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Cellular homeoproteins, SATB1 and CDP, bind to the unique region between the human cytomegalovirus UL127 and major immediate-early genes

Jialing Lee [a], Zachary Klase [b], Xiaoqi Gao [c], Jeremy S. Caldwell [c], Mark F. Stinski [d], Fatah Kashanchi [b], Sheng-Hao Chao [a,*]

[a] Expression Engineering Group, Bioprocessing Technology Institute, 20 Biopolis Way, #06-01 Centros, Singapore 138668, Singapore
[b] Department of Biochemistry and Molecular Biology, The George Washington University School of Medicine, Washington, DC 20037, USA
[c] Genomics Institute of the Novartis Research Foundation, 10675 John Jay Hopkins Drive, San Diego, CA 92121, USA
[d] Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA

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Abstract

An AT-rich region of the human cytomegalovirus (CMV) genome between the UL127 open reading frame and the major immediate-early (MIE) enhancer is referred to as the unique region (UR). It has been shown that the UR represses activation of transcription from the UL127 promoter and functions as a boundary between the divergent UL127 and MIE genes during human CMV infection [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 (6), 2826-2839; 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 (11), 9039–9052]. A putative forkhead box-like (FOX-like) site, AAATCAATATT, was identified in the UR and found to play a key role in repression of the UL127 promoter in recombinant virus-infected cells [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 (10), 5113–5123]. However, the cellular factors which associate with the UR and FOX-like region remain to be determined. We reported previously that pancreatic-duodenal homeobox factor-1 (PDX1) bound to a 45-bp element located within the UR [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 (16), 16111–16120]. Here we demonstrate that two additional cellular homeoproteins, special AT-rich sequence binding protein 1 (SATB1) and CCAAT displacement protein (CDP), bind to the human CMV UR *in vitro* and *in vivo*. Furthermore, CDP is identified as a FOX-like binding protein and a repressor of the UL127 promoter, while SATB1 has no effect on UL127 expression. Since CDP is known as a transcription repressor and a nuclear matrix-associated region binding protein, CDP may have a role in the regulation of human CMV transcription.

Keywords: Cytomegalovirus; Homeobox; MAR; PDX1; SATB1; CDP

Introduction

The human cytomegalovirus (CMV) major immediate early (MIE) promoter contains a very strong enhancer between −50 and −550 relative to the transcription start site of +1 with multiple binding sites for transcription factors such as NF-κB/rel, CREB/ATF, AP1, YY1, SP-1, RAR-RXR, ELK-1, and serum response factor (Meier and Stinski, 1996). 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) (Meier and Stinski, 1996). The UR represses expression of UL127 (Angulo et al., 2000; Lashmit et al., 2004; Lundquist et al., 1999). We and others reported that the UR functions as a boundary domain or an insulator, conferring repression on homologous as well as heterologous promoters (Angulo et al., 2000; Lashmit et al., 2004; Lundquist et al., 1999). Evidence suggests that the repression involves an

* Corresponding author. Fax: +65 6478 9561.
E-mail address: jimmy_chao@bti.a-star.edu.sg (S.-H. Chao).

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interaction between the UR and unknown cellular factors. DNase I protection assays using nuclear extracts prepared from human cells demonstrated multiple interactions between unknown cellular proteins and the UR (Ghazal et al., 1990). Putative cellular protein binding sites, such as those of nuclear factor 1 (NF-1), forkhead box-like (FOX), CCAAT displacement protein (CDP), and suppressor of Hairy-wing [Su(Hw)], were identified in the UR (Ghazal et al., 1990; Lashmit et al., 2004). Mutation of the putative NF-1, CDP, and Su(Hw) sites had no effect on the repressor. In contrast, mutation of the FOX-like site significantly reduced the repressive activity of the UR (Lashmit et al., 2004). However, the identity and function of the UR-associated cellular factors remain largely unknown.

Homeobox genes are developmental control genes encoding nuclear transcription factors that regulate morphogenesis and cell differentiation in multicellular organisms (Affolter et al., 1990; Gehring, 1992; Scott et al., 1989). The homeobox sequence encodes a 60-amino acid domain, the homeodomain, responsible for DNA binding and regulating gene expression (Gehring, 1987; Scott et al., 1989; Treisman et al., 1992). Besides regulating the transcription of cellular genes, a number of cellular homeoproteins regulate viral gene expression. These include pre B-cell leukemia transcription factor 1 and PBX-regulating protein-1 for Moloney murine leukemia virus (Chao et al., 2003), OCT-1 for herpes simplex virus (Pomerantz et al., 1992; Preston et al., 1988; Stern et al., 1989), Brn-3.0, Brn-3.2, and CDP for human papillomavirus (HPV) (Ai et al., 1999; Morris et al., 1994; Ndisdang et al., 1998; O'Connor et al., 2000), CDP and special AT-rich sequence binding protein 1 (SATB1) for mouse mammary tumor virus (MMTV) (Liu et al., 1997, 1999; Pattison et al., 1997; Zhu et al., 2000; Zhu and Dudley, 2002).

