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Full-length article

Amino acid 1–209 is essential for PDX-1-mediated repression of human CMV IE promoter activity

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Key words

CMV Immediately Early promoter; pancreatic duodenal homeobox factors-1; down regulation

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Abstract

Aim: To explore the different roles of pancreatic duodenal homeobox factors-1 (PDX-1) domains in PDX-1 mediated repression of human cytomegalovirus immediately early (CMV IE) promoter. **Methods:** A series of truncated PDX-1 mutants were constructed. The binding of PDX-1 and CMV IE promoter was identified by electrophoretic mobility shift assay (EMSA). The dual-reporter assay was applied to examine the repression activities of PDX-1 mutants on CMV IE promoter. In addition, RNAi technology was used to specifically knock down the endogenous PDX-1 expression. **Results:** The reporter assay indicated that compared to the mock controls (pEGFP-N2), overexpression of PDX-1 resulted in a 41% decrease of CMV IE promoter activity in the 293 cells ($P<0.05$) and 43% decrease in HeLa cells ($P<0.05$), and the repression levels of various truncated mutants played on CMV IE promoter were different. Specific knock down of the endogenous PDX-1 expression significantly restored the activity of CMV IE promoter. EMSA demonstrated that domain 3 is necessary for nuclear localization and DNA binding activity of PDX-1. However, binding of PDX-1 alone to CMV IE promoter was not sufficient to inhibit its transcriptional activity, and other domains of PDX-1 presented were also required. **Conclusion:** Our data suggested that the DNA binding activity of PDX-1 domain 3 and the cooperative binding of PDX-1 domain 1/2 with other proteins were required for PDX-1 mediated repression of CMV IE promoter.

Introduction

Pancreatic-duodenal homeobox factor-1 (PDX-1), also referred to as ipf-1[1], stf-1[2], and idx-1[3], is a homeodomain transcription factor with a molecular weight of 30 kDa, which was cloned by several laboratories. It plays a key role in regulating both pancreatic development and the differentiation of progenitor cells into the β-cell phenotype[4,5]. The expression of PDX-1 in the developing pancreas is maintained throughout development and provides both spatial and temporal contributions to the commitment of endoderm to a pancreatic phenotype[6]. Homozygous disruption of the PDX-1 gene results in pancreatic agenesis in mice and in humans[7–9]. Moreover, PDX-1 only expresses in pancreas islet β and δ cells in adults, which regulates the transcription of several genes that are essential for glucose-sensing and insulin production, including insulin[10,11], islet amyloid polypeptide[12,13], glucokinase[14], glucose transporter-2[15], and somatostatin[16]. The main DNA sequence that PDX-1 recognizes is TAAT, which is known as A-box.

PDX-1 protein contains four main domains. The first one is the transactivation domain (1–79 aa), which consists of three highly conserved sequences, subdomain A (13–22 aa), B (32–38 aa), and C (60–73 aa)[17]. These subdomains are required for the synergistic activation of insulin enhancer-mediated transcription by PDX-1 together with E2-5 and E47 proteins[10,18]. The second domain (80–149 aa) contains a pentapeptide motif FPWMK, which is very similar to the

Antennapedia class of the homeodomain proteins' motif YPWMK, and is absolutely essential for the cooperative binding of PDX-1 with Pbx to activate the somatostatin promoter in pancreatic cell lines[16,19]. The third domain is homeodomain (150–209 aa), which mediates the binding of PDX-1 with DNA and other proteins. It also mediates the nuclear localization of PDX-1, which is mainly due to an Antennapedia-like protein transduction domain (188–203 aa)[20,21]. However, the function of the last domain (210–282 aa) is still not clear.

Human cytomegalovirus (CMV) is a member of the β-herpes virus group, and is the viral agent most frequently associated with congenital infections in man. It has been reported that CMV infection is associated with many autoimmune diseases, such as lupus erythematosus[22], rheumatoid arthritis[23], and insulin-dependent diabetes mellitus (IDDM, type 1 diabetes)[24–26]. Expression of the human CMV immediately early (IE) gene is critical for productive viral replication. We used Patch search database (which searches for potential transcription factor binding sites in sequences with the pattern search program using TRANSFAC 6.0 public sites) to search potential transcription factor binding sites in CMV IE promoter and found many homeoprotein binding sites, including HOX, MEIS1, and PDX-1. Chao *et al*[27] have reported that PDX-1 can bind to CMV IE promoter and thus regulate its activity. They have identified a 45-bp element in CMV IE promoter, which contains multiple putative homeoprotein binding motifs, and also demonstrated the physical association between PDX-1 and the 45-bp CMV region using gel shift assays. They further determined that PDX-1 represses the CMV IE-promoter activity in HEK293 cells. However, they did not show the function that different domains of PDX-1 played in such interaction.

