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J. Lewis Stern, Westmead Millennium Institute
John Z Cao, Westmead Millennium Institute
Jiakew Xu, University of Western Australia
Edward Mocarski, Emory University
Barry Slobedman, Westmead Millennium Institute

Journal Title: Virology
Volume: Volume 378, Number 2
Publisher: Elsevier | 2008-09-01, Pages 214-225
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1016/j.virol.2008.05.024
Permanent URL: https://pid.emory.edu/ark:/25593/s3c6z

Final published version: http://dx.doi.org/10.1016/j.virol.2008.05.024

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Accessed January 2, 2020 3:44 PM EST
Repression of human cytomegalovirus major immediate early gene expression by the cellular transcription factor CCAAT displacement protein

J. Lewis Stern\textsuperscript{a,b,1}, John Z. Cao\textsuperscript{a,b,1}, Jiabe Xu\textsuperscript{c}, Edward S. Mocarski\textsuperscript{d}, and Barry Slobedman\textsuperscript{a,b,*}

\textsuperscript{a}Centre for Virus Research, Westmead Millennium Institute, PO Box 412, Westmead, New South Wales 2145, Australia
\textsuperscript{b}The University of Sydney, PO Box 412, Westmead, New South Wales 2145, Australia
\textsuperscript{c}Department of Orthopaedic Surgery, The University of Western Australia, Crawley WA 6009, Australia
\textsuperscript{d}Department of Microbiology and Immunology, Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30322, USA

Abstract

Initiation of human cytomegalovirus (HCMV) productive infection is dependent on the major immediate early (MIE) genes \textit{ie}1 and \textit{ie}2. Several putative binding sites for CCAAT displacement protein (CDP or CUX1) were identified within the MIE promoter/regulatory region. Binding assays demonstrated binding of CUX1 to MIE-region oligonucleotides containing the CUX1 core binding sequence ATCGAT and mutagenesis of this sequence abrogated CUX1 binding. Furthermore, CUX1 repressed expression of a luciferase reporter construct controlled by the MIE promoter, and mutation of CUX1 binding sites within the promoter diminished this repressive function of CUX1. In the context of virus infection of HEK293 cells transfected with the CUX1 expression vector, CUX1 showed evidence of association with the HCMV MIE regulatory region and inhibited the capacity of the virus to express \textit{ie}1 and \textit{ie}2 transcripts, suggesting that this cellular factor regulates MIE gene expression following virus entry. These data identify a role for CUX1 in repressing HCMV gene expression essential for initiation of the replicative cycle.

Keywords

Human cytomegalovirus; Immediate early genes; Repression of viral gene expression; CCAAT displacement protein

\textsuperscript{*}Corresponding author. Centre for Virus Research, Westmead Millennium Institute, PO Box 412, Westmead, NSW 2145, Australia.
Fax: +61 2 98459100. barry_slobedman@wmi.usyd.edu.au (B. Slobedman).
\textsuperscript{1}J.L.S. and J.Z.C. contributed equally to this work.
Introduction

Human cytomegalovirus (HCMV) is a beta herpes virus that infects a majority of the world’s population. Productive HCMV infection generally results in mild or clinically in apparent disease in most immunocompetent individuals but infection of the fetus during pregnancy can lead to devastating consequences including still birth, mental retardation and hearing impairment (Boppana et al., 2005; Pass, 2005; Yow et al., 1988). Host immune-mediated resolution of productive infection does not result in complete clearance of the virus from the body. Rather, the virus is able to establish a lifelong latent infection in primitive hematopoietic cells, including CD34+ cells and lineage committed myeloid progenitor cells and monocytes (Mocarski et al., 2007). During latency, the viral genome is maintained in a circular episomal configuration (Bolovan-Fritts et al., 1999) in a small proportion of cells at a level of 2–10 copies per cell (Slobedman and Mocarski, 1999), viral gene expression is restricted, and infectious virus is not detectable (Mocarski et al., 2007). Periodically, the virus is able to reactivate from latency, resulting in the synthesis of new, infectious virus. Reactivation occurs in response to unknown stimuli, but is linked to myeloid cell differentiation and growth or pro-inflammatory cytokines (Hahn et al., 1998; Taylor-Wiedeman et al., 1994) or culture of cells under conditions of allogeneic stimulation (Soderberg-Naucler et al., 1997). Virus reactivation in immunosuppressed individuals such as allogeneic transplant recipients causes significant morbidity and mortality as a consequence of disseminated infection and disease involving multiple organs including the lung, liver, eye and gastrointestinal tract (Mocarski et al., 2007).

During productive infection of permissive cell types viral gene expression occurs in an ordered cascade, of which a group of genes termed immediate early (IE) genes are the first to be expressed after virus entry. IE genes are expressed in the absence of prior de novo viral protein expression, and the most prominent expression at IE times post-infection of permissive cells occurs from the major immediate early (MIE) locus (DeMarchi et al., 1980; Wathen et al., 1981). The MIE region encompasses two differentially spliced transcripts with novel exons ie1 (UL123) and ie2 (UL122), that encode IE72 and IE86 proteins, respectively. This region also encodes a number of additional proteins. The MIE gene products are derived from a single alternately spliced transcript originating from a single transcriptional start site (Stenberg et al., 1985). The MIE regulatory region that controls expression of ie1/ie2 genes consists of several elements including a promoter, enhancer, unique region and distal modulator, which extend approximately 1150 bp upstream of the transcription start site (Meier and Stinski, 1996). Expression of MIE promoter-driven gene products are critical for efficient activation of the replicative cycle in permissive cells, as ie2 is essential for virus replication (Marchini et al., 2001) and at low multiplicities of infection, ie1 is necessary for proper initiation of the replicative cycle (Gawn and Greaves, 2002; Greaves and Mocarski, 1998).

In contrast to productive infection, latent infection is characterised by highly restricted viral gene expression with silencing of the MIE promoter-driven ie1/ie2 genes, and it has therefore been hypothesised that this repression may be an important step in the establishment or maintenance of latency in hematopoietic cells (Mendelson et al., 1996; Murphy et al., 2002; Taylor-Wiedeman et al., 1994). In this respect, several cellular proteins
known to act as repressors have been implicated as possible negative regulators of MIE gene activity (Sinclair and Sissons, 2006). These include transcription factors such as Ets-2 repressor factor (ERF) and Yin Yang 1 (YY1), which have been shown to repress the MIE regulatory region in transient transfection assays of non-permissive Ntera2 D1 (T2) cells (Bain et al., 2003; Liu et al., 1994), and also human Daxx (hDaxx) which can function to repress MIE gene expression (Preston and Nicholl, 2006; Woodhall et al., 2006). Chromatin modification enzymes appear important for this phenotype as the mechanism of action of ERF has been shown to involve the recruitment of histone deacetylases (HDACs) to the MIE regulatory region resulting in differentiation-dependent regulation of the MIE promoter (Wright et al., 2005). YY1 has also been shown to interact with HDACs (Yang et al., 1997) and hDaxx-mediated repression correlates with a repressive chromatin structure around the MIE promoter (Woodhall et al., 2006).

