The impact of SP1, SP3, and NF-κB proteins on the MIE promoter was further investigated. Surprisingly, overexpression of SP1 and SP3 showed little to no effect on the Luc expression driven by the HCMV MIE promoter and failed to rescue the inhibition caused by CREB1 (Fig. 6A). The overexpression of SP1 and SP3 proteins in the transfected cells was confirmed by Western blotting (Fig. 6B). SP1 knockdown assays were carried out by co-transfecting cells with an indicated siRNA (SP1-A, SP1-B, or SP1-C siRNAs) and a CMV-Luc reporter. The non-specific siRNA and a GL2 luciferase siRNA were used as a negative and a positive control, respectively. No significant effects on the MIE promoter activity were observed in the SP1 knock-down cells (Fig. 6C). The inhibition of SP1 expression by the three SP1 siRNAs was confirmed by Western blot (Fig. 6D). We also planned to examine the effects of SP3 knockdown on the activity of the MIE promoter. Four siRNAs targeting SP3 were obtained commercially. Unfortunately, the Western analysis showed that all four siRNAs failed to decrease the protein levels of SP3 in the transfected cells (data not shown).

The impact of NF-κB on HCMV MIE promoter was examined next. Overexpression of both NF-κB subunits, p50 and p65, repressed the MIE promoter by 40% (Fig. 6E). Co-transfection of p50 and p65 showed little to no effect on the inhibition caused by CREB1 overexpression (Fig. 6E). No changes in the levels of