In this article, we investigated the roles of the domains of PDX-1 in the regulation of CMV IE promoter. Here we constructed and expressed a series of mutants lacking one or more of the four domains of wild type PDX-1 and full-length PDX-1, carrying EGFP fused to the C-terminus of the gene. Electrophoretic mobility shift assay (EMSA) results from transfected HEK293 cells showed that domain 3 is necessary and sufficient for binding to the CMV IE promoter. Luciferase assays demonstrated that full-length PDX-1 can significantly down-regulate the Human CMV IE-dependent transcription both in HEK293 and HeLa cells. We concluded that PDX-1 domain 3 was necessary for binding to CMV IE promoter, but the inhibition function required other domains to be present. The presence of PDX-1 domain 2, and 3 at the same time was essential for the negative regulation, and domain 1 could stabilize the multiprotein complex formed by PDX-1 and other proteins. So, P123 (PDX1 domain 1, 2, & 3) caused greater inhibition than that of wild type PDX-1.

Materials and methods

Cell culture and transfection HEK293, HeLa, and β-TC-6 were obtained from American Type Culture Collection; 293 and HeLa were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, San Diego, CA, USA) containing 10% NCS; and β-TC-6 were grown in high glucose DMEM containing 15% FBS. Cells were grown to 50%–80% confluence in 24-well plates. Transient transfections were performed using lipofectMINE 2000 (Invitrogen, San Diego, CA, USA) according to the manufacturer's recommendation, and the nuclear was stained by 4',6-Diamidino-2-phenylindole (DAPI, Sigma Chemical Co, St Louis, MO. USA).

Plasmid construction The plasmid pIpf-1, which contains a murine PDX-1 gene, was a generous gift from Dr S Ferber (Institute of Endocrinology, Sheba Medical Center, Tel Hashomer, Israel). The full length PDX cDNA and PDX cDNA fragments were amplified from pIpf-1. The PCR products were digested by *Mun* I (designed in the 5' primers) and *Kpn* I (designed in the 3' primers), and were subcloned into pEGFP-N2 (Clontech) in the *EcoR* I and *Kpn* I sites. The resulting vectors were named pP1234-EGFP, pP123-EGFP, pP234-EGFP, pP12-EGFP, pP23-EGFP, pP34-EGFP, pP1-EGFP, pP2-EGFP, pP3-EGFP (numbers indicate the exact domains of PDX with EGFP fused at the C-terminus). The full-length luciferase Open Reading Frame (ORF) was digested from pGL3-Basic (Promega, Madison, WI, USA), and inserted into the multiple cloning sites of pcDNA3.0(+) using the *Xho* I and *Xba* I sites to generate pCMV-Luc.

RNA interference Oligonucleotides specifically targeted 430–448 of murine PDX-1 gene (designed by Wistar Institute siRNA selector) were cloned into the *Bgl* II and *Hind* III sites of the pSuper-vector to generate pSuper-Pdx i. The synthesized oligonucleotides containing *BamH* I and *Hind* III overhangs were as follows (the sequences shown in lowercases at 5' and 3' ends were *BamH* I and *Hind* III overhangs, and the sequence in the middle was the loop of the RNAi oligonucleotides): forward, 5'-gatccccCCCGAGGAAAACAAGAGGAttcaagagaATCCTCTTGTTTTCCTCGGGttttta-3'; reverse, 5'-agcttAAAAACCCGAGGAAAACAAGAGGATtctcttgaaTCCTCTTGTTTTCCTCGGGggg-3'.

Western blotting HEK 293 cells were transfected with the indicated expression plasmids, β-TC-6 cells were transfected with pSuper-Pdx i or pSuper-null, and cells were harvested after 24 h for western blot analysis. The samples were resolved on a 10% polyacrylamide gel, transferred to

nitrocellulose, blocked with 5% (*w*/*v*) milk powder in TBST (20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 0.05% (*v*/*v*) Tween 20), incubated with primary antisera to EGFP, mPDX-1, or mGAPDH (Santa Cruz Biotechnology) at a 1:200 dilution. After washing three times in TBST, they were incubated with horseradish peroxidase-conjugated secondary antibody at a 1:1000 dilution. Finally, the bands were detected using chemiluminescence (ECL; Amersham Biosciences).