The CCAAT displacement protein (CDP or CUX1) is a member of the conserved CDP/Cut family of homeoproteins found in higher order eukaryotes (Nepveu, 2001). CUX1 is a transcription factor expressed in a wide range of cells where it predominantly exhibits repressive activity (Mailly et al., 1996; Skalnik et al., 1991; van Gurp et al., 1999), although it has also been shown to be an activator of transcription in some cases (Harada et al., 2008; Truscott et al., 2007; Truscott et al., 2008; Truscott et al., 2003). CUX1 may control proliferation and differentiation (Luo and Skalnik, 1996; van Gurp et al., 1999; Vanden Heuvel et al., 1996), and plays an important regulatory role during myelopoiesis (Friedman, 2002; Friedman, 2007; Khanna-Gupta et al., 2001; Skalnik et al., 1991). CUX1 loses its DNA binding and repressive phenotype when progenitors differentiate into monocytes and macrophages (Martin-Soudant et al., 2000; Marziali et al., 1999). Transcriptional repression by CUX1 is thought to involve both inhibition of RNA polymerase II by occupying sites otherwise bound by activating factors (Barberis et al., 1987; Luo and Skalnik, 1996; Mailly et al., 1996), recruitment of histone methyltransferases (Nishio and Walsh, 2004), and like YY1, by recruitment of HDACs facilitating the formation of heterochromatin (Li et al., 1999; Mailly et al., 1996).

In this study we report that the HCMV MIE regulatory region contains an unusually high number of putative binding sites for CUX1 and show that this transcription factor is able to bind to core sequences within the MIE regulatory region in vitro. We also show that overexpression of CUX1 in HEK293 cells results in significant repression of luciferase expression driven by the MIE promoter/ regulatory region, and that mutation of identified CUX1 binding sites within this region diminishes the ability of CUX1 to repress MIE promoter activity. Importantly, following virus infection, CUX1 showed evidence of association with the MIE regulatory region and also inhibited the expression of HCMV ie1 and ie2, suggesting that this transcription factor may act as a regulator of HCMV gene expression required to initiate the replicative cycle.

Results

Identification of putative CUX1 binding sites in HCMV

Full length CUX1 contains four DNA binding domains and different potential binding sequences have been reported (Andres et al., 1994; Aufiero et al., 1994; Harada et al., 1995;
Moon et al., 2001; Nirodi et al., 2001). To determine the number of potential CUX1 binding sites within the HCMV major immediate early (MIE) gene regulatory region, motifs that had exact or similar matches to the CUX1 core consensus binding sites were mapped against a 2.1 kbp sequence encompassing the MIE regulatory region using the software tool MatInspector (Genomatix Software, Munchen, Germany). From this analysis, 13 putative distinct human CUX1 binding sites were identified (Fig. 1A). Three were located in the enhancer region, four in the unique region, four in the distal modulator and two upstream of the distal modulator. Nine putative binding sequences had core and matrix similarity scores of greater than or equal to 0.8 which is indicative of a strong binding potential to CUX1 while four had scores below 0.8, but greater than 0.7, which may also bind to CUX1.

The occurrence of putative binding motifs for transcription factors may occur due to random variation in genomic sequence; different transcription factor motifs occur randomly with different frequencies, depending on the nucleotide sequence. To ascertain whether the number of putative binding sites for CUX1 found in the MIE regulatory region was a non-specific feature of the viral genome, or whether it was restricted to specified regions of the viral genome, the entire genomic sequence of HCMV strain AD169 (accession x17403) was analysed for these motifs, and the number of binding sites was determined for 2000 bp segments across the HCMV genome (Fig. 1B). This analysis revealed that the number of putative CUX1 binding sites identified in the MIE regulatory region was not a common feature of the remainder of the viral genome. The majority of the viral genome contained very few putative CUX1 binding sites, with a vast majority of the 2000 bp genomic segments analysed containing 0, 1 or 2 putative binding sites. Notable exceptions being the segment including the MIE regulatory region (13 binding sites) and a region encompassing UL108–UL110 (13 binding sites). This analysis suggested that random occurrences of these motifs were unlikely to account for the relatively large number of putative binding sites within or proximal to the MIE regulatory region.

Detection of sequences within the HCMV MIE regulatory region that bind to CUX1

To test the identified sequences from the MIE regulatory region for CUX1 binding capability, an in vitro binding assay was performed. A subset of the sequences detected in our analysis of putative transcription factor binding sites of the MIE region were selected for further analysis and oligonucleotides encompassing these putative binding domains were synthesized. The eight sequences selected for further analysis were those with the highest degree of match with the core motifs reported to bind CUX1 (i.e. eight sequences with the highest core and matrix similarity scores). These oligonucleotides were termed CMV 1 (core and matrix similarity scores: 1 and 0.96, respectively), CMV 2 (1 and 0.95), CMV 3 (1 and 0.81), CMV 4 (1 and 0.92), CMV 5 (1 and 0.80), CMV 6 (1 and 0.77), CMV 7 (0.96 and 0.73) and CMV 8 (0.93 and 0.94). In addition to these 8 HCMV sequences, two positive control consensus CUX1 binding human sequences were included. These latter sequences were chosen based on published studies reporting the sequences that CUX1 binds with greatest affinity (Moon et al., 2001; Nirodi et al., 2001). Initial experiments in HEK293 cells indicated that basal levels of CUX1 were too low to carry out the in vitro transcription factor binding assays. Therefore, HEK293 cells were transfected with a CUX1 expression vector encoding the full length 5.2 kb cDNA for CUX1 driven by the adenovirus type 5 major-late
promoter (Neufeld et al., 1992). Efficiency of transfection of HEK293 cells was routinely greater than 90% (data not shown). At 48 h post-transfection nuclei were purified and lysed in the presence of a protease inhibitor cocktail. Biotin end-labelled double-stranded oligonucleotides containing each of the 8 putative MIE-region CUX1 binding sites or 2 known human CUX1 binding sites were incubated with or without nuclear protein extract, and then bound to streptavidin coated wells. After washing away unbound nucleotides and protein, wells were stained using an antibody specific for CUX1, followed by colorimetric detection.

In replicate experiments, both of the positive control consensus CUX1 binding human sequences showed higher levels of absorbance than a negative control which consisted of all reaction components but without the addition of any oligonucleotide sequences (Fig. 2A). In addition, when the positive control human cellular sequences were tested in the absence of nuclear extracts, the level of absorbance dropped to a level similar to that of the negative control. Results for three (CMV 1, CMV 2, and CMV 4) of the eight HCMV sequences gave positive staining of similar intensity to that of the positive control sequences, while the other five HCMV sequences were negative. The positions of the three HCMV MIE-region oligonucleotides which demonstrated positive binding to CUX1 are illustrated in Fig. 2B. CMV 1 is an oligonucleotide sequence located from −736 bp to −758 bp upstream of the MIE transcription start site within the unique region. CMV 4 is an oligonucleotide sequence located in the distal modulator −1006 bp to −1034 bp upstream of the start site. CMV 2 is an oligonucleotide sequence −1245 bp to −1267 bp upstream of the start site. Although the flanking sequences outside the core motifs contained within these oligonucleotides varied significantly, the oligonucleotides that gave positive signals in the binding assay (CMV 1, CMV 2 and CMV 4) all contained exact matches for one of the predicted CUX1 core binding site sequence (ATCGAT) (Moon et al., 2001), and were also those which yielded highest core and matrix similarity scores as a predictor of CUX1 binding potential.