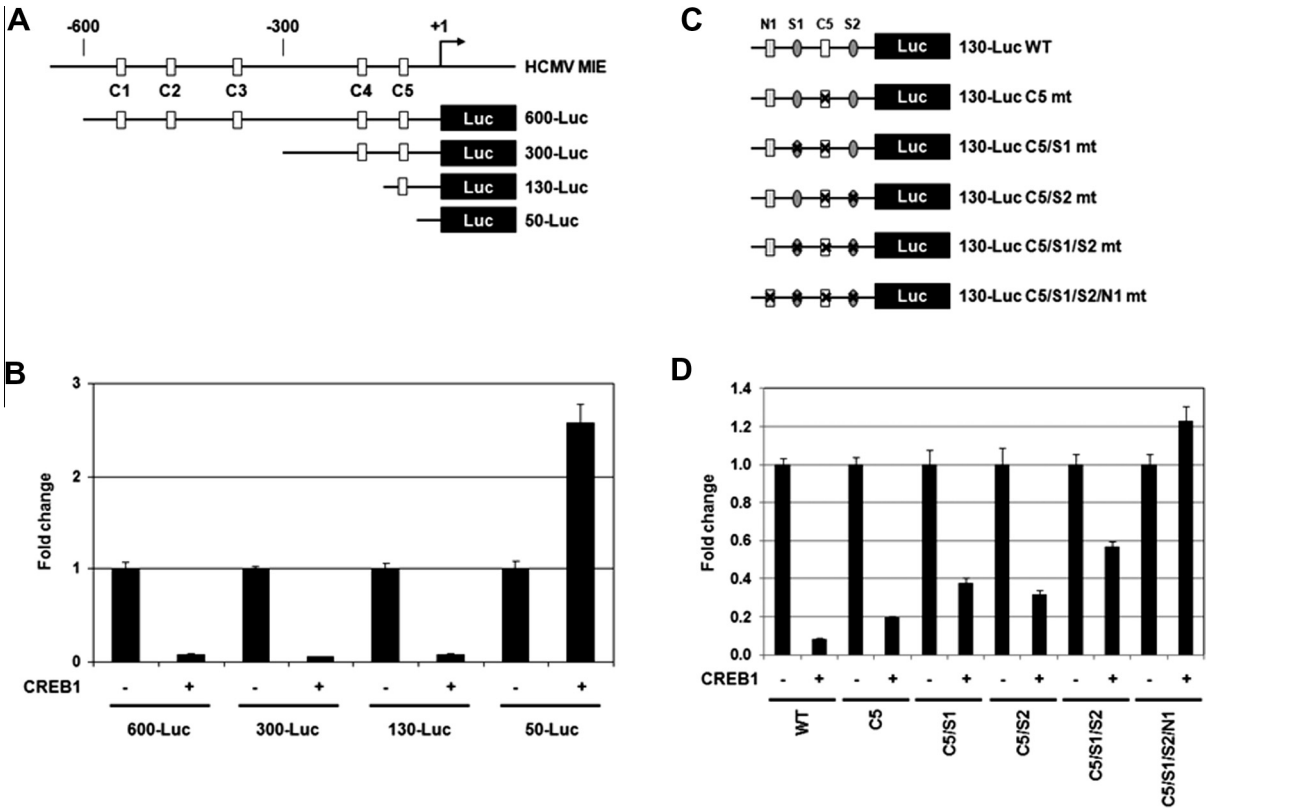

Fig. 4. Identification of the CREB1 responsive region in the HCMV MIE promoter. (A) Diagram of the Luc reporters, 600-Luc, 300-Luc, 130-Luc, and 50-Luc, with the deletions of the HCMV MIE promoter. The five putative CREB binding sites are denoted as C1–5. (B) Effects of CREB1 on the expression of Luc driven by the truncated HCMV MIE promoters. 293T cells were co-transfected with an indicated Luc reporter and an expression vector (i.e. empty vector or CREB1 plasmid). Luc activity was measured 2 days post-transfection. (C) Diagram of the 130-Luc reporters with mutations in CREB, SP1/3, and NF-κB binding sites. Locations of the CREB (i.e. C5), SP1/3 (i.e. S1 and S2), and NF-κB (N1) binding elements were indicated. (D) Effects of CREB1 overexpression on the WT and mutant 130-Luc reporters. 293T cells were transiently transfected with an indicated 130-Luc reporter and an expression vector (i.e. empty vector or CREB1 plasmid). Luc activity was measured 2 days post-transfection.

p50 and p65 proteins were observed in the CREB1-overexpressing cells (Supplementary Fig. 3). However, these assays could not reflect the impact of CREB1 overexpression on the activity of NF-κB. We thus continued to determine the NF-κB activity in the CREB1-transfected cells. Cells co-transfected with p50 and p65 plasmids were used as a positive control. Overexpression of p50/p65 induced a 31.7-fold increase in the NF-κB activity (Fig. 6F). Elevated expression of p50 and p65 in the transfected cells was confirmed by Western blot (Fig. 6G). In contrast, little or no change in the activity of NF-κB was detected in the CREB1-overexpressing cells (Fig. 6F; a 6% increase). We conclude that neither SP1/3 nor NF-κB proteins are involved in the inhibition of HCMV MIE gene mediated by CREB1. Further investigation is required to identify the unknown cellular factors that interact with the "CREB1 responsive region" between −130 and −50 and mediate the CREB1-dependent inhibition of the HCMV MIE genes.

4. Discussion

In this report, we show that overexpression of CREB1 inhibits the expression from the HCMV MIE enhancer-containing promoter (Fig. 1). In the context of the HCMV genome in permissive HFF cells, overexpression of CREB1 almost completely blocked transcription from the MIE promoter (Fig. 2). CAT expression in cells transfected with dl-223/-694 BAC DNA was almost completely inhibited which indicated that CREB1 inhibited viral expression with just the proximal enhancer present (Fig. 2). We later narrowed down the "CREB1 responsive region" between −130 and −50 position by the promoter deletion analysis (Fig. 4). Inhibition of transcription from the MIE promoter also occurred when all five CREB binding sites were mutated (Fig. 2). Recombinant HCMV infection of the CREB1-overexpressing cells showed fewer/smaller plaques and less mean corrected total cell fluorescence relative to cells transfected with the empty vector, which indicated that the entire viral replication cycle was inhibited (Fig. 3). For the viral infection assay, cells were first transfected with the CREB1 expression vector and then infected with the GFP-expressing recombinant HCMV 2 days after transfection. Since only ~50% of the cells were transfected with the CREB1 expression plasmids, approximately 50% of the un-transfected cells could be infected without the inhibitory affects of CREB1 overexpression. This suggests that the actual inhibitory efficiency of HCMV infection caused by CREB1 overexpression should be much higher than what was observed in Fig. 3, if 100% of the cells could be transfected with the CREB1 expression plasmid.

CREB1 functions as a transcriptional activator. Phosphorylation of CREB1 at Ser133 is essential for its transactivating activity and it has been reported that mutation at the Ser133 completely abolishes the transcriptional activity of CREB1 (Gonzalez and Montminy, 1989). In addition, the activity of CREB1 can be attenuated when associating with histone deacetylase 1 (HDAC1), which blocks the phosphorylation of CREB1 at Ser133 (Canettieri et al., 2003). However, our data indicates that the inhibition of the HCMV MIE promoter by CREB1 was not mediated through the direct CREB1 binding to the MIE enhancer-containing promoter. Mutagenesis showed that none of the previously identified CREB binding sites are required for the repression by CREB1 (Figs. 1 and 2B). We narrowed down the "CREB responsive region" to a region