Luciferase assay A dual luciferase assay was carried out according to manufacturer's instructions (Promega). For the overexpression assays, 293 or HeLa cells were cotransfected with pCMV-Luc, the indicated expression vectors (ie, pEGFP-N2, pP1234-EGFP, pP123-EGFP, pP234-EGFP, pP12-EGFP, pP23-EGFP, pP34-EGFP, pP1-EGFP, pP2-EGFP, pP3-EGFP), and pRL-TK (Promega, to normalize for transfection).

For the RNA interference assay, β-TC-6 cells were cotransfected with pCMV-Luc, pRL-TK, and pSuper-Pdx i or not. Cells were harvested 48 h after transfection and luciferase activity were measured as relative light unit with a luminomer (Luma LB9507, EG & G Berthold, Germany). All experiments were performed in triplicate.

The means±SD of a total number of analyzed samples are indicated on the figures. The significance of the effects of various treatments as compared with untreated control was evaluated by paired Student's *t*-test at the 95% confidence level.

Electrophoretic mobility shift assay A 3' biotin labeled 55-bp double-stranded CMV sequence (-598–-543, designed under the putative binding site search results and including Chao's 45-bp CMV region) was used as the DNA probe for EMSA: 5'-GGCATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCA-biotin-3' (only the forward sequence was shown). 293 cells were grown to 50%–80% confluence in 6-well plates, and transfected with 4 μg/well of pEGFP-N2, pP1234-EGFP, pP123-EGFP, pP234-EGFP, pP12-EGFP, pP23-EGFP, pP34-EGFP, pP1-EGFP, pP2-EGFP, or pP3-EGFP. Forty-eight hours later nuclear protein extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). EMSA were performed using LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's instructions.

Putative binding site search We used the program Patch (www.gene-regulation.com, formerly known as PatSearch) to analyze the CMV IE promoter. It had one putative PDX-1 homeoprotein binding site, and several putative homeoprotein family protein binding sites.

Results

Cellular location of PDX-1 mutants First, a series of expression vectors carrying the full-length or truncated (lacking one or more domains) PDX-1, with EGFP fused at the C-terminus, were constructed. A total of nine vectors were named as pP1234-EGFP, pP234-EGFP, pP123-EGFP, pP23-EGFP, p12-EGFP, pP34-EGFP, pP1-EGFP, pP2-EGFP, pP3-EGFP (Figure 1A). HEK293 cells were transfected with different expression vectors described above and pEGFP-N2 was used as control. Cellular localization of the expressed proteins was examined using fluorescence microscopy 24 h after transfection, and the molecular weight of the series mutants were identified by Western blot (Figure 1B, 1C). Domain 3 is necessary for nuclear localization of the expressed proteins and EGFP-N2, and those mutants without domain 3 were distributed all through the cells (Figure 2).

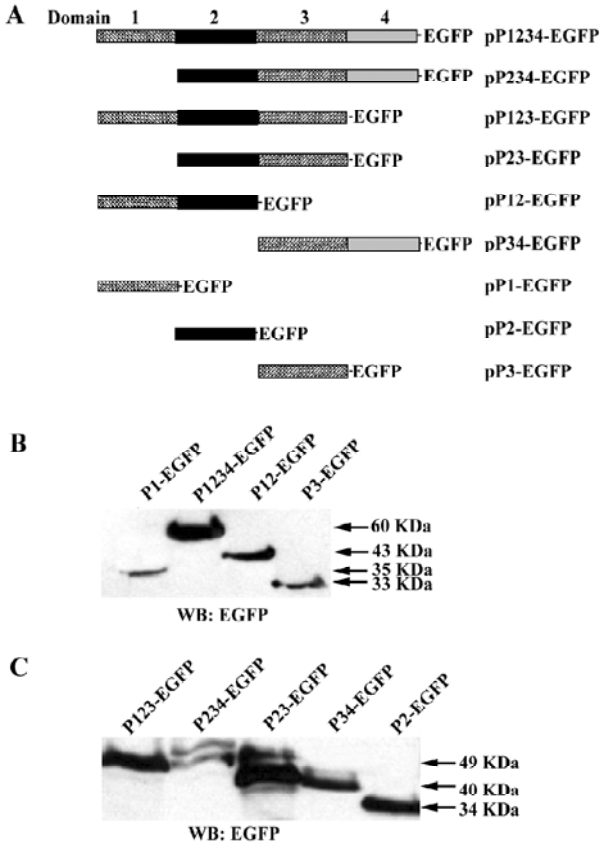
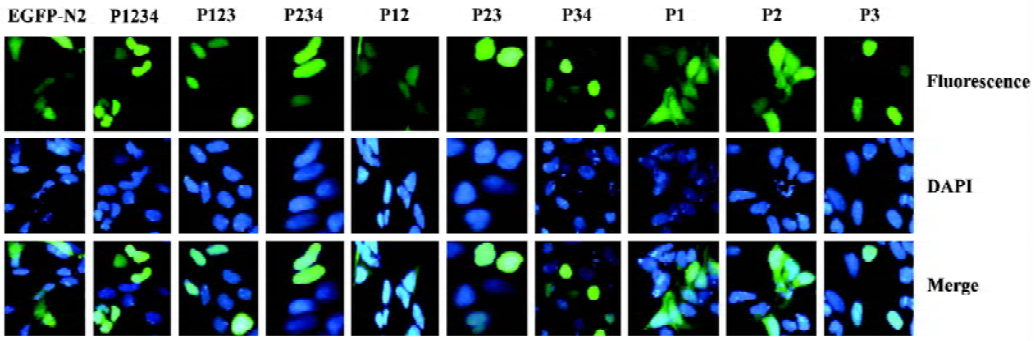