To determine whether the CUX1 core binding sequence ATCGAT was required for binding to CUX1 to the MIE region, this binding site was mutated in oligonucleotides CMV 1, CMV 2 and CMV 4 by reversing the positions of the 2 nucleotides A and T to generate oligonucleotides CMV 1 Mut, CMV 2 Mut and CMV 4 Mut. The mutated oligonucleotides were then compared with their respective unmodified parent oligonucleotide for ability to bind to CUX1 using an in vitro binding assay. As before, CMV 1, CMV 2 and CMV 4 demonstrated binding comparable to that of the 2 positive control human sequences (Fig. 2C). In contrast, mutation of the CUX1 core binding sequence in all 3 viral oligonucleotides abrogated this binding capacity, with absorbance readings reduced to a level comparable to those of the negative controls; which were reactions either without oligonucleotides, without nuclear extract, or inclusion of an HCMV oligonucleotide not containing any known CUX1 binding motif.

To determine the specificity of the three CUX1 binding sites identified within the MIE region, the parent oligonucleotides were competed against specific and non-specific unlabelled competitors using the in vitro binding assay (Fig. 3). In replicate experiments, the addition of increasing amounts of specific unlabelled competitors leads to statistically significant reductions in staining intensity when compared with staining of labelled
oligonucleotides without the addition of competitors. In addition, when unlabelled non-
npecific competitors were added, no statistically significant change in signal intensity was
observed. These results were consistently replicated in CMV 1, CMV 2 and CMV 4. It was
concluded that the sequence ATCGAT identified within 3 regions of the MIE regulatory
region facilitated the binding of CUX1.

**CUX1 inhibits luciferase expression driven by the MIE regulatory region**

Major immediate early gene expression is an essential feature of successful viral replication,
being required to initiate the characteristic gene expression cascade. Failure to express *iel*
and *ie2* genes from the MIE region results in a failure to initiate a productive infection
(Marchini et al., 2001; Mocarski et al., 1996). It has also been hypothesised that this failure
may be important in the establishment of latency (Shelbourn et al., 1989). To assess whether
CUX1 inhibited transcription driven by the MIE promoter, a luciferase reporter vector was
constructed, with luciferase expression driven by the MIE promoter encompassed within a
2.1 kbp sequence containing the MIE regulatory region. This reporter vector (pMIEP) was
transfected into HEK293 cells, together with a CUX1 expression vector (pMT2-CDP), and a
*β*-galactosidase expression vector driven by the SV40 promoter to normalise for endogenous
differences in basal transcription and transfection efficiency between treatments. The SV40
promoter does not contain any known motifs for CUX1 binding and was not itself affected
by transfection with the CUX1 expression vector (data not shown). In parallel, cells were
transfected with the parental vector (pMT), generated by excising the 5.2 kb CUX1 cDNA
from pMT2-CDP.

Nuclear protein extracts from 1.0×10⁷ transfected HEK293 cells at 48 h post-transfection
were subjected to SDS-PAGE and western blotted with a goat anti-CUX1 primary antibody
followed by a secondary anti-goat-HRP antibody. Bound antibody was visualised by ECL
(Fig. 4A). Low level endogenous CUX1 expression was detected in untransfected HEK293
cells and HEK293 cells transfected with the parental vector (pMT). In contrast, HEK293
cells transfected with the CUX1 expressing vector (pMT2-CDP) showed strong CUX1
expression. Previously identified isoforms of CUX1 were detected including the full length
200 kDa CUX1 (p200), the processed highly active 110 kDa CUX1 (p110) and the 75 kDa
CUX1 (p75) (Goulet et al., 2002; Moon et al., 2001). In addition to these characterised
isoforms, additional bands were identified at approximately 60 kDa and 40 kDa. These were
prominent only in the pMT2-CDP-transfected cells. To confirm equal protein loading of
each sample, the membrane was also reacted with antibody to the housekeeping protein
GAPDH. GAPDH protein at the predicted size of 37 kDa was detected at equal intensity for
all samples. Therefore it was concluded that CUX1 protein was successfully over-expressed
in HEK293 cells transfected with a CUX1 expressing vector.

To determine whether CUX1 influences MIE promoter-driven gene expression, luciferase
reporter assays were performed. HEK293 cells co-transfected with the MIE regulatory
region-driven luciferase vector (pMIEP), the *β*-galactosidase expression vector and either the
CUX1 expression vector (pMT2-CDP) or its parent vector (pMT) were lysed and luciferase
and *β*-galactosidase levels were determined. In comparison to the parent pMT, the CUX1
expression vector resulted in a significant decrease in luciferase expression when normalised
for endogenous differences in basal transcription and transfection efficiency using β-galactosidase levels (Fig. 4B). These data demonstrated that CUX1 repressed activity of the HCMV MIE promoter in transiently transfected cells.

The transcription factor binding site assay identified ATCGAT core sequences from within the HCMV MIE regulatory region as binding sites for CUX1. To test whether these binding sites were responsible for the observed repression of MIE promoter-driven luciferase expression by CUX1, the three ATCGAT motifs within the luciferase reporter vector (represented by oligonucleotides CMV 1, CMV 2 and CMV 4 in the transcription factor binding assay experiments) were mutated to TACGAT to generate pMIEPmut. The specificity of the mutations in pMIEPmut was confirmed by DNA sequencing. The impact of CUX1 on luciferase expression driven by this mutated luciferase vector or its parental luciferase vector was then examined. This analysis revealed that mutation of these three ATCGAT binding sites for CUX1 significantly alleviated the repression observed with the native luciferase reporter vector (P<0.05) (Fig. 4B). A construct which swapped the A and T at only a single site (encompassed by CMV 1), did not demonstrate a significant alleviation of the repressive effects of CUX1 in comparison to the parental construct (data not shown). These data demonstrate that the observed repression by CUX1 was mediated at least in part by the three ATCGAT sequences found within the HCMV MIE regulatory region represented by oligonucleotides CMV 1, CMV 2 and CMV 4.

To provide additional evidence that the repressive effect of CUX1 on the MIE regulatory region was due to expression of this protein, HEK293 cells were transfected as above, except that increasing concentrations of CUX1 expression construct (pMT2-CDP) were included, ranging from 100 ng to 375 ng. In each treatment the total amount of DNA was kept constant by altering the amount of parent vector (pMT) included in each transfection reaction. At 48 h post-transfection, cells were lysed and assessed for luciferase expression, with values normalised using β-galactosidase levels. Addition of increasing amounts of CUX1 resulted in a significant dose-dependent decrease in expression of luciferase driven from the MIE promoter (Fig. 5) indicating that the repressive effect observed was due to CUX1 expression.