It has been shown that SATB1 and CDP negatively regulate several viral and cellular genes through interaction with the histone deacetylases and histone lysine methyltransferase (Nishio and Walsh, 2004; Li et al., 1999; Snyder et al., 2001; Yasui et al., 2002). In addition, both SATB1 and CDP are also known as nuclear matrix-associated region (MAR) binding proteins (Banan et al., 1997; Liu et al., 1997). MARs are defined as AT-rich DNA sequences that are preferentially retained by the nuclear matrix and are frequently located near enhancer and promoter regions of genes (Cockerill and Garrard, 1986; Gasser and Laemmli, 1986; Webb et al., 1991). It has been demonstrated that MARs can function as insulator elements or boundary domains *in vivo* and can protect genes within a chromatin domain from stimulatory or repressive effects of flanking genomic regions (Antes et al., 2001; Bode et al., 2000; Namciu et al., 1998). Furthermore, Mi-2 protein, which associates with SATB1 *in vivo*, has been shown to regulate the activity of insulators (Hirose et al., 2002; Yasui et al., 2002). Therefore, SATB1 and CDP can play an important role in viral transcription by repressing the gene expression or binding to a boundary domain.

In a previous study, we identified a cellular homeoprotein, pancreatic-duodenal homeobox factor-1 (PDX1), that binds between nucleotides −593 and −549 located in the UR (Chao et al., 2004). Electrophoretic mobility shift assays (EMSAs) using nuclear extracts from 293 cells detected additional cellular DNA-binding proteins associated with the UR (Chao et al., 2004). To better understand the mechanism of the UR-mediated repression and insulation, it is important to identify the cellular proteins associated with the UR. Here we report that two cellular MAR binding proteins, SATB1 and CDP, interact with the UR of the human CMV *in vitro* and *in vivo*. CDP is also identified as the cellular factor associated with the putative FOX-like site, which is required for repression of the UL127 promoter (Lashmit et al., 2004). We show that CDP, but not SATB1 or PDX1, represses the UL127-dependent transcription in 293 and 293T cells. Collectively, our study demonstrates that SATB1 and CDP bind to the boundary domain between human CMV MIE and UL127 genes and identifies CDP as a cellular repressor which regulates transcription of the UL127 gene.

Results

SATB1 and CDP associate with the UR

CDP, a cellular homeoprotein as well as a MAR-binding protein, binds to the HPV silencing element and blocks HPV transcription (O'Connor et al., 2000). In addition, CDP co-operates with another MAR-binding homeoprotein, SATB1, and represses transcription from the MMTV LTR (Liu et al., 1999). We previously identified a putative HPV silencing motif between −591 and −584 located within the human CMV UR (Lundquist et al., 1999). To determine if SATB1 and CDP bind to the UR, EMSAs were carried out using nuclear extracts prepared from 293 cells and the human CMV −593 to −549 DNA probe (Fig. 1). As shown in Fig. 2A, two specific DNA–protein complexes, C1 and C2, were detected. Incubation of nuclear extracts with antibodies raised against PDX1 and CDP proteins either supershifted or prevented the formation of the C1 complex with no effect on the C2 complex, respectively (Fig. 2A). No C2 complexes were detected with anti-SATB1 antibodies (Fig. 2A). Anti-SABT1 antibodies also caused a slight shift on C1 complexes. Incubation with anti-Brn-3.0 or anti-SP1 antibodies showed no detectable effects (Fig. 2A). These results indicate that SATB1 and CDP bind to the 45-bp region between −593 and −549 of the UR.

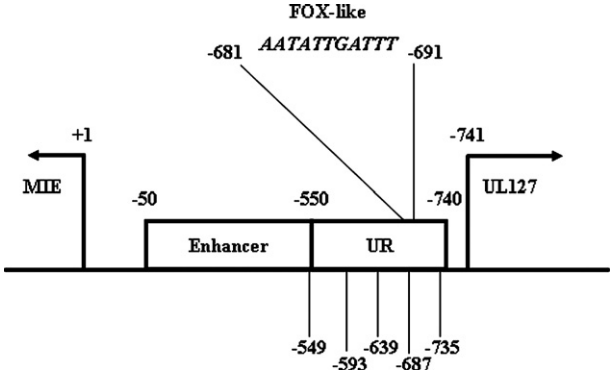

Fig. 1. Diagram of the human CMV MIE promoter containing the upstream UR. Locations of the enhancer (−50 to −550), UR (−550 to −740), FOX-like site (−681 to −691), and the transcription start sites of MIE (+1) and UL127 (−741) are indicated.