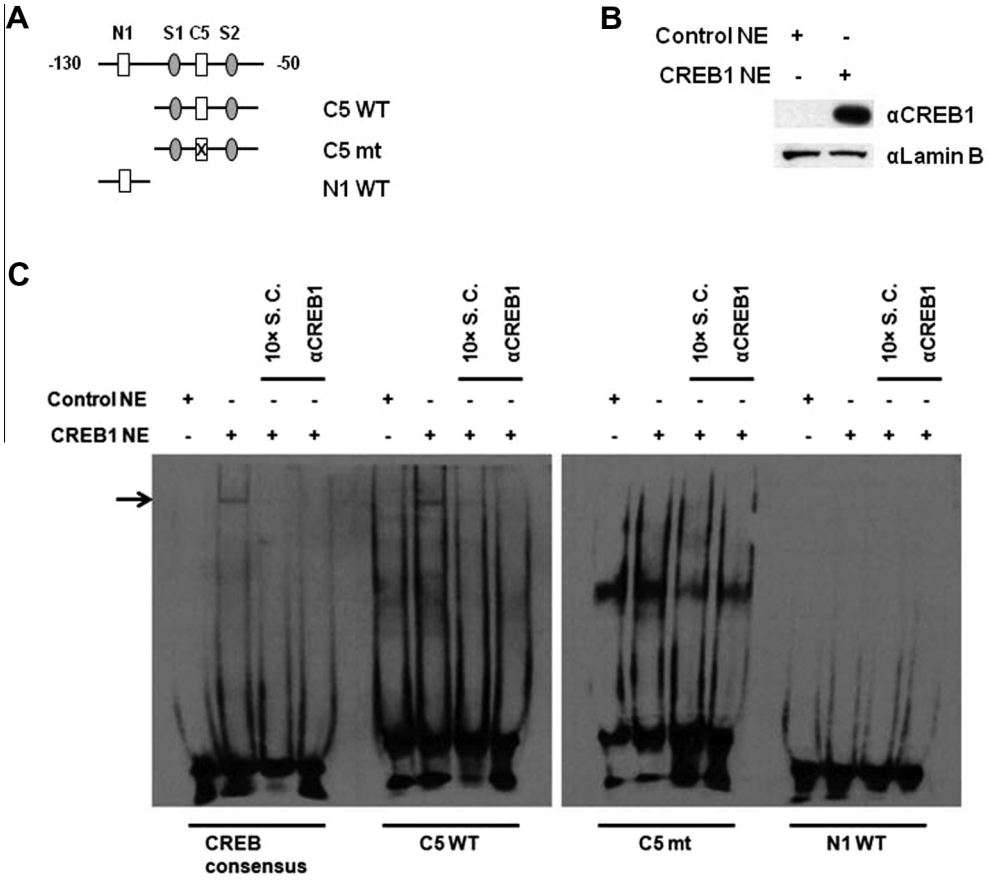

Fig. 5. CREB1 binds to the C5 CREB binding site located between −130 and −50 of the MIE promoter. (A) Diagram of the oligonucleotides used in EMSA. Locations of the CREB (i.e. C5), SP1/3 (i.e. S1 and S2), and NF-κB (N1) binding sites present between −130 and −50 were indicated. (B) The expression levels of CREB1 in the nuclear extracts (NEs) prepared from the mock- and CREB1-transfected cells were examined by Western blot. The nuclear protein, Lamin B, was used as a loading control. (C) EMSA was carried out using the NEs (i.e. Control or CREB1 NEs) and the indicated biotin-labeled DNA probes. Formation of the specific CREB1-DNA complex (marked by an arrow) was confirmed by incubating with 10× excess un-labeled DNA probes [i.e. specific competitors (S. C.)] or an anti-CREB1 antibody.

between −130 and −50 of the HCMV MIE proximal promoter by deletion analyses (Fig. 4). Only one putative CREB1 binding element (i.e. C5, Fig. 4A) was identified within this region. Results obtained from EMSAs confirmed the C5 site as a CREB1 binding element, while no binding between CREB1 and the DNA region was detected when the C5 site was mutated (Fig. 5). After destroying the only putative CREB element in 130-Luc, CREB1 still exhibited strong repression of the MIE promoter as shown in cell-based experiments (Fig. 4 and 130-Luc C5 mt). Taken together, our results suggested that overexpression of CREB1 affected the expression of the MIE genes without directly binding to the MIE enhancer-containing promoter.

Our analyses demonstrated that the two putative SP1/3 binding sites located between −130 and −50 region were responsible for the CREB1-mediated inhibition of the HCMV MIE genes (Fig. 4). In addition, both SP1 and SP3 were among the candidate genes identified by microarray analysis (Supplementary Table 3). Previous studies revealed the importance of these two putative SP1/3 sites in HCMV replication (Isomura et al., 2005). Mutation of both putative SP1/3 sites significantly inhibits the transcription of the MIE gene and blocks viral replication (Isomura et al., 2005). Results obtained from EMSA demonstrated the *in vitro* binding between SP1/3 proteins and the binding sites, and identified SP1 as the major binding protein (Isomura et al., 2005). In addition, HCMV infection results in the increased mRNA and protein levels of SP1 but shows no effects on SP3 (Isomura et al., 2005; Yurochko et al., 1997). However, no functional assays were carried out to directly determine the effects of SP1/3 proteins on the MIE gene expression. Our microarray analysis showed that overexpression of CREB1 repressed the expression of SP1 and SP3 genes, suggesting a possible connection between SP1/3 proteins and the CREB1-mediated inhibition (Supplementary Table 2). However, overexpression of SP1 or SP3 failed to up-regulate the expression driven by the MIE promoter and did not prevent the CREB1-mediated repression of the MIE promoter (Fig. 6A). In addition, knockdown of SP1 did not have significant effects on the MIE promoter (Fig. 6C). Although SP1 and SP3 may not be the most critical transcription factor for up-regulation of the HCMV MIE transcription, our results demonstrate that the site at or overlapping the SP1/3 binding elements are required for the CREB1-dependent inhibition.