**CUX1 inhibits transcription of viral ie1 and ie2 during HCMV infection**

The ability of CUX1 to reduce luciferase reporter expression driven by the MIE regulatory region suggested that this protein may have a negative influence on the expression of the HCMV genes which are under the control of the MIE regulatory region. To test the effect of CUX1 on MIE gene expression in the context of a virus infection, we sought to overexpress CUX1 during HCMV infection of HEK293 cells. These cells are not fully permissive to HCMV infection, but do support virus entry and immediate early gene expression (Compton et al., 2003), and so therefore provide not only a model for transient transfection experiments, but also a model to examine the impact of CUX1 on *in vivo* MIE gene expression (i.e. from the virus). To confirm that these cells were infectable with HCMV, we utilised a GFP-tagged virus RC2940, derived from HCMV strain Towne. This recombinant virus contains a fusion between IE2 and GFP, and consequently functions to report on IE2 gene expression during infection. Cells were exposed to virus at an MOI of 3. At 2 h P.I. and
48 h P.I., cells were harvested and analysed by flow cytometry for GFP expression. This analysis showed that between 2 h P. I. and 48 h P.I. the number of GFP positive HEK293 cells increased from 0.3% to 25%, demonstrating successful infection, and at the very least, a capacity for de novo MIE gene expression in these cells (Fig. 6).

To determine whether CUX1 was able to inhibit MIE promoter-driven ie1 and ie2 transcription, HEK293 cells were transfected with the CUX1 expression construct (pMT2-CDP) or its parent vector (pMT), followed 48 h later by infection with HCMV at an MOI of 3. At 6, 24 and 72 h post-infection, RNA was extracted and subjected to DNase I digestion and quantitative RT-PCR for CUX1 and viral ie1 and ie2 transcription. Transcript levels for each gene were normalised to two housekeeping genes GAPDH and LDHA. In a total of 4 independent replicate experiments, there was a significant increase in the expression of CUX1 transcripts in cells transfected with the CUX1 expressing vector, relative to cells transfected with the parental vector. This overexpression of CUX1 was maintained throughout the time course of infection, and although there was a decrease in CUX1 expression in pMT2-CDP-transfected cells over time between 6 h to 72 h P.I., this change was not statistically significant (P>0.05) (Fig. 7A).

At all three time points post-infection ie1 mRNA expression in cells transfected with the CUX1 expression vector prior to infection with HCMV was lower than that of parental vector-transfected cells that had been infected with HCMV in parallel (Fig. 7B). Similarly, CUX1 overexpression reduced the expression of ie2 transcripts at all time points tested (Fig. 7C). The decrease in both ie1 and ie2 expression by CUX1 was statistically significant at all times post-infection. These data provide evidence that CUX1 is able to repress ie1 and ie2 mRNA expressed by the virus following infection.

**CUX1 associates with the MIE regulatory region in vivo**

Using the in vitro transcription factor binding assay, three CUX1 binding sites were identified within the MIE regulatory region. We therefore sought to determine whether the association of CUX1 to these three sites occurred in the context of a viral infection using a chromatin immunoprecipitation (ChIP) assay. HEK293 cells transfected with the CUX1 expressing vector pMT2-CDP for 48 h were infected with the Toledo strain of HCMV at a MOI of 3 for a period of 24 h. Cross-linked, sonicated chromatin was then subjected to immunoprecipitation against an anti-CUX1 antibody and a negative control normal rabbit IgG, followed by PCR using primers which flanked the 3 CUX1 binding sites identified earlier by the in vitro transcription factor binding assay. Strong amplification of all 3 regions was detected following immunoprecipitation with anti-CUX1 antibody, but not with the isotype control (Fig. 8). These data provide evidence that CUX1 associates with the MIE regulatory region during HCMV infection.

**Discussion**

HCMV replication is dependent on the expression of ie1 and ie2 gene products expressed from the MIE region of the viral genome. The MIE promoter is controlled by a complex upstream regulatory region which contains binding sites for a broad range of cellular transcription factors which act as either activators or repressors of MIE promoter-driven
gene expression (Bain et al., 2003; Hennighausen and Fleckenstein, 1986; Huang et al., 1996; Hunninghake et al., 1989; Jeang et al., 1987; Lang et al., 1992; Liu et al., 1994; Sambucetti et al., 1989; Shelbourn et al., 1989; Zhang et al., 1991; Zweidler-Mckay et al., 1996). This study provides the first evidence that a cellular transcription factor CUX1, can bind to sequences from the HCMV MIE regulatory region in vitro and inhibit MIE promoter activity. Three sites within the MIE regulatory region, all of which contained the binding motif ATCGAT, were shown to bind to CUX1, and these sites at least partially accounted for the capacity of CUX1 to repress the MIE promoter. In addition, overexpression of CUX1 in HCMV-infected cells was shown to significantly repress ie1 and ie2 mRNA expression by the virus, suggesting that CUX1 may play a role in modulating the outcome of HCMV infection.

The demonstration that CUX1 binds in vitro and in vivo to sequences from HCMV raises the possibility that this transcription factor may play a role in other cell types. A study by Meier and Stinski (1997) showed that in comparison to its parent virus, a virus deleted of the region from 750 bp to 1140 bp upstream of the MIE gene transcriptional start site displayed very similar, low level ie1 gene expression in a non-permissive myeloid progenitor cell line THP-1 (Meier and Stinski, 1997). Thus, deletion of this region, which contains one of the three positive binding sites for CUX1 identified in our study, does not appear to be required for the repression of IE gene expression in THP-1 cells. However, two of the three oligonucleotides found to bind CUX1 in our study were outside this region, and may therefore represent redundant functional sites for CUX1 binding and repression. In an analysis of the repressive capacity of CUX1, Mailly et al. (1996) demonstrated that repression domains from CUX1 retained the capacity to function even when bound at a large distance from the transcription initiation site. In their study, insertion of a 1.6 kb fragment of neutral spacer DNA between the site of CUX1 repression domain binding and a herpes simplex virus tk promoter-driven reporter gene did not result in a reduction of the repressive effects of CUX1 in comparison to a reporter construct in which binding occurred immediately adjacent to the promoter. Thus, CUX1 can exert a repressive effect at a relatively large distance, which is consistent with a functional role for the CUX1 binding sites we demonstrated as far as 1.2 kb upstream from the MIE transcription start site.

In an analysis of control of the UL127 promoter, which lies within the MIE regulatory region, mutations in two other putative CUX1 binding sites at position −650 to −642 and −635 to −627 (relative to the MIE transcription start site) had little or no effect on UL127 promoter activity (Lashmit et al., 2004). The impact of these mutations on MIE promoter activity was not tested in this study, but it remains possible that additional CUX1 binding sites within the MIE region may play a biologically important role in the control of MIE gene expression. Indeed, our finding that mutation of the three MIE regulatory region ATCGAT sequences only partially abolished CUX1-mediated repression of the MIE promoter supports the notion that either additional CUX1 binding sites or other regulatory elements may be biologically active in this context and/or that the nature of the mutation we introduced into each CUX1 binding site did not result in complete inactivation of CUX1 binding. Extension of these studies to include more extensive mutation of ATCGAT sequences, as well as the application of additional transcription factor binding assay techniques such as electrophoretic mobility shift assay (EMSA) may help to define the full

_Virology_. Author manuscript; available in PMC 2017 June 26.
repertoire of CUX1 binding sites which play biologically significant roles in modulating MIE promoter activity. Recently, Lee et al. (2007) identified several regions within the MIE unique region (UR) which could bind to CUX1, including several which were not identified in our study. In addition, they demonstrated that CUX1 overexpression in transiently transfected HEK293 or 293T cells repressed expression from the UL127 promoter, but that overexpression of CUX1 did not repress expression from the MIE promoter. The vector used to assess the impact of CUX1 on MIE promoter activity utilised pRL-CMV (or CMV-R-Luc), which is a commercially acquired reporter construct with luciferase expression under the control of HCMV MIE promoter/enhancer sequences. However, this construct includes only the first 735 bp upstream of the MIE transcriptional start site, and so does not contain the three ATCGAT sites further upstream which were included in our reporter construct, and which we identified as being able to bind to CUX1 to cause repression of the MIE promoter. The lack of CUX1-mediated repression of the MIE promoter reported by Lee et al. may therefore be a consequence of this difference in the reporter constructs used for these studies.