Figure 1. The full-length PDX-1 and its truncated mutants. (A) Plasmid of PDX-1 and its eight truncated mutants. Numbers mean the plasmid containing the exact domains of PDX with EGFP fused at the C-terminate. (B,C) HEK293 cells were transfected with different expression vectors and harvested after 24 h for Western blot analysis.

PDX-1 downregulated human CMV IE promoter activity in both 293 and HeLa cell Using the Patch program, we

A



B

	cyto	nuclea
EGFP-N2	+	+
P1234	-	+
P123	-	+
P234	-	+
P12	+	+
P23	-	+
P34	-	+
P1	+	+
P2	+	+
P3	-	+

Figure 2. Cellular localizations of Pdx-1 and its mutants in HEK293 cell line. HEK293 cells were transfected with different expression vectors and pEGFP-N2. Observation 24 h post-transfection demonstrated that only those containing domain 3 possessed the ability of nuclear localization, EGFP-N2 and those without domain 3 were distributed all through the cells. (A) Fluorescence micrographs of the transfection results and DAPI staining. (B) The summarized data of the cellular localizations.

found a 55-bp region (-598 to -543, which included the whole sequences of Chao's 45-bp CMV region) in the human CMV-IE promoter carrying one putative PDX-1 homeoprotein binding sites, and several other putative binding sites of homeoprotein family members, such as HOXA and MEIS1. This suggested that PDX-1 might interact with CMV IE promoter and regulate its transcription.

To study the regulation of CMV IE promoter by PDX-1, 293 and HeLa cells were co-transfected with CMV IE luciferase reporter vector (pCMV-Luc) and PDX-1 expression vector pP1234-EGFP, respectively. Compared to the mock controls (pEGFP-N2), overexpression of PDX-1 resulted in a 41% decrease of CMV promoter activity in the 293 cells ($P<0.05$) and 43% decrease in the HeLa cells ($P<0.05$)(Figure 3A).

Specific knockdown of the endogenous PDX-1 expression significantly decreased its repression on CMV IE promoter Further evidence supporting the role for PDX-1 came from experiments using RNAi techniques to abolish PDX-1 expression. PDX-1 shRNA expression vectors were generated in the pSuper-background and driven by H1 promoter (Figure 3B). As shown in Figure 3C, β-TC-6 cells were cotransfected with pCMV-Luc and pSuper-Pdx i or pSuper-null constructs and achieved 40%–50% transfection efficiency (shown by EGFP reporter vector). 24 h after transfection, a 1.6-fold ($P<0.05$) increase in pCMV-Luc reporter was observed in β-TC-6 cells in the presence of pSuper-Pdx i construct, when compared to the cells transfected with pSuper-null construct. In addition, the expression of the PDX-1 protein was significantly reduced compared to cells transfected with the empty RNAi vector (50% reduction) (Figure 3D). Because only half of the cells were transfected, we estimated the efficiency of shRNA-mediated reduction of PDX-1 might be up to 70% in transfected cells. These results suggest that it is feasible to apply RNAi technology to study the repression of PDX-1 on CMV IE promoter *in vitro*.