Studies on the importance of the NF-κB binding sites located in the enhancer region on the regulation of MIE gene expression are controversial. Transient transfection experiments demonstrate the NF-κB sites as the key components of the HCMV MIE enhancer (Hunninghake et al., 1989). Unexpectedly, mutation of all the NF-κB sites has little to no effect on CMV replication in the cultured fibroblasts (Benedict et al., 2004). A recent study, however, showed that mutations in MIE enhancer binding sites for NF-κB partially block phorbol myristate acetate-activated MIE gene expression in quiescently infected human pluripotent NTera2 cells (Liu et al., 2010). However, none of the studies examined the effect of NF-κB proteins on the HCMV MIE promoter. In this study, we identified the NF-κB binding site located between −130 and −50 region to be within the "CREB1 responsive region".

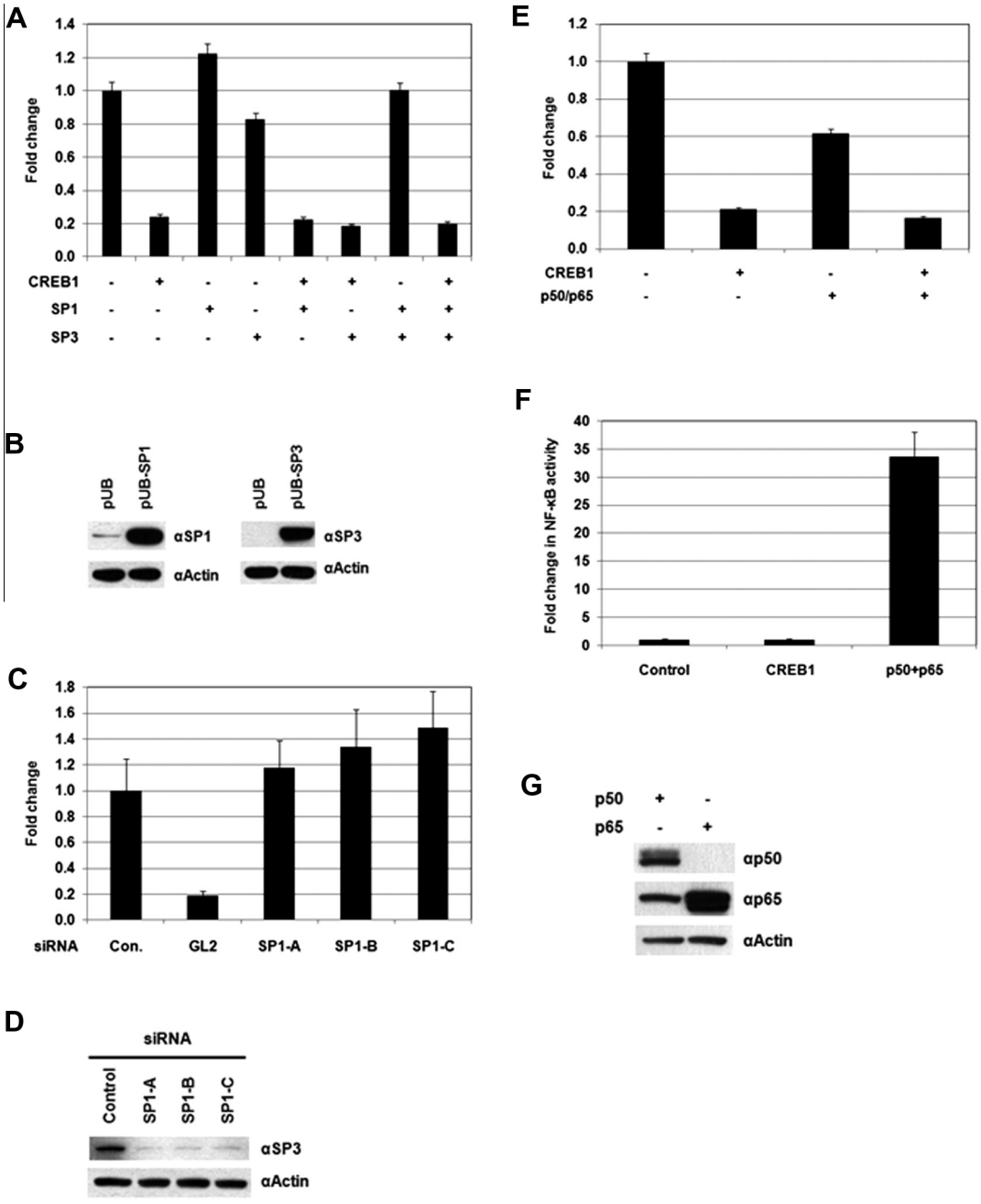

Fig. 6. SP1, SP3, and NF-κB proteins are not required for the CREB1-mediated inhibition of MIE gene expression. (A) 293T cells were transfected with CMV-Luc and the indicated expression vectors (i.e. CREB1, SP1, or SP3). Luc activity was measured 2 days post-transfection. (B) Cell lysates prepared from the SP1- and SP3-transfected cells (pUB-SP1 and pUB-SP3, respectively) were analyzed by Western blotting. Cells transfected with an empty vector (i.e. pUB) were used as a control. Actin was utilized as the loading control. (C) 293T cells were co-transfected with CMV-Luc and an indicated siRNA. The GL2 siRNA against luciferase served as a positive control while the non-specific siRNA (i.e. con.) was used as a negative control. (D) To determine the effectiveness of the SP1 siRNAs, cell lysates prepared from the cells transfected with a specific SP1 siRNA were analyzed by Western blotting. Cells transfected with a non-specific siRNA were used as a control. Actin was utilized as the loading control. (E) 293T cells were transfected with CMV-Luc and the indicated expression vectors (i.e. CREB1, p50, or p65). Luc activity was measured 2 days after transfection. (F) 293T cells were transfected with the indicated expression plasmids. Cells transfected with an empty vector were used as a negative control (i.e. Control). NF-κB activities of the transfected cells were measured as described in Materials and Methods. (G) 293T cells were transiently transfected with a p50 or a p65 expression vector. Cell lysates prepared from the transfected cells were analyzed by Western blotting.

However, our data do not suggest a functional connection between CREB1-mediated MIE inhibition and NF-κB protein. Overexpression of CREB1 showed no effects either on the protein level or the activity of NF-κB (Supplementary Fig. S3 and Fig. 6F). Interestingly, we demonstrated that overexpression of NF-κB exhibited moderate inhibition on the HCMV MIE promoter (Fig. 6E). Similar results were obtained on mouse CMV. Benedict et al. (2004) reported that mouse embryo fibroblasts genetically deficient for p65/RelA actually showed elevated levels of mouse CMV replication. Taken together, our results clearly demonstrate that the region between −130 and −50 of the MIE promoter is required for the CREB1-mediated transcriptional inhibition of the HCMV MIE genes. Although this region contains two SP1/3 and one NF-κB binding sites, the putative binding proteins (i.e. SP1, SP3, and NF-κB) of the elements are not involved in the CREB1-mediated inhibition.