The full length CUX1 protein contains four distinct DNA binding domains, at least two of which are required to affect DNA binding. Three of these DNA binding domains are composed of repeats, termed Cut repeats (CR) 1–3, while the fourth is a Cut homeodomain (HD). CUX1 exists in a number of different isoforms which have different DNA binding characteristics due to the presence or absence of different combinations of CR1–3 and HD. Full length CUX1, p200, exhibits rapid but transient binding that seems to make use of the CR1CR2, while the proteolytically cleaved p110 and p90 isoforms contain CR2CR3HD, and p75 contains CR3HD, each appear to be capable of binding more stably than the full length protein. It remains to be determined which CUX1 isoforms bind to the MIE region, as we detected expression of multiple isoforms in the transfected HEK293 cells used in our binding studies. Our western blot analysis also detected a number of smaller products which may be additional CUX1 isoforms expressed in HEK293 cells. It is not clear if these proteins contain DNA binding domains or if they have any role in the observation reported in this study.

CUX1 exhibits some flexibility in its sequence requirements for binding (Nepveu, 2001). The main consensus binding site for p110 is ATCGAT, and this palindromic motif mediates stable DNA binding by CR2, CR3 and the HD. Full length CUX1 binding appears to be mediated by CGAT or CAAT repeats. Interestingly this ATCGAT motif is similar to a R1 motif that occurs 16 times within the modulator region of the MIE regulatory region, TATCG (Huang et al., 1996; Meier and Stinski, 1997; Thrower et al., 1996). Huang and co-workers have shown that an MIE motif pair, ATCG-N6-CGAT, strikingly similar to the motifs that bound CUX1, was able to bind a protein that repressed transcription driven by the MIE promoter in undifferentiated THP-1 cells, and that this phenotype was absent in differentiated THP-1 cells (Huang et al., 1996). While Huang et al. (1996) did not report identification of the protein responsible, this motif pair is similar to the motifs that are bound by full length CUX1 p200 or the p110 isoform, suggesting that this repression may have been mediated by CUX1. Bullock et al. (2002) also found that the R1 element was bound by a protein that is antigenically similar to Drosophila BEAF-32 (Bullock et al., 2002).

Interestingly, the CUX1 consensus binding site matches that reported for BEAF-32 (Orian et
al., 2003), further supporting the notion that the R1 repeat element may contain an active binding site for CUX1.

CUX1 has been shown to repress viral gene expression or replication of viruses such as human papilloma virus and mouse mammary tumor virus by binding to critical viral regulatory sequences (Ai et al., 1999; Narahari et al., 2006; O’Connor et al., 2000; Pattison et al., 1997; Sato et al., 2007; Zhu and Dudley, 2002; Zhu et al., 2000), although the mechanism of repression by CUX1 has not been fully elucidated. CUX1 has also been shown to bind to adenovirus packaging sequences, where it may play a role in viral DNA packaging (Erturk et al., 2003). One mode by which CUX1 is capable of repressing transcription is by recruiting HDACs thereby facilitating the formation of heterochromatin (Li et al., 1999; Mailly et al., 1996). As CUX1 is a transcriptional repressor which is expressed in myeloid cells, and during latent infection of myeloid cells the MIE region is associated with markers of transcriptional inactivity such as non-acetylated histones and heterochromatin protein 1 (Murphy et al., 2002; Reeves et al., 2005), it is tempting to speculate that CUX1 may be a factor contributing to HCMV latency in these cells. Moreover, CUX1 is capable of recruiting HDACs (Li et al., 1999), and the activity of CUX1 is down-modulated at terminal stages of myeloid differentiation to macrophages (Martin-Soudant et al., 2000; Marziali et al., 1999), when these cells can become permissive to HCMV replication, further correlating with a possible role of this transcription factor during HCMV latency or reactivation.

CUX1 is an important transcription factor involved in both cellular differentiation and cell cycle control (Bodmer et al., 1987; Coqueret et al., 1998; Vanden Heuvel et al., 1996). Our experiments showing that CUX1 reduces MIE gene expression during virus infection do not rule out the possibility that global effects of CUX1 on host cell gene expression may have indirect effects on viral gene expression. However, our data providing evidence that CUX1 may bind to sequences found within the MIE regulatory region, together with our demonstration of a reduction of MIE promoter-driven gene expression in transient cotransfection experiments suggests that the effects of CUX1 on MIE gene expression during virus infection are likely to be specific.

Assessing the impact of CUX1 on the expression of other viral genes, the potential for recovery of MIE repression if CUX1 levels drop in infected cells, and determining whether CUX1 repression of MIE gene expression inhibits virus replication following infection of fully permissive cell types will be important components of future studies to determine the role of CUX1 in regulating permissiveness to productive infection. While our initial assessment of infected, fully permissive HFFs indicated no significant changes in CUX1 mRNA levels compared with mock-infected cells (data not shown), examination of CUX1 function in this cell type will be required to determine the extent of any impact on the full virus replicative cycle. Similarly, as monocytes and hematopoietic progenitor cells are a reservoir for latent infection, extension of this work to include analysis of CUX1 expression and function in primary myeloid lineage cells in the context of HCMV latent infection and reactivation and cellular differentiation state will be important to determine whether this transcription factor regulates viral latency.
Materials and methods

Cells and virus

Human embryonic kidney 293 (HEK293) cells (ATCC #CRL-1573) and human foreskin fibroblast cells (HFFs) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) at 37 °C with 5% CO₂. HCMV RC2940 is an IE2 green fluorescent protein (GFP)-tagged virus prepared from a plaque-purified derivative of the Towne varRIT3 strain of HCMV.