PDX-1 domain 3 necessary for binding to CMV IE promoter but inhibition function also required PDX-1 domain 1/2 be present To explore the potential role of PDX-1 in the regulation of CMV IE promoter, we applied EMSA to evaluate the binding status of wild type or truncated mutant PDX-1 to CMV IE promoter. First, we transfected HEK293 cells with the following expression vectors: pEGFP-N2, pP1234-EGFP, pP123-EGFP, pP234-EGFP, pP12-EGFP, pP23-EGFP, pP34-EGFP, pP1-EGFP, pP2-EGFP, and pP3-EGFP. Nuclear extracts of HEK 293 cells transfected with each of the expression vectors were then incubated with the 55-bp biotin-labeled CMV IE promoter probe. The results showed that there were major DNA-protein complexes when the CMV IE promoter probe incubated with P1234, P123, P234, P23, P34, or P3 nuclear extracts, all of which carried domain 3. However, no

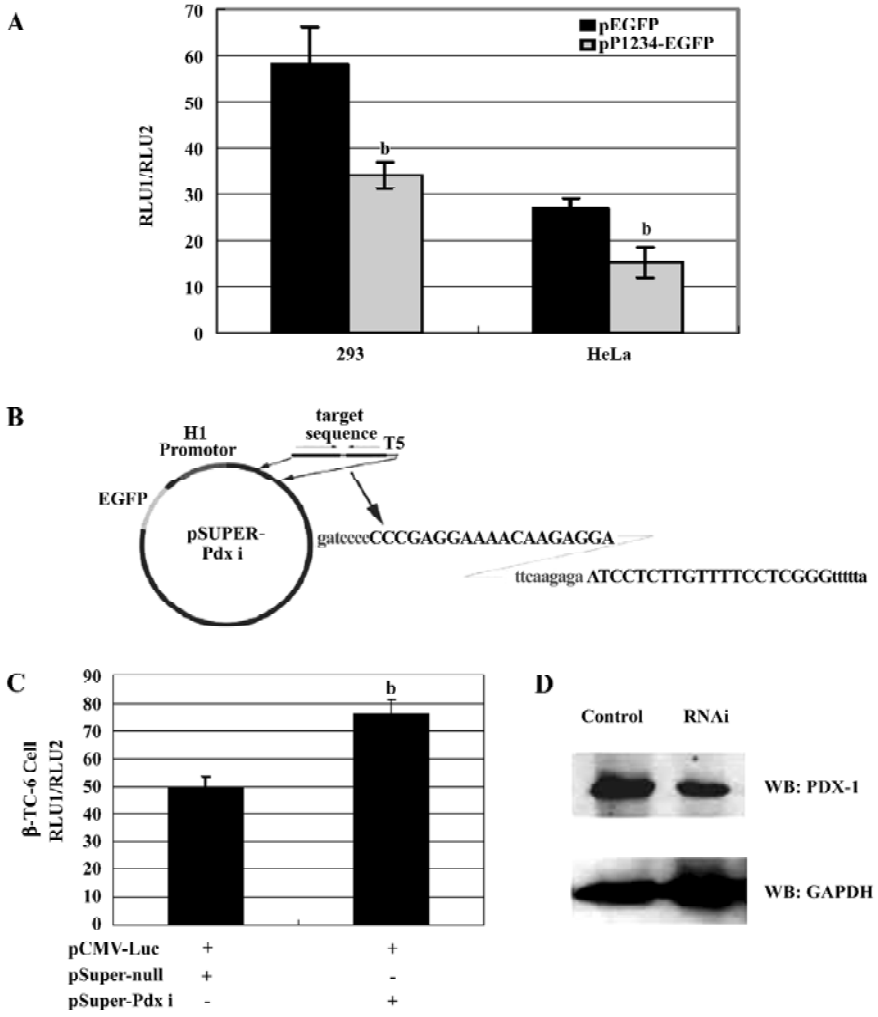


Figure 3. Overexpression and RNAi experiments indicated PDX-1 could down-regulate CMV IE promoter efficiently. (A) Both HEK293 and HeLa cells were cotransfected with pCMV-Luc and pEGFP-N2 or pP1234-EGFP. Overexpression of PDX-1 resulted in a 41.41% (P<0.05) decrease of promoter activity in 293 cell lines, and a 43.42% (P<0.05) in HeLa cell lines. (B) Schematic representation of pSuper-Pdx i. (C) β-TC-6 cells were cotransfected with pCMV-Luc and pSuper-null or pSuper-Pdx i. A 1.6-fold (P<0.05) increase in pCMV-Luc reporter was observed in β-TC-6 cells in the presence of pSuper-Pdx i construct. (D) The expression of PDX-1 in transfected β-TC 6 cells was checked by Western blot with anti-PDX-1 antibody. The same blot was also probed with antibody to GAPDH as intra control (low panel). ^bP<0.05.