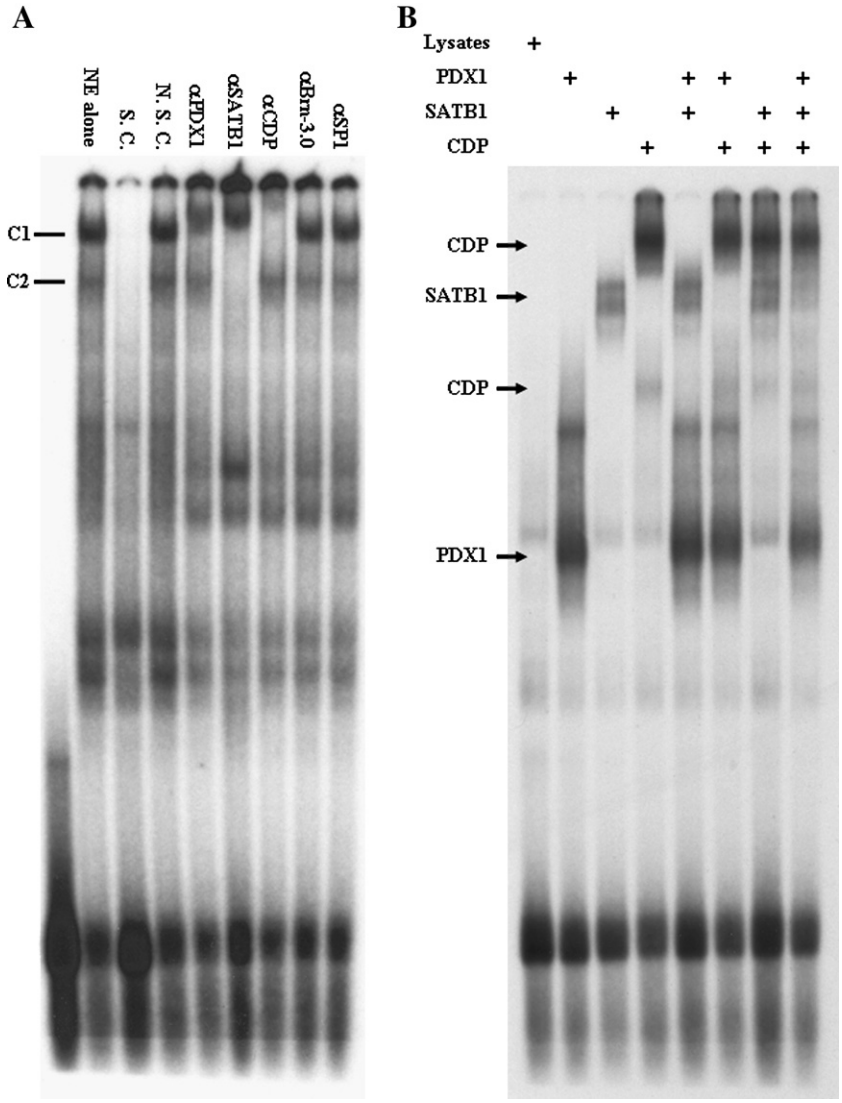

Fig. 2. SATB1 and CDP associate with the 45-bp PDX1-binding region. (A) EMSAs were performed using nuclear extracts (NE) of 293 cells and the radiolabeled CMV −593 to −549 DNA probe. "C1" and "C2" denote the specific DNA–protein complexes formed in the presence of the human CMV DNA probe and the nuclear extracts. While the unlabeled specific competitor (i.e. unlabeled −593 to −549 oligonucleotide; S.C.) was included, both DNA–protein complexes were disrupted. No effects on C1 or C2 were observed with unlabeled nonspecific competitor (i.e. unlabeled SP1 consensus oligonucleotide; N.S.C.). Nuclear extracts of 293 cells were incubated with antibodies against PDX1, CDP, SATB1, Brn-3.0 or SP1 prior to addition of the DNA probe. The anti-SP1 antibody was used as a negative control. (B) EMSAs were carried out using the CMV −593 to −549 probe and *in vitro* translated PDX1, SATB1, and CDP proteins produced by a coupled reticulocyte lysate system. The first lane contains reticulocyte lysates alone and does not include an expression construct, thus demonstrating the binding of endogenous complexes in lysates. The arrows indicate the binding of PDX1, SATB1, and CDP proteins.

To confirm the above results, EMSAs were carried out using *in vitro* synthesized SATB1 and CDP proteins and the CMV −593 to −549 DNA probe (Fig. 2B). Similar to the positive control PDX1, SATB1 and CDP were associated with the 45-bp region. Two DNA–protein complexes were observed in the reactions containing *in vitro* synthesized SATB1 (Fig. 2B). Since SATB1 can form a homodimer (Galande et al., 2001), it is possible that SATB1 binds to the 45-bp DNA as a monomer and homodimer. We also detected two specific DNA–CDP complexes (Fig. 2B). The presence of three CDP isoforms, p200, p110, and p75, has been reported (Moon et al., 2001; Goulet et al., 2002). Judging from the migrated positions of the DNA–protein complexes, this result suggested that p200 and p75 CDP isoforms bound to the 45-bp element, while the p200 isoform displayed stronger DNA binding activity (Fig. 2B).

EMSAs were performed with combinations of PDX1, SATB1, and CDP proteins. PDX1, CDP, and SATB1 bind independently to the UR and not necessarily as a heterodimer or heterotrimer (Fig. 2B). However, the presence of both PDX1 and CDP lessened binding of SATB1 (Fig. 2B, last lane), suggesting that PDX1-CDP might compete with SATB1 for the same DNA binding sites.

PDX1, SATB1, and CDP bind to multiple sites within the UR

The 45-bp region between −593 and −549 has a high AT content (73%; Fig. 1). Likewise, the rest of the UR is also AT-

rich (consisting of a 68% AT content). Since PDX1, SATB1, and CDP proteins tend to bind to AT-rich regions, an association between the UR and the three proteins was further investigated. The UR was divided equally into 4 fragments (i.e. −735 to −688, −687 to −640, −639 to −594, and −593 to −549) for DNA probes in EMSAs with *in vitro* synthesized protein (Fig. 1). In addition to the −593 to −549 region, all three proteins bound to −735 to −688 but with a lower apparent affinity (Fig. 3). CDP also associated with the CMV −687 to −640 element whereas none of these proteins bound to −639 to −594 (Fig. 3).