To construct RC2940, plasmid pON2940 was first constructed as follows: A 6.7 kb Sall-EcoRI IE-region fragment from plasmid pON303G (Cherrington and Mocarski, 1989) was ligated between the Sall and EcoRI sites of plasmid pGEM3zf(+) (Promega) to produce plasmid pON2903. pON2903 was PCR-amplified with primer primer ie p4 (5’-TCCTTTCAAGGTGATCASTCAACCGCCGTGCTCT-3’) and primer ie p7 (5’-GGATCCCTGAGACTTGTTCCTCAG-3’). PCR products were T/A cloned into pGEMT-easy (Promega) to produce pON2937. pON2903 was PCR-amplified with primer sp6 (5’-ATTTAGGTGACACTATAG-3’) and primer ie p8 (5’-GGATCCTAGTGAAGAATCGGAAAG-3’). PCR products were T/A cloned into pGEMT-easy (Promega) to produce pON2938. pGFP-mut2 (Cormack et al., 1996) was PCR-amplified with primer GFP Bam 5’ (5’-GGATCCATGAGTAAAGGAGAAGA-3’). PCR products were T/A cloned into pGEMT-easy (Promega) to produce pON2939. A 700 bp BamHI/PstI from pON2937 and a 1000 bp BamHI/SphI from pON2938 were ligated into the PstI and SphI sites of pGEMT-easy (Promega) to make pON2941. A 712 bp BamHI fragment from pON2939 was ligated into the BamHI site of pON2941 to make pON2942. A 1.4 kb Sfi/Ise fragment from pON2942 was ligated into the Sfi/Ise site of pON2903 to make pON2940. HFFs were then electroporated with 20 μg of EcoRI/SalI-linearized pON2940. Cells were then seeded into two T25 flasks, incubated overnight and then infected with HCMV CR208 virus at a multiplicity of infection (MOI) of 5. CR208 is a Towne-derived iei-defective virus which exhibits a major growth defect at low MOIs (Greaves and Mocarski, 1998). After 7 days, supernatant was collected and progeny virus titers were determined on iei-expressing ihfie1.3 cells (Greaves and Mocarski, 1998). To enrich rescued viruses (which would now contain the IE2–GFP fusion), progeny virus was used to infect HFFs at an MOI of 0.1 and supernatant viruses were harvested at 100% cytopathic effect (CPE). To purify RC2940 virus, three rounds of plaque purification were performed using HFFs overlaid with 0.5% agarose. The nature of the fusion was confirmed by DNA sequencing. RC2940 expresses high levels of GFP during productive infection of HFFs, with virus titers and plaquing efficiency comparable to that of the parental Towne varRIT3 strain of HCMV (J. Xu, D. Formankova, and E. S. Mocarski, unpublished results).

Expression vectors

pMIEP is a luciferase reporter construct produced by excising a 2.1 kbp section of the HCMV genomic sequence encompassing the MIE regulatory region (positions +100 to −2000 with respect to the start of MIE gene transcription). This fragment was directionally cloned into the pGL3-Basic vector (Promega) such that luciferase expression is driven by the
MIE promoter. The three ATCGAT binding sites within the MIE regulatory region of the construct pMIEP were mutated using the Stratagene Quick Change II site directed mutagenesis kit to generate the construct pMIEPmut. Primers used to construct the mutations were: CMV1_F: CAATATTGATTCAATGTATATTACGATATGCATTGGCATGTG; CMV1_R:CACATGGCAATGCAATCGAATATACATTTGAAAT-CAATATTG; CMV2_F:CCAGGTTGATCCTACGATAGGGAGCG; CMV2_R: CGCTCCCTATCGTAGATTTTCAAATTACGATTTGTCATTATCGGCC; and CMV4_F: GGCATATGGA-CAATACATTTCAATTAGATGTGAGGCG; CMV4_R: CMV4_R: GGCATATGGA-CAATACATTTCAATTAGATGTGAGGCG; CMV4_R: GGCATATGGA-CAATACATTTCAATTAGATGTGAGGCG. pMT2-CDP is a construct expressing human CUX1 protein consisting of a 5.2 kb full length CUX1 cDNA under the control of the major adenovirus late promoter and kindly provided by Dr Ellis Neufeld of the Children’s Hospital, Boston (Neufeld et al., 1992). pMT is a derivative of pMT2-CDP, prepared by excising the 5.2 kb cDNA expressing the full length CUX1 protein. pSV is a β-galactosidase expressing construct (Promega) driven by the SV40 early promoter and enhancer and was used as an internal control to normalise transfection efficiency.

**Transient transfections and luciferase assays**

For transient transfections, six well transfection plates were seeded with HEK293 cells in DMEM with 10% FCS and at 80% confluency, each well was co-transfected with 750 ng of the pMIEP reporter construct, 250 ng of the pSV construct and 1 μg of either pMT2-CDP or pMT constructs using 6 μl of Fugene 6 (Roche) in 100 μl of DMEM per well. After 48 h of expression, cells were lysed and assayed using the manufacturer’s protocols of the Dual-Light Assay (Applied Biosystems).

**Nuclear protein extraction and western blot analysis**

To extract nuclear proteins for the western blot, 1.5 ml of a solution containing 10 mM HEPES (pH 7.9), 15 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, dH₂O and protease inhibitor cocktail was added to 10⁷ HEK293 cells. After 10 min incubation on ice, 25 μl of 0.2% NP-40 was added followed by brief vortexing and centrifuging at 20,000 × g for 10 s. The nuclear pellet was then washed once in the above solution before being resuspended in 100 μl of a solution containing 50 mM HEPES (pH 7.9), 50 mM KCl, 0.1 mM EDTA, dH₂O and protease inhibitor cocktail. This was followed by the addition of 100 μl of a solution containing 50 mM HEPES (pH 7.9), 0.8 M KCl, 0.1 mM EDTA, dH₂O and protease inhibitor cocktail. The mixture was then incubated for 30 min at 4 °C and centrifuged at 20,000 × g for 10 min. The supernatant containing the nuclear extracts was then used for the western blot analysis.

Samples were boiled for 10 min in the presence of Reducing Agent (Biorad) and nuclear extracts subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to PVDF membrane and stained with 0.1% amido black. The membrane was then incubated overnight at 4 °C in a blocking solution containing TBS (200 mM Tris base, 1.4 M NaCl, pH 7.6) with 5% skim milk powder. The membrane was then reacted with a CUX1 specific antibody (C-20, Santa Cruz Biotechnology) diluted 1:400 in blocking solution for 1 h at room temperature. The membrane was then washed three times for 5 min in TBS–T (TBS and 0.1% Tween), followed by one wash with TBS for 2 min. To detect
specific binding to CUX1 protein, a secondary antibody of anti-goat-HRP (Dako) diluted 1:4000 in blocking solution was added to the membrane and incubated for 30 min at room temperature, followed by the above wash steps. Bound antibody was detected with enhanced chemiluminescence (ECL) (Amersham Biosciences) and exposure to X-ray film.

To reprobe the membrane, it was first stripped by soaking in a solution containing 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl (pH 6.7) for 30 min at 70 °C with gentle agitation. The membrane was then washed twice in TBS–T and once in TBS. The housekeeping gene GAPDH was detected using an antibody specific to GAPDH (Santa Cruz Biotechnology) diluted 1:1000 in blocking solution. This was followed by a secondary antibody of anti-rabbit-HRP (Dako) diluted 1:12000 in blocking solution. Bound antibody was detected with ECL (Amersham Biosciences) and exposure to X-ray film.