obvious DNA-protein complexes could be observed when the probe was incubated with EGFP, P12, P1, or P2 nuclear extracts, in which domain 3 was absent (Figure 4). To further confirm the binding specificity of the complex to CMV IE promoter, we performed a competition assay with unlabeled CMV IE promoter probe. The competitor oligonuleotides specifically reduced the formation of the DNA-protein complex (Figure 4A, lane 5). These results indicated that the protein complex bound specifically to the CMV IE promoter. We concluded from the observations that domain 3 of the PDX-1 protein is necessary and sufficient to confer DNA binding activity.

We further investigated the regulation of CMV IE promoter by different PDX-1 mutants using luciferase reporter assay in HEK293 cells. Each construct was cotransfected with pCMV-Luc and pRL-TK. The results showed that overexpression of P123 caused a 56.70% (P<0.05) decrease in promoter activity compared with normal CMV IE promoter activity, a 38.52% (P<0.05) decrease in P234 transfection, and a 27.28% decrease (P<0.05) in P23 transfection (Figure 5). No significant effects were observed when P12, P34, P1, P2, P3 overexpressed. According to the functions of each domain, we concluded that the absence of DNA binding domain (PDX-1 domain 3) could result in the total loss of repression activities.

We noticed that PDX-1 domain 3 was sufficient for the binding activities of PDX-1 proteins, and P34, P3 showed specific DNA-protein complexes in EMSA. However, no promoter repression was observed in the transfection of P34 and P3, which suggested that binding of PDX-1 protein to the CMV promoter alone was not sufficient to inhibit CMV transcriptional activity. Furthermore, P23 caused a 27.28% decrease while P123 caused greater inhibition, which was close to wild type PDX-1. And we found that the DNA-