To confirm the binding between PDX1, SATB1, CDP, and the UR, EMSAs were carried out using nuclear extracts of 293 cells and antibodies against PDX1, SATB1, and CDP. Similar results were obtained and confirmed the association between, PDX1, SATB1, CDP, and −735 to −688, as well as the interaction between CDP and −687 to −640 (data not shown). However, none of the proteins bound to −639 to −594 which were consistent with the results presented in Fig. 3 (data not shown). Collectively, the EMSAs demonstrate that additional PDX1, SATB1, and CDP binding sites are located in the UR of human CMV MIE promoter.

PDX1, SATB1, and CDP bind to the UR in vivo

The EMSAs demonstrated *in vitro* interactions between PDX1, SATB1, CDP, and the UR (Figs. 2 and 3). We used a

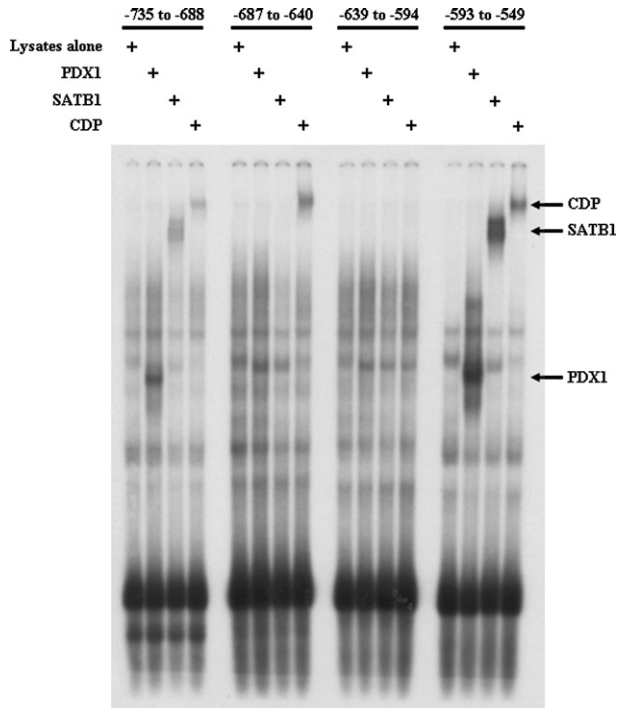

Fig. 3. *In vitro* synthesized PDX1, SATB1, and CDP proteins bind to multiple sites located within the UR of human CMV. The UR of human was divided into four segments extending from position −735 to −688, −687 to −640, −639 to −594, and −593 to −549 (see Fig. 1). EMSAs were performed by incubating the *in vitro* synthesized PDX1, SATB1, and CDP proteins, and the indicated CMV DNA probe. The lanes containing reticulocyte lysates alone, which do not include any expression plasmid, served as negative controls. The arrows indicate the binding of PDX1, SATB1, and CDP proteins.

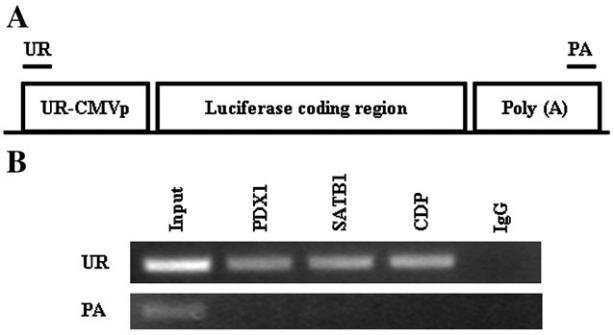

Fig. 4. Association between PDX1, SATB1, CDP, and the UR *in vivo*. (A) Schematic representation of the CMV-F-Luc reporter gene integrated in 293-CMV-F-Luc stable cells. Locations of the human CMV MIE promoter (CMVp), the coding region of firefly luciferase, the SV40 poly (A) signal [Poly (A)], and two primer sets (i.e. UR and PA) for ChIP assays are indicated. (B) Cell lysates prepared from 293-CMV-F-Luc cells were analyzed by ChIP with the control mouse IgG (IgG), anti-PDX1, anti-SATB1, or anti-CDP antibodies as described in Materials and methods.

CMV-F-Luc integrated cell line, 293-CMV-F-Luc (Fig. 4A) for chromatin immunoprecipitation (ChIP) assay of proteins bound to the UR. Cross-linked chromatin fragments were immunoprecipitated with anti-PDX1, -SATB1, or -CDP antibodies. Normal mouse IgG was a negative control. All three proteins were detected to associate with the UR (Fig. 4B). None of the proteins bound to the SV40 poly(A) region, which was about ~3 kb downstream of the UR (Figs. 4A and B). We conclude that PDX1, SATB1, and CDP associate with the UR of human CMV *in vitro* and *in vivo* (Figs. 2–4).

PDX1 and CDP bind to the FOX-like site located within the UR

A putative FOX-like site, AATATTGATTT, located between −681 and −691 (Fig. 1), was identified as a repressive element in the UR and found to be essential in repression of the UL127 promoter in recombinant virus-infected cells (Lashmit et al., 2004). Two members of FOX protein family, FOXa1 and FOXa2, were unable to associate with the putative site (Lashmit et al., 2004). Up to date, the identity of the cellular protein(s) binding to the site has not been determined. Since our results demonstrated the association between PDX1, SATB1, CDP, and the UR *in vitro* and *in vivo* (Figs. 2–4), we next examined if any of the three homeoproteins could bind to the FOX-like element.