**In vitro transcription factor binding assay**

The in vitro transcription factor binding assay was performed using the Novagen NoShift Transcription Factor Assay Kit (Merck KGaA). Briefly, biotinylated oligonucleotides (Sigma-Proligo) were re-annealed by adding 10 μg of sense and anti-sense strands with 100 μl of 0.5x SCC (Sigma) which was then heated for 10 min at 100 °C and diluted to a concentration of 10 pmol/μl. A mixture consisting of 5 μl 4x NoShift Binding Buffer, 1 μl of Poly(dI–dC)9Poly(dI–dC), 1 μl of Salmon Sperm DNA, 5 μl of nuclear extract and 7 μl of nuclelease-free H2O was prepared and added to 1 μl of the double-stranded biotinylated oligonucleotide followed by incubation on ice for 30 min. Two human consensus CUX1 binding sequences were included as positive controls: POS 1 (5′-TCGAGACGATATCGATAAGCTTCTTTTC) (Moon et al., 2001) and POS 2 (5′-GGGATCGATCTGGAACTCCGGGATCGATCTGGAACTCC) (Nirodi et al., 2001). Sequences tested for CUX1 binding within the HCMV MIE region upstream of the transcription site (nucleotide position +1) were CMV 1, which was located at position −738 to −759 (5′-TTCAA-TGTATAGATCGATATGCA); CMV 2, position −1246 to −1268 (5′-CCAGGT-TGATCCATCGATAGGGA); CMV 3, position −219 to −247 (5′-CCAT-GGTAATAGCGATAGC); CMV 4, position −1007 to −1035 (5′-ATTTTCAATATCGATTTTTCCAATATCG); CMV 5, position −1030 to −1058 (5′-ATATCGCCATCTCTATCGGCGATAAACAC); CMV 6, position −697 to −725 (5′-TATATAACCAATGATATGGCTAATG); CMV 7, position −436 to −464 (5′-TATGGGAACATACGTCATTATTGACGTCA); CMV 8, position −180 to −208 (5′-GCTATCCACGCCATTTGATGTACTGCCAA). A sequence located +118 to +138 downstream of the transcription start site with no predicted CUX1 binding site, NEG (5′-TCTATAGGCCG-TACTTACGTC), was included as a negative control. Sequences used for mutation studies consisted of parent sequences with the A and T nucleotides swapped at the predicted core CUX1 binding site as indicated by the underlines: CMV 1Mut (5′-TTCAATGATAGATCGA-TATGCA); CMV 2Mut (5′-CCAGGTGATCTCTATCGGATAGGGA); CMV 4Mut (5′-ATTTTCAATATCGATTTTTCCAATATCG). The mixtures were then added to a freshly prepared Streptavidin coated 96 well plate. Following incubation for 1 h at 37 °C, wells were washed three times with washing buffer and 100 μl of the goat anti-CUX1 antibody (C-20, Santa Cruz Biotechnology) was added diluted to 1:500 in the antibody dilution buffer.
Following 1 h incubation and three washes with washing buffer, 100 μl of a secondary anti-goat-HRP antibody (Dako) was added at 1:1000 dilution. The plate was then incubated for 30 min at 37 °C before being washed five times in washing buffer. 100 μl of TMB substrate was then added to each well and incubated in the dark at room temperature for 30 min. The reaction was stopped by adding 100 μl of 1 M HCl. The absorbance for each well was measured at 450 nm with a luminometer within 30 min.

**RNA extraction and quantitative RT-PCR**

RNA extractions were carried out according to the manufacturer’s instructions using Ambion’s RNAqueous kit (Ambion). Eluted RNA was precipitated with 0.1 volumes of 5 M ammonium acetate and 3 volumes of 100% ethanol. The precipitated RNA was quantitated using a Bionalayser (Agilent Technologies). RNA was then treated with RQ1 DNase I (Promega) followed by reverse transcription with random hexamers using SuperScript III reverse transcriptase (Invitrogen). Removal of RNA complementary to the cDNA was achieved by adding 2 units of RNase H (Invitrogen) and incubating at 37 °C for 20 min.

PCR was carried out using SYBR GreenER qPCR SuperMix (Invitrogen) in a Stratagene Mx3005P qPCR machine. The PCR reaction thermal profile was as follows: 1 × cycle: 2 min at 50 °C, 2 min at 95 °C; 50 × cycles: 15 s at 95 °C, 45 s at 45 °C; 1 × cycle: 1 min at 95 °C, 30 s at 55 °C, 30 s at 95 °C. Two housekeeping genes GAPDH and LDHA were included to assess relative changes in gene expression between the different treatments. The amplification efficiencies for each primer pair were determined by creating standard curves with 10-fold serial dilutions of pooled cDNA samples. The log of the relative target quantity was plotted versus the cycle threshold (C_T) values. A dissociation curve was gene-rated for each primer pair to demonstrate the amplification of a single product. All primers were designed using the Primer3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The sequences used for primers were as follows: GAPDH-F: 5′-TCACCAGGG-CTGCTTTTAAC, GAPDH-R: 5′-GACAAGCTTCCCGTTTCTCAG; LDHA-F: 5′-TGGGAGTTCACCACCATAAGC, LDHA-R: 5′-AGCACTCTCAACCACCACCTGCT; CUX1-F: 5′-TGTCAGCTACCCACCTCTC, CUX1-R: 5′-CCCCACCT-CATTTTTCACCTG; IE1: IEP3G: 5′-CAATACACTTCATCTCCTCGAAAGG, IEP3-BII: 5′-TATGTGTGTGGTATCCTCCTCAG; IE2: IEP5E-F: 5′-GGAGCCTCACAAGAATTGCAC, IEP5E-R: 5′-CCTGGTTGGTGAGAAGATG.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assays were performed using the EZ-ChIP kit following the manufacturer’s protocol (Millipore). Chromatin was obtained from HEK293 cells transfected with pMT2-CDP for 48 h and infected for 24 h with the Toledo strain of HCMV at an MOI of 3. The chromatin of 1×10⁶ cells was then sonicated to DNA sizes of between 200 bp and 1000 bp and used for each immunoprecipitation with normal rabbit IgG (Millipore) or rabbit anti-CUX1 antibodies (M-222, Santa Cruz Biotechnology). M-222 is a polyclonal antibody that recognizes amino acids 1111–1332 mapping at the C-terminus of CUX1 of mouse origin. Immunoprecipitated DNA was analysed by PCR using primers specific to the MIE region (CMV1_F: 5′-CAATAGCCAATATTTATGC, CMV1_R: 5′-CMV1_R:
5′GCGATTCTGTGTGTCGCAAA; CMV2_F: 5′ATTTGCGACACACAGAATCG,
CMV2_R: 5′CGCGATAGTGGTGTTTATCG; CMV4_F: 5′CGCTCTCCAGGTA-
CTGATCC, CMV4_R: 5′CCCCTCCGTGTTGTAGGTTA).

Acknowledgments

The authors would like to thank Ellis Neufeld and David Yao for the CUX1 expression construct pMT2-CDP and Dana Formankova for assistance with RC2940 construction. J.Z.C. was the holder of an Australian Postgraduate Award and Westmead Millennium Foundation Stipend Enhancement Award. This work was supported by Australian National Health and Medical Research Council grants #301942, #301943 and #358399.

References


Virology. Author manuscript; available in PMC 2017 June 26.