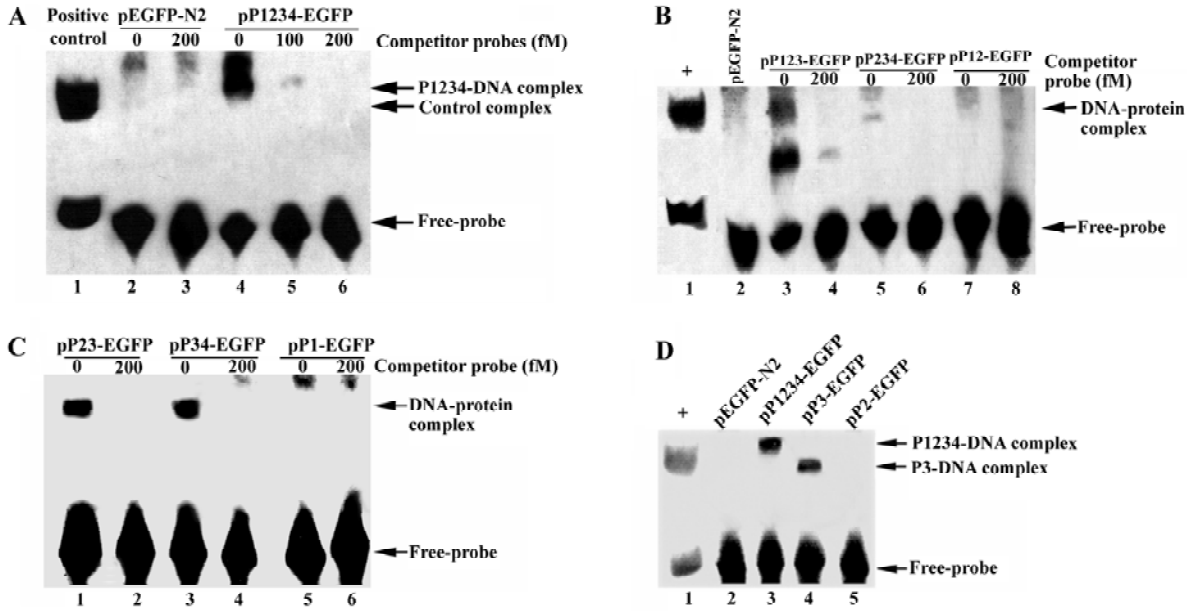


Figure 4. Characterization of PDX-1 and its truncated mutants binding to CMV IE promoter. 293 cells were transfected with indicated vectors and nuclear extracts were collected. (A) Lane 1, EMSA kit positive control (Epstein-Barr Nuclear Antigen, 67–73 kDa (37) and EBNA binding probe, 60 bp); Lanes 2 and 3, pEGFP-N2 transfected 293 cell extract or containing 200 fM unlabeled CMV competitor oligos; Lane 4, p1234 transfected 293 cell extract; Lanes 5 and 6, p1234 transfected 293 cell extract containing 100 fM or 200 fM unlabeled CMV competitor probes. Arrow shows the specific DNA-protein complex. (B) Extracts from P123-EGFP, P234-EGFP or P12-EGFP transfected cells were assayed (with or without 200 fM unlabeled competitor probes). (C) Extracts from P23-EGFP, P34-EGFP, P1-EGFP transfected cells were assayed (with or without 200 fM unlabeled competitor probes). (D) Extracts from pEGFP-N2, P1234-EGFP, P3-EGFP, P2-EGFP transfected cells were assayed.

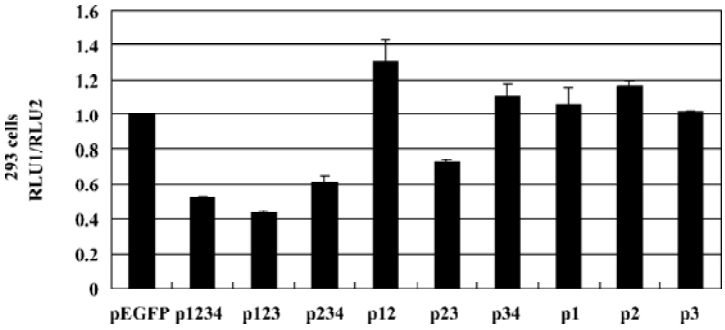


Figure 5. The effect of PDX-1 and its mutants to the CMV IE promoter activity. 293 cotransfected cells with pCMV-Luc, an expression plasmid (ie, pEGFP-N2, pP1234-EGFP, pP123-EGFP, pP234-EGFP, pP12-EGFP, pP23-EGFP, pP34-EGFP, pP1-EGFP, pP2-EGFP, or pP3-EGFP), and pRL-TK in 24-well plates. The overexpression of P1234 caused a 47.60% ($P<0.05$) decrease in promoter activity; P123 caused a 56.70% ($P<0.05$) decrease; P234 caused a 38.52% ($P<0.05$) decrease; and P23 caused a 27.28% ($P<0.05$) decrease. No significant effects were observed when P12, P34, P1, P2, P3 were overexpressed.

protein complex formed by probe DNA and P3 nuclear extracts was much smaller than that of P1234 nuclear extracts. Based on our knowledge of the functions of the four domains, we hypothesized that there should be a multi-protein complex rather than PDX-1 alone acting as a repression unit, which inhibits the expression activity of CMV IE promoter. And the putative multi-protein complex might contain P300, Pbx, Prep1, E2-5, and E47, *etc*, which were known to interact with PDX-1 through domain 1 and domain 2.

Discussion

The human CMV IE promoter is one of the most commonly used promoters for overexpressing recombinant genes in a wide range of mammalian cells. Previous studies have found that a cellular homeoprotein, PDX-1, could bind to CMV IE promoter and negatively regulated its transcriptional activity in HEK 293 cell lines[27]. In the present article, we constructed eight PDX-1 truncated mutants in order to determine the molecular mechanism of PDX-1 that regulates

CMV IE promoter. Through the dual-reporter and EMSA assay, we confirmed that domain 3 of PDX-1 is essential for its nuclear localization and DNA binding activity. However, PDX-1 domain 3 and domains 34 showed no transcriptional repression activities in luciferase assay. These results indicated that PDX-1 binding alone was not sufficient to repress the transcription activity of CMV IE promoter.

Further study showed that both domain 1 and domain 2 contributed to PDX-1's repressive activity to the CMV IE promoter in the presence of domain 3. And the bands of DNA-protein complex formed by probe DNA and P3-transfected cellular nuclear extracts is much smaller than that formed by probe DNA and full-length PDX-1-transfected cellular nuclear extracts. As it has been reported that PDX-1 domain 1 and domain 2 interact with other proteins such as P300, Pbx, Prep1, E2-5, and E47[10,18,28–30], we suspected that a multi-protein complex involved in protein interaction with domain 1 and 2 was required for the negative regulation of CMV IE promoter mediated by PDX-1.