EMSAs were carried out using the DNA probes containing three repeats of wild-type or mutant FOX-like sites [i.e. Fox(wt) and Fox¯a, respectively] as described previously (Lashmit et al., 2004). Three specific DNA–protein complexes (indicated as D1–3; Fig. 5A) were detected when the Fox(wt) probe was incubated with the nuclear extracts prepared from HeLa cells. None of the three specific complexes were formed when Fox¯a was used as the probe (Fig. 5A). To identify the cellular proteins present in the DNA–protein complexes, a specific antibody was incubated with the nuclear extracts and DNA probe. The positions of D1 complexes were slightly supershifted when anti-PDX1 and anti-SATB1 antibodies were included in the reactions, while incubation with anti-CDP antibody prevented

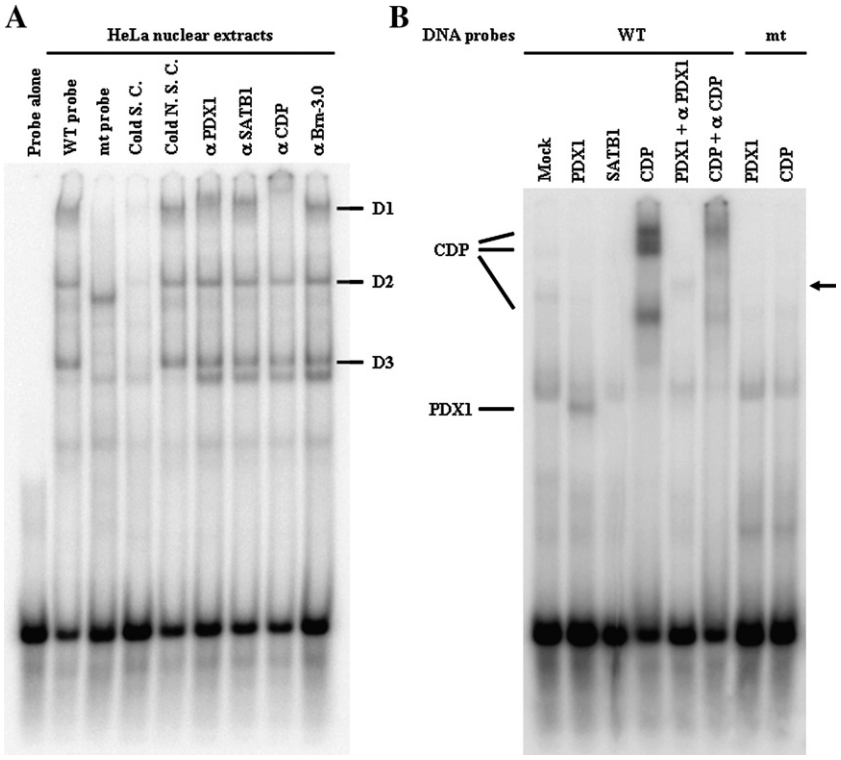

Fig. 5. PDX1 and CDP bind to the FOX-like site. (A) EMSAs were performed using the nuclear extracts prepared from HeLa cells. Fox(wt) and Fox¯a (WT and mt, respectively) were used as the DNA probes. Three specific DNA–protein complexes, "D1", "D2", and "D3", were detected when the Fox(wt) probe was included in the reaction. Incubation of the unlabeled specific competitor (S.C.) and nonspecific competitor (N.S.C.) confirmed the specificity of the three DNA–protein complexes. Nuclear extracts of HeLa cells were incubated with antibodies against PDX1, CDP, SATB1, or Brn-3.0 prior to addition of the DNA probe. The anti-Brn-3.0 antibody was used as a negative control. (B) EMSAs were performed using *in vitro* translated PDX1, SATB1, and CDP proteins (produced by a coupled reticulocyte lysate system) and a FOX-like DNA probe (WT or mt). The first lane contains reticulocyte lysates alone and does not include an expression construct. The binding of PDX1 and CDP proteins is indicated. Incubation of anti-PDX1 antibodies caused a supershift on the PDX1–Fox(wt) complex (indicated by an arrow), while inclusion of anti-CDP antibodies inhibited the formation of all three CDP–Fox(wt) complexes.

the formation of D1 complexes (Fig. 5A). No effects on the D1 complex were observed when incubated with anti-Brn-3.0 antibody (Fig. 5A). Similar results were obtained from an identical set of EMSAs using the nuclear extracts prepared from 293 cells (data not shown).

EMSAs using *in vitro* synthesized PDX1, SATB1, and CDP proteins were carried out to confirm the interaction between the proteins and FOX-like site. PDX1 as well as all three isoforms of CDP bound to the Fox(wt) DNA, but not to the Fox¯a probes (Fig. 5B). Stronger binding between CDP proteins and Fox(wt) was observed (Fig. 5B). Incubation of the anti-PDX1 antibody led to a supershift on the PDX1–Fox(wt) complex (indicated by an arrow; Fig. 5B), while the formation of the specific CDP–DNA complexes was inhibited when the anti-CDP antibody was included (Fig. 5B). In contrast, no association between Fox (wt) and STAB1 was detected (Fig. 5B). Collectively, PDX1 and CDP, but not SATB1, were identified as the cellular binding proteins for the putative FOX-like site.