Fig. 1.
Putative CUX1 binding sites in HCMV. (A) Map showing the location of the putative CUX1 binding sites identified within a 2.1 kbp sequence of the HCMV genome (strain AD169) encompassing the MIE regulatory region, which includes the ie1/ie2 promoter, enhancer, unique region and distal modulator. Arrow indicates the transcriptional start site at nucleotide position 173,730. Filled ovals indicate putative CUX1 binding sites identified by MatInspector software to have high core and matrix similarity values (≥0.8) while open ovals indicate putative CUX1 binding sites with core and matrix similarity values <0.8 but >0.7. (B) Number of putative CUX1 binding sites counted for 2000 bp segments across the HCMV strain AD169 genome. A schematic representation of the HCMV AD169 genome is aligned beneath binding site counts, with the position of the major immediate early region (MIE) shown. Also shown are major features of the genome including the terminal repeat long and short segments (TRL and TRS, respectively), internal repeat long and short segments (IRL and IRS, respectively), and the unique long (UL) and unique short (US) segments.
Fig. 2.
Binding of CUX1 to sites within the MIE regulatory region identified by transcription factor binding assay. (A) Graph depicting the binding activity of double-stranded oligonucleotide sequences to CUX1 using a colorimetric transcription factor binding assay. The average absorbance readings at 450 nm from three independent replicate experiments utilising extracts from CUX1 expressing HEK293 cells incubated with 10 pmol of oligonucleotides containing putative CUX1 binding sites from the HCMV MIE regulatory region (CMV 1–CMV 8) or consensus human CUX1 binding sequences (POS 1 and POS 2) are shown, with a negative control (No oligo) in which no oligonucleotides were added to the reaction mixture. Black bars show absorbance readings when nuclear extract from 2.5×10^5 CUX1 expressing HEK293 cells was added, while the grey bars show readings for the same sequences when nuclear extracts were omitted. Significant differences to the no extract control were determined using a 1-tailed, paired Students t-test and are indicated as follows:
*P<0.05. Error bars are ±standard error of the mean (SEM). (B) Location of oligonucleotide sequences (CMV 1, CMV 2 and CMV 4) within or proximal to the MIE regulatory region which exhibited binding to CUX1. (C) Effect of mutation of CUX1 binding sites within the MIE regulatory region using a colorimetric transcription factor binding assay. Oligonucleotides containing putative CUX1 binding sites from the HCMV MIE regulatory region (CMV 1, CMV 2 and CMV 4) or these oligonucleotides with a 2 base pair swap within the putative CUX1 binding site (CMV 1 Mut, CMV 2 Mut and CMV 4 Mut). Positive controls were oligonucleotides with consensus human CUX1 binding sequences (POS 1 and POS 2) and negative controls were reactions with no addition of oligonucleotides (No oligo), reactions using an HCMV MIE-region oligonucleotide containing no CUX1 binding site (Neg) and reactions where nuclear extracts were omitted. The average absorbance readings at 450 nm from four independent replicate experiments are shown. Significant differences to no extract controls were determined using a 1-tailed, paired Students t-test and are indicated as follows: *P<0.05. Error bars are ±standard error of the mean (SEM).
Fig. 3.
Binding of CUX1 to sites within the MIE regulatory region in the presence of competitor binding sequences. Graphs depicting the binding activity of doublestranded oligonucleotide sequences of the putative CUX1 binding sites (A) CMV 1, (B) CMV 2 and (C) CMV 4 (10 pmol of each) incubated with unlabelled specific competitors at increasing concentrations and unlabelled non-specific competitors using a colorimetric transcription factor binding assay. Non-specific competitors were an unlabelled HCMV MIE-region oligonucleotide containing no CUX1 binding sites (Neg) at a concentration of 100 pmol, and 100 pmol of an
unlabelled mutant oligonucleotide with an A–T base swap within the putative CUX1 binding site in each of the 3 oligonucleotides (CMV 1 Mut, CMV 2 Mut and CMV 4 Mut). Negative control was a reaction where extract was omitted (No Extract). The mean absorbance readings at 450 nm normalised to reactions where no competitor was added from three to five independent experiments are shown. Significant differences were determined using a 1-tailed, paired Students t-test and are indicated as follows: *P<0.05, **P<0.005, ***P<0.0005, ****P<0.00005, *****P<0.000005. Error bars are ±SEM.
Fig. 4.
Overexpression of CUX1 represses transcription driven by the HCMV MIE regulatory region. (A) Western blot of nuclei extracted from untransfected HEK293 cells, or HEK293 cells transfected with either a CUX1 expression construct (CUX1) or its parent vector (pMT). Nuclear proteins were separated by SDS-PAGE and transferred to PVDF membrane before incubation with an anti-CUX1 antibody and binding visualised with ECL. The membrane was subsequently stripped of bound antibody and re-probed for the housekeeping gene GAPDH to assess any differences in loading between wells. (B) HEK293 cells transfected with a luciferase expression construct driven by a 2.1 kb region of the HCMV genome containing the MIE regulatory region (pMIEP) or a mutant construct whereby the three CUX1 binding sequences containing the core motif ATCGAT were mutated to TACGAT (pMIEPmut), were co-transfected with either a CUX1 expression construct (CUX1) or parental construct (pMT). Cells were also transfected with a β-galactosidase expressing vector under the control of the SV40 promoter, which was used to normalise between treatments for differences in transfection efficiency and endogenous differences in global transcription. Luciferase expression levels are shown relative to parental vector-transfected cells from 3 independent replicate experiments. Significant differences were
determined using a 1-tailed, paired Students t-test and are indicated as follows: *$P<0.05$, **$P<0.005$. Error bars are ±SEM.
Fig. 5.
CUX1 dose-dependent repression of MIE regulatory region-driven gene expression.
HEK293 cells were transfected with a luciferase expression construct driven by 2.1 kb of the
HCMV MIE regulatory region, along with varying amounts of the CUX1 expression vector
or its parent vector pMT. Cells were also transfected with a β-galactosidase expression
vector to normalise between treatments for differences in transfection efficiency and
endogenous differences in global transcription. Mean luciferase values (±SEM) are shown
relative to parental vector-transfected cells from 3 independent replicate experiments.
*P<0.05, one factor ANOVA.
Fig. 6.
Infection of HEK293 cells with HCMV IE2–GFP-tagged virus RC2940. Flow cytometric analysis of HEK293 cells either mock-infected or RC2940-infected (MOI=3) at 2 h and 48 h post-infection. The percentage of GFP positive cells at each time point after infection is shown.
Fig. 7.
Overexpression of CUX1 inhibits ie1 and ie2 transcription during HCMV infection.
HEK293 cells were transfected with a CUX1 expression construct, or its parent vector pMT, and infected 48 h later with HCMV (MOI=3). At 6, 24 and 72 h post-infection, RNA extracted from cells was subjected to quantitative RT-PCR for either (A) CUX1, (B) ie1 or (C) ie2 transcripts. Data were normalised to the mRNA expression by the housekeeping genes GAPDH and LDHA. Expressions of CUX1, ie1 and ie2 transcripts are shown relative to parental vector-transfected cells from 4 independent replicate experiments. Significant differences to pMT control were determined using a 1-tailed, paired Students t-test and are indicated as follows: *P<0.05, **P<0.005. Error bars are ±SEM.
Fig. 8. Association of CUX1 with the HCMV MIE region during viral infection by ChIP assay. HEK293 cells were transfected with a CUX1 expression vector for 48 h before being exposed to HCMV for 24 h. Cell lysates were analysed by ChIP assay with control rabbit IgG (Isotype) and anti-CUX1 antibody (α-CUX1), followed by PCR using HCMV MIE-region primers encompassing the CUX1 binding sites within sequences CMV 1, CMV 2 and CMV 4. A PCR control was cell lysate with no antibody selection (Input). A 100 bp DNA ladder is shown (Marker).