Chao *et al*[27] first discovered a similar phenomenon. They constructed 10 mutations of a 45-bp CMV IE promoter as we have described (Figure 6). They found that site 6 and site 10 might be PDX-1 binding sites, and the mutations of either of the two sites resulted in partial loss of PDX-1 negatively-regulate activity to CMV IE promoter. Furthermore, they found that, although site 9 showed no relationship with PDX-1 binding activity, PDX-1 lost its negatively regulating activity when it mutated.

In our work, we used the Patch program to search the CMV IE promoter sequence and found that site 10, which is in the (-) chain (Figure 6), is the only PDX-1 binding site in this sequence and the complementary sequence of site 9 in the (+) chain (Figure 6) is the cAMP response element binding protein (CREB) binding site.

It has been reported that transcriptional activity of PDX-1 required recruitment of P300[28]. The C-terminal of P300 interacted directly with PDX-1 between amino acids 1 and 143, which is exactly PDX-1 domain 1 and 2[29], and this protein complex was supposed to be located at site 10 in the (-) chain in CMV IE promoter. It's well known that CREB-P300/CBP complex is essential for CMV IE promoter transcription activity[31–33], which should be located at site 9, the CREB binding site, in the (+) chain in CMV IE promoter. So there might be a possibility that PDX-1, P300, CBP, and CREB could form a complex as PDX-1-P300/ CBP-CREB, binding to both (-) and (+) chain. Such a multi-protein complex could protect double-strand DNA from melting, and thus decrease transcriptional activity. However, site 9 of CMV IE promoter is the first CREB binding site. The forming of PDX-1-P300/ CBP-CREB complex could also inhibit the CREB-P300/CBP complex slipping to the next CREB binding site, and it is known that the slipping was essential for the CMV IE promoter transcriptional activity. So this may explain why loss of domain 1 decreased the repression efficiency of PDX-1 upon CMV IE promoter.

According to our findings that P23 could also reduce the expression driven by CMV IE promoter, there might be another mechanism depended on PDX-1 domain 2. One of the major proteins that interacted with PDX-1 is PBX1, which has been reported to form PBX1/E2A complex interacting directly with P300/CBP complex[34]. So, our model above could also explain the repression function of domain 2.

To test our model we further constructed a CMV IE promoter mutant in which the CREB and the PDX-1 binding sites are at the same chain of DNA (+ chain). The mutant was also inserted into the multiple cloning sites of pGL3-Basic to generate pCMV+/+-Luc, and was co-transfected with pP1234-EFGP and pRL-TK. The luciferase assay results showed that PDX-1 has weak repression activity to CMV+/+ promoter (10.73% decreased), although the EMSA assay indicated the same binding activity of PDX-1 protein to CMV+/+ promoter probe (data not shown).

The 55-bp fragment used in our study is located at the CMV IE gene promoter unique region. And the importance of this unique region in human CMV IE transcription and viral replication has been investigated previously. Recombinant CMV containing deletions from positions -640 to -583, which did not contain the CREB and PDX-1 binding site, showed no significant effects on IE transcription[35]. However, a deletion of the sequence between -521 and -579, which including the preferred CREB and PDX-1 binding site, showed significant multiplicities of infection (moi)-dependent increases in recombinant CMV replication. And it had been reported that the 47-bp segment has consensus-binding sites for CREB/ATF, SP1, and YY1[36]. Therefore, these previous studies and our results suggested the possible in-

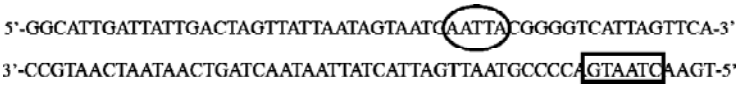


Figure 6. The 55-bp CMV IE promoter in our study, which contains several homeobox protein binding sites. The circle indicates the CREB binding site, and the rectangle indicates the only one PDX-1 binding site in this sequence (analyzed by Patch).

volvement of PDX-1 in the CMV replication by downregulating the CMV IE promoter. And for the other cells that have no PDX-1 expression, perhaps other homeodomain proteins such as Isl, MEIS1, HOXA may bind to this region and have a similar effect to CMV IE promoter. However, more work should be carried out to explore the exact model of PDX-1 mediated downregulation of CMV IE promoter, and to discover whether the virus applied this method to shut down the expression of IE gene.

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