CDP represses UL127-dependent transcription

Functional importance of the interaction between PDX1, CDP, and the FOX-like site was investigated next. 293 cells were transiently co-transfected with the UL127-firefly luciferase (UL127-F-Luc) reporter and an expression plasmid to over-express PDX1, SATB1, or CDP. Since SATB1 does not associate with the FOX-like element (Fig. 5), SATB1 should not affect UL127 expression and therefore can be used as a negative control for this experiment. As expected, SATB1 did not cause any detectable effects on UL127-driven transcription (Fig. 6A). A 43% decrease in luciferase activity was observed when CDP was overexpressed in 293 cells (Fig. 6A). In contrast, over-expression of PDX1 did not lead to any significant effects (Fig. 6A). An identical set of cell-based assays was performed using 293T cells. Overexpression of CDP resulted in a 74% decrease in UL127-dependent transcription while the expression of luciferase remained unchanged when cells were transfected with the PDX1 or SATB1 plasmids (Fig. 6A). The results indicate that CDP negatively regulates the transcription driven by the human CMV UL127 promoter.

Overexpression studies were complemented with gene knockdown experiments to address the influence of CDP on the UL127 transcription events. We obtained 11 siRNAs against CDP and evaluated their effects by quantitative real-time polymerase chain reaction. Only 3 out of 11 siRNAs showed a greater than 2-fold decrease in the mRNA levels of CDP (data not shown). Two most efficient siRNAs, CDP-P1 and CDP-P2, which caused 2.14- and 3.5-fold decreases in CDP mRNAs respectively, were chosen for the cell-based assays. Three off-target CDP siRNAs (i.e. CDP-N1, CDP-N2, and CDP-N3),

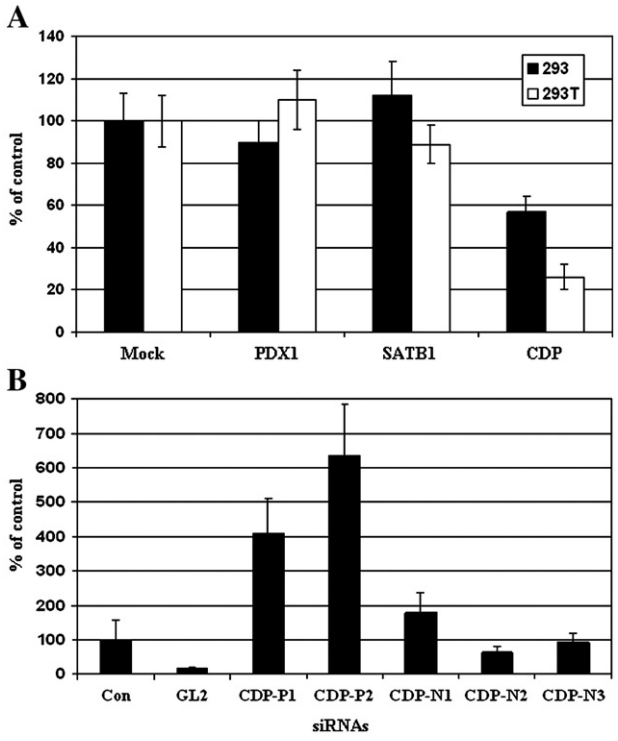

Fig. 6. CDP represses UL127-dependent transcription. (A) 293 and 293T cells were transiently transfected with UL127-F-Luc and an indicated expression plasmid (pUB mock, pUB-PDX1, pUB-SATB1, or pUB-CDP plasmids). RSV-R-Luc served as an internal control plasmid. The activity of *Renilla* and firefly luciferases was determined by Dual-Glo assays 2 days post-transfection. (B) 293T cells were transiently transfected with UL127-F-Luc and an indicated siRNA. RSV-R-Luc was used to normalize transfection efficiency. The activity of *Renilla* and firefly luciferases was measured by Dual-Glo assays 3 days post-transfection.

which had no significant effects on CDP mRNA levels, were also included. 293T cells were transiently transfected with UL127-F-Luc and a siRNA. The siRNA targeting F-Luc (i.e. GL2 siRNA) was used as a positive control (Fig. 6B). Treatment with the CDP-P1 and CDP-P2 siRNAs resulted in up to 4- to 6-fold increases in luciferase activity, while the off-target controls showed little or no effects on UL127 expression (Fig. 6B). The siRNA assays further confirm that CDP plays a role in repressing the UL127 promoter.

Effects of SATB1 and CDP on CMV MIE expression were also investigated. 293T cells were transiently transfected with a CMV MIE reporter and an indicated expression vector. As reported previously, PDX1 significantly repressed luciferase expression driven by the MIE promoter (Fig. 7) (Chao et al., 2004). However, overexpression of SATB1 and CDP had no detectable effects on the MIE-dependent transcription (Fig. 7). This result demonstrates that CDP exhibits specific repression on the UL127 promoter.

Discussion

The human CMV UR represses expression from the UL127 promoter and functions as a boundary domain by blocking activation from the MIE enhancer (Angulo et al., 2000; Lashmit et al., 2004; Lundquist et al., 1999). UR-mediated repression involves an interaction with host-encoded regulatory factors. Unknown cellular nuclear proteins that bind to the UR upstream of the human CMV MIE promoter were detected 17 years ago (Ghazal et al., 1990). A putative FOX-like site, located within the UR (Fig. 1), was found to be essential in repression of the UL127 promoter (Lashmit et al., 2004). However, little is known about the identity of the UR- and FOX-like-associated cellular factors and their regulatory function. We identified a cellular homeoprotein, PDX1, which associated with a specific 45-bp element located in the UR between nucleotides −593 and −549 (Chao et al., 2004). In this study, we report that two other cellular homeoproteins, SATB1 and CDP, bind to the UR. Furthermore, CDP is identified as a FOX-like binding protein and functions as a repressor of UL127. However, SATB1 does not demonstrate any significant effect on the UL127 promoter.