We previously showed that the CREB binding site at −137 in the proximal enhancer (i.e. C4 in this study, Fig. 1A) is important for viral replication in HFF cells (Lashmit et al., 2009). We further co-transfected cells with a short hairpin RNA against CREB1 and a dl-223/-694 HCMV BAC DNA. Knockdown of CREB1 inhibited the transcription of the MIE genes driven by the proximal enhancer (i.e. dl-223/-694) (Lashmit et al., 2009). In this study, we found not only the CREB site at −137 but all the five CREB sites are not important for CREB1-mediated repression of the MIE promoter (Figs. 1 and 2). Paradoxically and surprisingly, overexpression of CREB1 potently blocked the MIE transcription driven by the entire or the proximal enhancers (Fig. 2, WT and dl-223/-694). Although it may not be easy to define CREB1 as an activator or inhibitor of HCMV, our previous and current results clearly demonstrate an essential role of CREB1 in the regulation of HCMV transcription and replication. Therefore, in order to re-activate the transcription of the MIE genes, it is likely that the protein level of CREB1 in the cells must be maintained within a narrow range. When the CREB1 level in cells is higher or lower than this tightly regulated range, CREB1 could interfere with the transcription of the MIE genes.

As a transcription factor, overexpression or knockdown of CREB1 are expected to have a broad effect on the expression profiles of the genes and proteins in cells. In the attempt to identify the genes involved in the CREB1-mediated inhibition of the MIE genes, microarray analysis was carried out to investigate the gene expression in the CREB1-overexpression cells. We showed here that the expression of thousands of cellular genes were affected by CREB1 overexpression (Supplementary Table 2). Based on our results, CREB1 can block HCMV MIE gene expression indirectly, without binding to the MIE promoter/enhancer. We propose that overexpression of CREB1 may lead to up-regulation of the specific repressor(s), resulting in inhibition of MIE expression. Alternatively, overexpression of CREB1 may down-regulate the level of the specific activators to induce MIE expression. It is worthwhile to identify the cellular factors required in the CREB1-mediated inhibition of the MIE promoter to broaden our understanding of the molecular mechanism of HCMV re-activation. However, it would be challenging to thoroughly elucidate the regulatory mechanism of HCMV inhibition caused by CREB1.

An increase in the mRNA level of PDX1, an inhibitor of the HCMV MIE promoter, was detected in our microarray data (Supplementary Tables 2 and 3) (Chao et al., 2004). However, no putative PDX1 binding elements were found in the "CREB1 responsive region". Several studies demonstrate the involvement of histone acetylation in regulation of the MIE genes (Murphy et al., 2002; Nevels et al., 2004). It has been shown that Ets-2 repressor factor (ERF) interacts with HDAC1 and mediates repression of the MIE genes (Wright et al., 2005). Using a ChIP-on-Chip assay, a recent report shows that HDAC3 interacts not only with the MIE promoter but also with the entire MIE locus (Huang et al., 2011). Future investigation is required to determine if there are any functional connections between PDX1/HDACs and CREB1, and if PDX1/HDACs regulate the MIE gene expression through the "CREB1 responsive region".

