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Improving transcriptional activity of human cytomegalovirus major immediate-early promoter by mutating NF-κB binding sites

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ABSTRACT

Many mammalian gene expression vectors express the transferred genes under the control of the cytomegalovirus (CMV) major immediate-early promoter (MIEP). The human MIEP has been known as the strongest promoter in mammalian cells and utilized widely in mammalian expression systems. There are four NF-κB binding sites (named as κBs) in the human MIEP. In this study, we have constructed multiple mutated MIEPs by changing the natural κBs in the human MIEP into the high-affinity artificial sequences that were *in vitro* selected by using systematic evolution of ligands by exponential enrichment (SELEX) and predicted by bioinformatics. With various transcriptional activity evaluations, we found three mutated MIEPs with the transcriptional activity higher than the wild-type MIEP, which should be useful and widely applicable in many mammalian transgene expression fields such as gene engineering, gene therapy and gene editing. This study provides a useful approach for promoter engineering in biotechnology. This study also produced a series of mutated MIEPs with various transcriptional activities, which may be used for the fine control of gene expression output in the future synthetic biology.

1. Introduction

The ectopic gene expression in mammalian cells is indispensable for life scientific research and biomedical application. The expression level of a foreign gene in a mammalian host cell is dependent on the transcriptional activity of its upstream promoter in the expression vector. Therefore, using a strong promoter is critical for the high-level expression of an interested gene in mammalian cells [1]. Almost three decades ago, the major immediate-early promoter (MIEP) of human cytomegalovirus (HCMV) was found as a natural mammalian promoter with high transcriptional activity [2].

Many ectopic gene expression vectors express the transferred genes under the control of MIEP. In general, MIEP and the human EF-1α promoter have been known as the strongest promoters in various mammalian cells [2–4]. Comparison revealed that the murine MIEP sometimes directed higher gene expression than the human MIEP [5,6]; however, the full-length human MIEP plus intron A gives the highest levels of protein expression in transient and stable transfections in the engineered 293EBNA and CHO-K1-S cells [7]. When the human and mouse MIEPs were used to produce the monoclonal antibody (mAb) in the Chinese hamster ovary (CHO) cell, the human MIEP had the expression level higher than the mouse MIEP [8]. Originally, the human MIEP has been identified to display high transcriptional activity in a wide range of cultured cells [9]. The human MIEP can drive the ectopic gene expression in broad tissues in transgenic animals [10,11].

MIEP also provides a common adaptable basic promoter for constructing various gene vector systems [12]. For example, the PDX1 binding site in the human MIEP was shown to be a repressor and removal of the site by mutation increased expression by up to four-fold in transient luciferase experiments [13]. Positive effects are also observed after inserting binding sites for ZFP-2392v upstream of the human MIEP [14]. The CpG dinucleotide sites are prone to methylation, removing the CpG dinucleotide sites in MIEP improved the expression stability of transferred gene during long

Abbreviations: CMV, cytomegalovirus; MIEP, major immediate-early promoter; SELEX, systematic evolution of ligands by exponential enrichment; HCMV, human cytomegalovirus; CHO, Chinese hamster ovary; mAb, monoclonal antibody; NF-κB, Nuclear factor κB; CREB, cAMP-response element binding protein; AP1, Activating protein 1; RAR, retinoic acid receptor; SRF, serum response factor; TSS, transcription start site; DMEM, Dulbecco's modified Eagle's medium; hG-CSF, human granulocyte colony stimulating factor; GLuc, Gaussia luciferase; SEAP, secreted alkaline phosphatase; EGFP, enhanced green fluorescence protein; MFI, mean fluorescence intensity; SD, standard deviation; mut MIEPs, mutated MIEPs; wt MIEP, wild-type MIEP.

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term culture [15,16]. Several previous studies have added a MIEP to the 5′ end of a cellular promoter to increase its transcriptional activity [1,17–22].

The full-length human MIEP contains many binding sites of various transcription factors. For instance, it harbors one or several DNA binding sites of nuclear factor κB (NF-κB), cAMP-response element binding protein (CREB), activating protein 1 (AP1), retinoic acid receptor (RAR), SP-1, serum response factor (SRF), and ELK-1 [23–26]. There are four NF-κB binding sites (named as κBs) in the human MIEP, three of which have the same sequence (Site 1, 2 and 4). Some studies indicate that the κBs are responsible for both the basic and modulated transcriptional activity of human MIEP [27]. Meanwhile, some researchers believe that these κBs are dispensable for MIEP activation in multiple types of cells [28,29]. Additionally, the contribution of these κBs to the transcriptional activity is related to their distance to transcription start site (TSS). Site 4 and 2 play the main role and Site 3 plays an intermediate role, while Site 1, which has the same sequence as Site 4 and 2 but far distance to TSS, has no influence on the NF-κB-mediated regulation of the promoter [30]. Some researchers showed that Site 2 and 3 function as the major and minor responsible site, respectively [31]. Although it is difficult to clarify their exact roles, these κBs together are critical for the high transcriptional activity of MIEP.