EMSA-supershifts indicated that CDP was present in the −593 to −549 DNA–protein complexes (Fig. 2A, C1 complexes). The results also suggested that SATB1 was the only protein component in C2 complexes; however, it was not clear if SATB1 was also complexed with PDX1 and CDP (Fig. 2A). *In vitro* synthesized PDX1, SATB1, and CDP were used in gel shift assays. While these proteins associated with the UR, no heterotrimers or heterodimers bound to the UR (Fig. 2B). Posttranslational modifications may be required for the formation of oligomers and such modifications may be absent in the *in vitro* translated proteins. Another possibility is that there may be additional cellular proteins present in the C1 complexes and these unknown proteins may be required for the formation of C1 complexes.

The UR of human CMV blocks the MIE enhancer from having an effect on the transcription of the UL127 gene during productive infection (Angulo et al., 2000; Lashmit et al., 2004; Lundquist et al., 1999). The repressive function of the UR may explain how the human CMV MIE enhancer can selectively activate expression of the MIE genes but not the UL127 gene during productive infection. MARs can function as boundary domains or insulator elements *in vivo* (Namciu et al., 1998). SATB1 and CDP homeoproteins are also known as MAR-

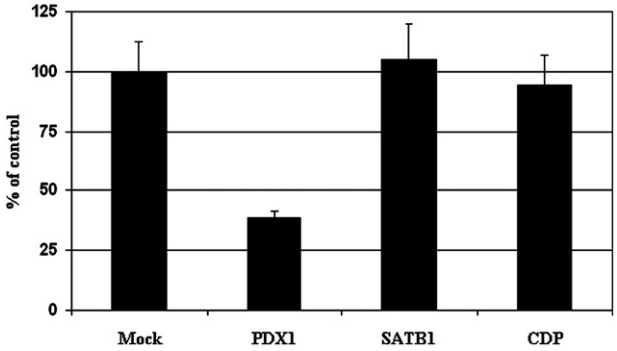

Fig. 7. Effects of SATB1 and CDP on the CMV MIE-dependent transcription. 293T cells were transiently transfected with CMV-R-Luc, pUB-F-Luc, and an indicated expression plasmid (pUB mock, pUB-PDX1, pUB-SATB1, or pUB-CDP plasmids). pUB-PDX1 served as a positive control while pUB-F-Luc was used to normalize transfection efficiency. The activity of *Renilla* and firefly luciferases was determined by Dual-Glo assays 2 days post-transfection.

binding proteins, suggesting that both proteins may be important for regulating the UR activity (Banan et al., 1997; Liu et al., 1997). It has been reported that MARs can function as insulator elements or boundary domains *in vivo* (Antes et al., 2001; Bode et al., 2000; Namciu et al., 1998). In addition, MARs are frequently located near enhancer and promoter regions of genes (Cockerill and Garrard, 1986; Gasser and Laemmli, 1986; Webb et al., 1991). The association between the MAR-binding proteins SATB1, CDP, and the UR as well as the genomic localization of the UR (next to the MIE enhancer and promoter) suggest that the UR may indeed function as a boundary domain.

We demonstrate the interaction between PDX1, CDP, and the putative FOX-like region. EMSAs indicate that CDP is a FOX-like binding protein. Using the *in vitro* synthesized CDP, all three CDP isoforms show similar DNA binding affinity to the FOX-like site (Fig. 5). CDP overexpression and knock-down assays demonstrate the repressive function of CDP (Fig. 6). An up to 6-fold increase in luciferase expression (driven by the UL127 promoter) was observed when cells were transfected with the CDP siRNA (Fig. 6B). Previously, 14- to 31-fold increases in UL127-dependent transcription were reported when the putative FOX-like site was deleted or mutated (Lashmit et al., 2004). Therefore, we further examined the effects of the CDP-P2 siRNA (which was used in this study) on the protein levels of CDP in the cells. Treatment of the CDP-P2 siRNA resulted in 50% and 20% decreases in p110 and p75 CDP isoforms, respectively, while no inhibitory effects on p200 CDP isoforms were detected (data not shown). Since the siRNA fails to completely knock down the protein synthesis of CDP, a modest effect on the relief of UL127 repression would be expected. However, we can not rule out the possibility that additional cellular factors may be involved in the repression. In contrast, no association between SATB1 and the putative FOX-like site was detected in EMASs (Fig. 5). In agreement with the results of EMSAs, we could not detect any functional involvement of SATB1 in UL127-mediated expression in the cell-based assays (Fig. 6A).

A putative CDP binding site was identified in the UR. We previously showed the mutation of the CDP site had no effect on UL127-dependent transcription (Lashmit et al., 2004). However, it remains to be determined if the putative site indeed serves as a genuine CDP binding element. Therefore, the possible involvement of CDP in UL127 regulation can not be excluded if solely based on the previous mutation study. Future experiments, such as EMSAs and ChIPs, will be required to examine the interaction between CDP and the putative CDP site in detail. Although the region located between −681 and −691 in the UR was previously referred to as a putative FOX binding site, our current results clearly demonstrated that this element functions as a CDP binding site.