The CREB1 knockout mouse has been generated. The knockout animal appeared normal and did not show any impairment of growth or development (Hummler et al., 1994). In our study, we noticed that overexpression of CREB1 did not kill the cells or cause any detectable cytopathic effect during the time course of our experiments (Supplementary Fig. 1). These findings suggest that CREB1 could be an ideal target for a gene therapy-based strategy to inhibit HCMV infection and replication. Protein/mRNA delivery-based approaches could also be utilized to deliver CREB1 protein/mRNA to the target cells or tissues. Novel technologies, such as virus-like particles and nanoparticles, have been recently developed for efficient and specific delivery (Davis et al., 2008; Schott et al., 2011). Since the infection of HCMV is a deadly threat to people who are immunocompromised, such as HIV-infected persons and organ transplant recipients, our finding of the potent HCMV inhibition by CREB1 overexpression may contribute to the development of better and more efficient anti-HCMV therapy.

Acknowledgements

We would like to thank Dr. Arnold Rabson for providing HTLV plasmids, Dr. Hirotoshi Tanaka for providing the p50 and p65 expression vectors, and Dr. Jeffery Meier for the HCMV BACs and plasmids with the five CREB sites mutated. We thank Drs. Yuansheng Yang, Guixin Du, and Nirmal Dutta for critical review of the manuscript, and Dr. Xuezhi Bi and Qing Wei Cheang for expert technical assistance. This work was supported by the Agency for Science, Technology and Research, Singapore (SHC) and the National Institutes of Health, USA (MFS).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.11.012.

References

Angulo, A., Kerry, D., Huang, H., Borst, E.M., Razinsky, A., Wu, J., Hobom, U., Messerle, M., Ghazal, P., 2000. Identification of a boundary domain adjacent to the potent human cytomegalovirus enhancer that represses transcription of the divergent UL127 promoter. J. Virol. 74, 2826–2839.

Benedict, C.A., Angulo, A., Patterson, G., Ha, S., Huang, H., Messerle, M., Ware, C.F., Ghazal, P., 2004. Neutrality of the canonical NF-κB-dependent pathway for human and murine cytomegalovirus transcription and replication in vitro. J. Virol. 78, 741–750.

Bresnahan, W.A., Boldogh, I., Chi, P., Thompson, E.A., Albrecht, T., 1997. Inhibition of cellular Cdk2 activity blocks human cytomegalovirus replication. Virology 231, 239–247.

Burgess, A., Vigneron, S., Brioudes, E., Labbe, J.C., Lorca, T., Castro, A., 2010. Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance. Proc. Natl. Acad. Sci. USA 107, 12564–12569.

Canettieri, G., Morantte, I., Guzman, E., Asahara, H., Herzig, S., Anderson, S.D., Yates 3rd, J.R., Montminy, M., 2003. Attenuation of a phosphorylation-dependent activator by an HDAC-PP1 complex. Nat. Struct. Biol. 10, 175–181.

Chao, S.H., Price, D.H., 2001. Flavopiridol inactivates P-TEFb and blocks most RNA polymerase II transcription in vivo. J. Biol. Chem. 276, 31793–31799.

Chao, S.H., Fujinaga, K., Marion, J.E., Taube, R., Sausville, E.A., Senderowicz, A.M., Peterlin, B.M., Price, D.H., 2000. Flavopiridol inhibits P-TEFb and blocks HIV-1 replication. J. Biol. Chem. 275, 28345–28348.

Chao, S.H., Harada, J.N., Hyndman, F., Gao, X., Nelson, C.G., Chanda, S.K., Caldwell, J.S., 2004. PDX1, a cellular homeoprotein, binds to and regulates the activity of human cytomegalovirus immediate early promoter. J. Biol. Chem. 279, 16111–16120.

Davis, M.E., Chen, Z.G., Shin, D.M., 2008. Nanoparticle therapeutics: an emerging treatment modality for cancer. Nat. Rev. Drug Discov. 7, 771–782.

Fish, K.N., Depto, A.S., Moses, A.V., Britt, W., Nelson, J.A., 1995a. Growth kinetics of human cytomegalovirus are altered in monocyte-derived macrophages. J. Virol. 69, 3737–3743.

Fish, K.N., Stenglein, S.G., Ibanez, C., Nelson, J.A., 1995b. Cytomegalovirus persistence in macrophages and endothelial cells. Scand. J. Infect. Dis. Suppl. 99, 34–40.

Fortunato, E.A., Sanchez, V., Yen, J.Y., Spector, D.H., 2002. Infection of cells with human cytomegalovirus during S phase results in a blockade to immediate-early gene expression that can be overcome by inhibition of the proteasome. J. Virol. 76, 5369–5379.

Gonzalez, G.A., Montminy, M.R., 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59, 675–680.

Graham, F.L., van der Eb, A.J., 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52, 456–467.