We have ever selected NF-κB RelA binding sequences by using a SELEX-sequencing technique [32], in which we identified many 10-mer dsDNA sequences with the binding affinity higher than the natural κBs of 5′-GGGACTTTCC-3' [33]. Because there are four κBs in the human MIEP and three of them have the sequence of 5′-GGGACTTTCC-3′, we conceived that the transcriptional activity of the human MIEP may be further improved by replacing these natural κBs with the *in vitro* selected high-affinity sequences. In this study, we fabricated multiple mutated MIEPs (mut MIEPs) by changing different natural κBs in the wild-type MIEP (wt MIEP) into two *in vitro* selected high-affinity sequences. In this way, we found three mut MIEPs that had the transcriptional activity higher than the wt MIEP. These newly found mut MIEPs should be useful in gene engineering, gene therapy and gene editing.

2. Materials and methods

2.1. Cultivation of cells

HepG2, HeLa and 293T cells were cultured in the Dulbecco's modified Eagle's medium (DMEM). CHO cells were cultured in DMEM/F12 1:1 culture medium. K562 cells were cultured in RPMI1640 culture medium. NFS-60 cells were cultured in RPMI1640 culture medium supplemented with 15 ng/mL of human granulocyte colony stimulating factor (hG-CSF) (*E.coli* derived) (Peprotech). All media contained 10% fetal calf serum, 100 units/mL of penicillin and 100 g/mL of streptomycin. All cells were cultured at 37 °C in 5% CO_2. All cells were obtained from the Cell resource center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

2.2. Preparation of promoters

The human MIEP cloned in pEGFP-N1 (Clontech) was mutated by replacing the natural κBs (Table S1) with two *in vitro* selected high-affinity sequences (T and P; Table S2). The mut MIEPs were fabricated by using fusion PCR amplification with designed primers (Table S3). The PCR reaction with the TaKaRa Ex Taq HS Polymerase was performed as: 94 °C for 3 min, 30 cycles of 94 °C for 40 s, 57 °C for 40 s and 72 °C for 30 s, and 72 °C for 5 min. The PCR reaction with the Prime STAR HS DNA Polymerase (Takara) was performed as: 30 cycles of 95 °C for 10 s and 68 °C for 1 min. The full length DNA fragments of two tentative promoter s, T1P2 and T1P24, were prepared by chemically synthesized.

2.3. Construction of plasmids

The vector pGL4.10 that can express firefly luciferase (Fluc) (Promega) was employed to clone promoters by using the sites of *Kpn*I and HindIII (Fermentas). The vector pGluc was constructed for expressing secretory Gaussia luciferase (GLuc) by replacing the IRES and EGFP sequences of pIRES-EGFP (Clontech) with the Gluc gene from pGluc-Basic (NEB). Promoters were cloned in pGluc by using the sites of *Ase*I and *Nhe*I (Takara). The vector pEZX-ePG04 (GeneCopoeia) can express secretory GLuc and secreted alkaline phosphatase (SEAP), in which GLuc is used as a reporter gene under the control of interested promoters and SEAP as an internal control. The pEZX vector was constructed by cloning the interested promoters into pEZX-ePG04 by using the sites of *Eco*RI and HindIII (Takara). The vector pEGFP-N1 (Clontech) can express the enhanced green fluorescence protein (EGFP) in the cytoplasm. The pEGFP vector was constructed by cloning the interested promoters into pEGFP-N1 by using the sites of *Ase*I and *Nhe*I (Takara). The vector pGCSF was constructed by cloning a chemically synthesized full length hG-CSF gene sequence fused with 6×His Tag at the N terminal (Sangon) into pIRES-EGFP by using the sites of *Bam*HI and *Eco*RI (Takara). Promoters were cloned in pGCSF by using the sites of *Ase*I and *Nhe*I (Takara). All plasmids were verified by sequencing (Fig. S1 and Fig. S2).

2.4. Evaluation of promoter activity with intracellular reporter

Cells (Table S4) were seeded at a density of 1×10^5 cells per well in 24 well plates and cultivated for 12 h. Cells were then cotransfected with pGL4.10 (0.5 μg/well) containing the interested promoters or blank pGL4.10 (0.5 μg/well) (as negative control) and internal control pGL4.75 (0.05 μg/well) by using Lipofectamine 2000 for 5 h. The media were then discarded and the cells were cultured with fresh media for more time. Cells were collected for detecting Fluc by using the Dual-Luciferase Reporter Assay System (Promega). The optical density was read with SynergyHT (BioTek). The promoter activity was assessed by the relative Fluc activity normalized against the Renilla luciferase activity.

2.5. Evaluation of promoter activity with single-secretory reporter

Cells (Table S4) were seeded at a density of 1×10^5 cells per well in 24 well plates and cultivated for 12 h. Cells were then transfected with the pGluc (0.8 μg/well) containing the interested promoters or blank pGluc (as negative control) by using Lipofectamine 2000 for 5 h. Then the media were discarded and cells were cultured for more time with fresh medium. The culture media were collected for measuring the Gluc activity by using the reagents for detecting the GLuc activity included in the Dual-Luciferase Reporter Assay System (Promega) (referred as the GLuc reporter assay hereafter). The total protein concentration of culture media were determined by the Bradford Protein Assay Kit (Beyotime). The optical density was read with SynergyHT (BioTek). The promoter activity was assessed with the relative GLuc activity normalized against the total protein concentration.

2.6. Evaluation of promoter activity with dual-secretory reporter

Cells (Table S4) were seeded at a density of 1×10^5 cells per well in 24-well plates and cultivated for 12 h. Cells were then transfected with pEZX (0.8 μg/well) containing the interested promoters or blank pEZX by using Lipofectamine 2000 for 5 h. Then the media