Besides associating with MARs and regulating the expression of cellular genes, CDP is also involved in viral transcription. CDP negatively regulates HPV-dependent transcription by binding to specific DNA elements located in viral promoters and long control regions, thus inhibiting HPV replication (Ai et al., 1999; O'Connor et al., 2000; Pattison et al., 1997). CDP can also block the transcription of MMTV through the interaction with the negative regulatory regions located in the long terminal repeats of the virus (Liu et al., 1997; Zhu et al., 2000; Zhu and Dudley, 2002). Our results represent the first work demonstrating a possible role of CDP in transcriptional regulation of human CMV.

Collectively, identification of SATB1 and CDP as the cellular UR-binding factors provides a molecular mechanism which may help to explain why the UR of human CMV functions as a boundary element. CDP, but not SATB1, inhibits UL127 expression specifically. Neither SATB1 nor CDP show any significant effect on MIE-dependent transcription. The effects of the UR and the UR-binding proteins, PDX1, SATB1, and CDP, on the MIE promoter during latency require further investigation.

Materials and methods

Cells

HeLa, 293, and 293T cells were obtained from American Type Culture Collection. A CMV-firefly luciferase stable cell line, 293-CMV-F-Luc, was generated as described before (Chao et al., 2004).

Plasmids and plasmid construction

The CMV-CDP plasmid (i.e. pRc/CMV CDP) was kindly provided by Dr. Dudley (Zhu et al., 2000), and then subcloned into pUB6 (Invitrogen) to generate pUB-CDP, in which the expression of CDP was driven by the human ubiquitin C promoter. The coding region of CDP was subcloned into the pCITE vector (Novagen) to generate the pCITE-CDP plasmid. The SATB1 cDNA was obtained from internal clone collections and used to create pUB-SATB1 and pCITE-SATB1. The pUB-firefly luciferase (pUB-F-Luc), pUB-PDX1, pCITE-PDX1, and UL127-F-luc (i.e. pLC3+) plasmids were constructed as described previously (Chao et al., 2004; Lashmit et al., 2004). The pCITE constructs were used in *in vitro* reactions (TNT Quick Coupled Transcription/Translation Systems, Promega) to generate the *in vitro* synthesized proteins. The respiratory syncytial virus-*Renilla* luciferase (RSV-R-Luc) and CMV-R-Luc plasmids were purchased from Promega (pRL-RSV and pRL-CMV, respectively).

EMSA

Various DNA fragments corresponding to the UR of the human CMV MIE promoter were used as DNA probes for EMSAs. The CMV −593 to −549 DNA contains the 45-bp PDX1 binding elements (5′-GGCATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCA) (Chao et al., 2004), whereas CMV −735 to −688 (5′-TTGAATCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATA), CMV −687 to −640 (5′-TAAATCAATATTGGCTATTGGCCATTGCATACGTTGTATCTATATCATAATATGTACA), and CMV −639 to −594 (5′-TATATGTACATTTATATTGGCTCATGTCCAATAT-

GACCGCCATGTTGGCATTGATT) elements contain the remaining parts of the UR. The Fox(wt) and Fox⁻a DNA probes contain three repeats of wild-type and mutant FOX-like sequences, respectively (Lashmit et al., 2004). EMSAs were performed as described previously (Chao et al., 2003, 2004). Anti-PDX1, anti-SATB1, anti-CDP, anti-Brn-3.0, and anti-SP1 antibodies were purchased from Santa Cruz Biotechnology, Inc.

ChIP

ChIP assay was performed using the EZ ChIP™ kit according to the manufacturer's protocol (Upstate Biotechnology). The chromatins (which were sonicated to the DNA sizes between 200 and 1000 bp) of 2.5×10^6 293-CMV-F-Luc cells were used per immunoprecipitation with normal mouse IgG (Upstate Biotechnology), anti-PDX1, -SATB1, or -CDP antibodies. Immunoprecipitated DNA was analyzed by PCR by using specific primers to the UR (5′- ATGTACGGGCCAGATATACG-3′ and 5′-GGTCATGTACTGGGCATAAT-3′) and the SV40 poly(A) signal (5′-AATCAGCCATACCACATTTG-3′ and 5′-AGATACATTGATGAGTTTGG-3′).

Transfection and luciferase assays

293 and 293T cells were grown to 50–80% confluence in 96-well plates. Cells were transiently co-transfected with UL127-F-luc and an expression plasmid (pUB-PDX1, pUB-SATB1, or pUB-CDP). Transfection was carried out using Fugene 6 (Roche) as described in the manufacturer's manual. RSV-R-Luc was used as an internal control. Firefly and *Renilla* luciferases were measured at 48 h post-transfection using the Dual-Glo assay system (Promega). Luciferase signals were quantitated by an Infinite™ 200 microplate reader (Tecan). To examine the effects of PDX1, SATB1, and CDP overexpression on the CMV MIE promoter, 293T cells were transiently transfected with CMV-R-Luc, pUB-F-Luc (as an internal control), and an expression vector. The activity of the luciferases was determined as described above.

A set of siRNAs targeting CDP as well as the non-specific and firefly luciferase (i.e. GL2) siRNAs were purchased from Dharmacon. Two other sets of CDP siRNAs were obtained from Qiagen and Ambion. 293T cells were grown to 50–80% confluence in 96-well plates and transfected with UL127-F-Luc, RSV-R-Luc (the internal control) and a siRNA (i.e. non-specific, GL2, or CDP siRNAs). Transfection was carried out using Lipofectamine 2000 (Invitrogen) as described in the manufacturer's manual. Firefly and *Renilla* luciferases were measured at 72 h post-transfection.

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