Huang, Y., Tang, Q., Nguyen, M., Dulal, K., Wang, W., Zhu, H., 2011. Histone deacetylase 3, not histone deacetylase 2, interacts with the major immediate early locus of human cytomegalovirus. Virol. J. 8, 151.

Hummler, E., Cole, T.J., Blendy, J.A., Ganss, R., Aguzzi, A., Schmid, W., Beermann, F., Schutz, G., 1994. Targeted mutation of the CREB gene: compensation within the CREB/ATF family of transcription factors. Proc. Natl. Acad. Sci. USA 91, 5647–5651.

Hunninghake, G.W., Monick, M.M., Liu, B., Stinski, M.F., 1989. The promoter-regulatory region of the major immediate-early gene of human

cytomegalovirus responds to T-lymphocyte stimulation and contains functional cyclic AMP-response elements. J. Virol. 63, 3026–3033.
Ibanez, C.E., Schrier, R., Ghazal, P., Wiley, C., Nelson, J.A., 1991. Human cytomegalovirus productively infects primary differentiated macrophages. J. Virol. 65, 6581–6588.
Isomura, H., Stinski, M.F., Kudoh, A., Daikoku, T., Shirata, N., Tsurumi, T., 2005. Two Sp1/Sp3 binding sites in the major immediate-early proximal enhancer of human cytomegalovirus have a significant role in viral replication. J. Virol. 79, 9597–9607.
Kapasi, A.J., Spector, D.H., 2008. Inhibition of the cyclin-dependent kinases at the beginning of human cytomegalovirus infection specifically alters the levels and localization of the RNA polymerase II carboxyl-terminal domain kinases cdk9 and cdk7 at the viral transcriptosome. J. Virol. 82, 394–407.
Keller, M.J., Wheeler, D.G., Cooper, E., Meier, J.L., 2003. Role of the human cytomegalovirus major immediate-early promoter's 19-base-pair-repeat cyclic AMP-response element in acutely infected cells. J. Virol. 77, 6666–6675.
Kirby, H., Rickinson, A., Bell, A., 2000. The activity of the Epstein-Barr virus BamHI W promoter in B cells is dependent on the binding of CREB/ATF factors. J. Gen. Virol. 81, 1057–1066.
Lang, D., Fickenscher, H., Stamminger, T., 1992. Analysis of proteins binding to the proximal promoter region of the human cytomegalovirus IE-1/2 enhancer/promoter reveals both consensus and aberrant recognition sequences for transcription factors Sp1 and CREB. Nucleic Acids Res. 20, 3287–3295.
Lashmit, P.E., Lundquist, C.A., Meier, J.L., Stinski, M.F., 2004. Cellular repressor inhibits human cytomegalovirus transcription from the UL127 promoter. J. Virol. 78, 5113–5123.
Lashmit, P., Wang, S., Li, H., Isomura, H., Stinski, M.F., 2009. The CREB site in the proximal enhancer is critical for cooperative interaction with the other transcription factor binding sites to enhance transcription of the major intermediate-early genes in human cytomegalovirus-infected cells. J. Virol. 83, 8893–8904.
Lee, J., Klase, Z., Gao, X., Caldwell, J.S., Stinski, M.F., Kashanchi, F., Chao, S.H., 2007a. Cellular homeoproteins, SATB1 and CDP, bind to the unique region between the human cytomegalovirus UL127 and major immediate-early genes. Virology 366, 117–125.
Lee, J., Lau, J., Chong, G., Chao, S.H., 2007b. Cell-specific effects of human cytomegalovirus unique region on recombinant protein expression. Biotechnol. Lett. 29, 1797–1802.
Lin, H.C., Hickey, M., Hsu, L., Medina, D., Rabson, A.B., 2005. Activation of human T cell leukemia virus type 1 LTR promoter and cellular promoter elements by T cell receptor signaling and HTLV-1 Tax expression. Virology 339, 1–11.
Liu, B., Stinski, M.F., 1992. Human cytomegalovirus contains a tegument protein that enhances transcription from promoters with upstream ATF and AP-1 cis-acting elements. J. Virol. 66, 4434–4444.
Liu, X., Yuan, J., Wu, A.W., McGonagill, P.W., Galle, C.S., Meier, J.L., 2010. Phorbol ester-induced human cytomegalovirus major immediate-early (MIE) enhancer activation through PKC-delta, CREB, and NF-kappaB desilences MIE gene expression in quiescently infected human pluripotent NTera2 cells. J. Virol. 84, 8495–8508.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.
Lundquist, C.A., Meier, J.L., Stinski, M.F., 1999. A strong negative transcriptional regulatory region between the human cytomegalovirus UL127 gene and the major immediate-early enhancer. J. Virol. 73, 9039–9052.
Meier, J.L., Stinski, M.F., 1996. Regulation of human cytomegalovirus immediate-early gene expression. Intervirology 39, 331–342.
Millhouse, S., Kenny, J.J., Quinn, P.G., Lee, V., Wigdahl, B., 1998. ATF/CREB elements in the herpes simplex virus type 1 latency-associated transcript promoter interact with members of the ATF/CREB and AP-1 transcription factor families. J. Biomed. Sci. 5, 451–464.
Mocarski Jr., E.S., 2002. Immunomodulation by cytomegaloviruses: manipulative strategies beyond evasion. Trends Microbiol. 10, 332–339.
Murphy, J.C., Fischle, W., Verdin, E., Sinclair, J.H., 2002. Control of cytomegalovirus lytic gene expression by histone acetylation. EMBO J. 21, 1112–1120.
Nevels, M., Paulus, C., Shenk, T., 2004. Human cytomegalovirus immediate-early 1 protein facilitates viral replication by antagonizing histone deacetylation. Proc. Natl. Acad. Sci. USA 101, 17234–17239.
Ouchida, R., Kusuhara, M., Shimizu, N., Hisada, T., Makino, Y., Morimoto, C., Handa, H., Ohsuzu, F., Tanaka, H., 2003. Suppression of NF-kappaB-dependent gene expression by a hexamethylene bisacetamide-inducible protein HEXIM1 in human vascular smooth muscle cells. Genes Cells 8, 95–107.
Prosch, S., Wendt, C.E., Reinke, P., Priemer, C., Oppert, M., Kruger, D.H., Volk, H.D., Docke, W.D., 2000. A novel link between stress and human cytomegalovirus (HCMV) infection: sympathetic hyperactivity stimulates HCMV activation. Virology 272, 357–365.
Pumfery, A., de la Fuente, C., Berro, R., Nekhai, S., Kashanchi, F., Chao, S.H., 2006. Potential use of pharmacological cyclin-dependent kinase inhibitors as anti-HIV therapeutics. Curr. Pharm. Des. 12, 1949–1961.
Rideg, K., Hirka, G., Prakash, K., Bushar, L.M., Nothias, J.Y., Weinmann, R., Andrews, P.W., Gonczol, E., 1994. DNA-binding proteins that interact with the 19-base pair (CRE-like) element from the HCMV major immediate early promoter in differentiating human embryonal carcinoma cells. Differentiation 56, 119–129.
Salvant, B.S., Fortunato, E.A., Spector, D.H., 1998. Cell cycle dysregulation by human cytomegalovirus: influence of the cell cycle phase at the time of infection and effects on cyclin transcription. J. Virol. 72, 3729–3741.
Sanchez, V., Spector, D.H., 2006. Cyclin-dependent kinase activity is required for efficient expression and posttranslational modification of human cytomegalovirus proteins and for production of extracellular particles. J. Virol. 80, 5886–5896.
Sanchez, V., McElroy, A.K., Yen, J., Tamrakar, S., Clark, C.L., Schwartz, R.A., Spector, D.H., 2004. Cyclin-dependent kinase activity is required at early times for accurate processing and accumulation of the human cytomegalovirus UL122-123 and UL37 immediate-early transcripts and at later times for virus production. J. Virol. 78, 11219–11232.
Schott, J.W., Galla, M., Godinho, T., Baum, C., Schambach, A., 2011. Viral and non-viral approaches for transient delivery of mRNA and proteins. Curr. Gene. Ther. 11, 382–398.
Sinclair, J., Sissons, P., 1996. Latent and persistent infections of monocytes and macrophages. Intervirology 39, 293–301.
Sinzger, C., Grefte, A., Plachter, B., Gouw, A.S., The, T.H., Jahn, G., 1995. Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissues. J. Gen. Virol. 76 (Pt 4), 741–750.
Staak, K., Prosch, S., Stein, J., Priemer, C., Ewert, R., Docke, W.D., Kruger, D.H., Volk, H.D., Reinke, P., 1997. Pentoxifylline promotes replication of human cytomegalovirus in vivo and in vitro. Blood 89, 3682–3690.
Stinski, M.F., Isomura, H., 2008. Role of the cytomegalovirus major immediate early enhancer in acute infection and reactivation from latency. Med. Microbiol. Immunol. 197, 223–231.
Wright, E., Bain, M., Teague, L., Murphy, J., Sinclair, J., 2005. Ets-2 repressor factor recruits histone deacetylase to silence human cytomegalovirus immediate-early gene expression in non-permissive cells. J. Gen. Virol. 86, 535–544.
Yoshimura, T., Fujisawa, J., Yoshida, M., 1990. Multiple cDNA clones encoding nuclear proteins that bind to the tax-dependent enhancer of HTLV-1: all contain a leucine zipper structure and basic amino acid domain. EMBO J. 9, 2537–2542.
Yurochko, A.D., Hwang, E.S., Rasmussen, L., Keay, S., Pereira, L., Huang, E.S., 1997. The human cytomegalovirus UL55 (gB) and UL75 (gH) glycoprotein ligands initiate the rapid activation of Sp1 and NF-κB during infection. J. Virol. 71, 5051–5059.
Zydek, M., Hagemeier, C., Wiebusch, L., 2010. Cyclin-dependent kinase activity controls the onset of the HCMV lytic cycle. PLoS Pathog. 6, e1001096.