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Glucocorticoids facilitate the transcription from the human cytomegalovirus major immediate early promoter in glucocorticoid receptor- and nuclear factor-I-like protein-dependent manner

Maki Inoue-Toyoda [a, 1], Kohsuke Kato [a, b, 1], Kyosuke Nagata [c, *], Hiroyuki Yoshikawa [a, b]

[a] Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan
[b] Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan
[c] University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan

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ABSTRACT

Human cytomegalovirus (HCMV) is a common and usually asymptomatic virus agent in healthy individuals. Initiation of HCMV productive infection depends on expression of the major immediate early (MIE) genes. The transcription of HCMV MIE genes is regulated by a diverse set of transcription factors. It was previously reported that productive HCMV infection is triggered probably by elevation of the plasma hydroxycorticoid level. However, it is poorly understood whether the transcription of MIE genes is directly regulated by glucocorticoid. Here, we found that the dexamethasone (DEX), a synthetic glucocorticoid, facilitates the transcription of HCMV MIE genes through the MIE promoter and enhancer in a glucocorticoid receptor (GR)-dependent manner. By competitive EMSA and reporter assays, we revealed that an NF-I like protein is involved in DEX-mediated transcriptional activation of the MIE promoter. Thus, this study supports a notion that the increased level of hydroxycorticoid in the third trimester of pregnancy reactivates HCMV virus production from the latent state.

1. Introduction

Human cytomegalovirus (HCMV), a member of herpesvirus, is common and usually asymptomatic in healthy children and adults including pregnant women. Initiation of HCMV productive infection is dependent on the expression of the major immediate early (MIE) genes. The virus establishes a lifelong latent infection and is periodically reactivated from latency, producing infectious viruses [1]. Congenital HCMV infection, which occurs *in utero* via the placenta, may cause fatal and neonatal death, developmental defects, or serious clinical sequelae. It is reported that the virus is reactivated more often in the third trimester than in the second or the first of pregnant women [2]. In this reactivation process, the transcriptional regulation of the MIE genes is one of the most important key events for both HCMV life cycle and clinical results.

The transcription of the MIE genes is driven by the MIE regulatory region encompassing approximately 2 kbp region upstream of the transcription start site including a core promoter, enhancer, unique region, and distal modulator [3]. The MIE regulatory region contains binding sites for a diverse set of cellular transcription factors [3–8]. Adrenal glucocorticoid hormones such as cortisol are known to play significant roles in the regulation of inflammatory signals thorough gene regulation [9]. The glucocorticoid-bound glucocorticoid receptor (GR) binds to its response element (GRE) and regulates transcription. It was previously reported that the production of HCMV progeny viruses was facilitated in dexamethasone (DEX)-treated cells but not in other hormones-treated cells [10]. The expression level of MIE proteins was increased by DEX treatment in HCMV-infected cells. These results suggested that DEX treatment facilitates HCMV virus production through activation of the MIE gene expression. Recently, it was reported that glucocorticoids trigger reactivation of HCMV from HCMV-latently infected myeloid cells and increases the incidence of HCMV infection in liver transplant patients when both donor and recipient are HCMV seropositive [11]. Furthermore, it is suggested that HCMV infection in the third trimester of pregnant women is probably due to elevation of the plasma hydroxycorticoid level [12]. However, it has never been clarified whether DEX-mediated signal pathway is directly involved in the transcriptional regulation of the MIE genes.

* Corresponding author. Fax: +81 29 853 3942.
E-mail address: knagata@md.tsukuba.ac.jp (K. Nagata).
[1] These authors contributed equally to this work.

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Here, we found that the DEX treatment facilitates the transcription of the HCMV MIE genes through the MIE promoter and enhancer region in a GR-dependent manner. By competitive EMSA and reporter assays, we revealed that an NF-I-like protein is involved in the DEX-mediated transcriptional activation of the MIE promoter.

2. Materials and methods

2.1. Cell culture, virus preparation, and infection

BJ-TERT cells were maintained in a mixed medium (4:1) of Dulbecco modified Eagle medium (DMEM, Nissui) and Medium 199 (Gibco-Life technology) (DMEM-Medium 199) containing 10% (v/v) fetal bovine serum (FBS) and 1 mM sodium pyruvate. HeLa cells were maintained in DMEM containing 10% (v/v) FBS. Infection experiments and propagation of HCMV AD169 strain were basically performed as follows: BJ-TERT cells in DMEM-Medium 199 mixed medium containing 2% FBS were infected with AD169 strain at appropriate infectious units. After incubation for 1 h at 37 °C, DMEM-Medium 199 containing 10% FBS was added and further incubated for desired periods. Infectious units of AD169 strain were determined by indirect immunofluorescent analyses with anti-HCMV IE72 antibody (Millipore) using BJ-TERT cells.

2.2. Plasmid transfection and luciferase assay

HeLa cells were transfected with pMIEP-Luc reporter plasmid using GeneJuice® (Novagen) in combination with a control plasmid DNA expressing secreted alkali phosphatase (SEAP). At 24 h after transfection, the cell culture medium was collected, and the SEAP activity was measured using SEAP assay kit (TOYOBO) for evaluating the transfection efficiency. At the same time, the culture medium was exchanged with fresh one containing desired concentrations of DEX and/or RU486, one of GR antagonists, and cells were further incubated for 24 h. Cells were washed with PBS and lysed in a cell lysis buffer (25 mM Tris–HCl [pH 7.9], 10% glycerol, and 0.1% TritonX-100) by three freezing–thawing cycles. The cell lysates and a luciferase substrate (Promega) were mixed, and the luciferase activity was measured by Lumat LB9506 (BERTHOLD). Data were normalized by the SEAP activity.

2.3. Reverse transcription and quantitative PCR analyses

Total RNA was prepared from cells using RNeasyR Mini Kit (QIAGEN) in combination with DNaseI. The concentration of RNA in each sample was determined using a Nano Drop Lite spectrophotometer (Thermo Scientific). To analyze the level of the MIE gene and *β-actin* mRNAs, cDNA was synthesized from the total RNA prepared from AD169-infected BJ-TERT cells using RevaTra Ace reverse transcriptase (TOYOBO) and oligo-dT primer. To analyze the level of mature and pre-mature *luciferase* mRNAs, cDNAs were synthesized from total RNA prepared from pMIEP-Luc-509 plasmid DNA-transfected HeLa cells using RevaTra Ace reverse transcriptase and oligo-dT primer or a reverse primer corresponding to complementary sequence within the intron located at immediately upstream of SV40 polyA signal in PGV-B plasmid, 5′-TCAGTAGTTTAACACATTATACACTT-3′ (R2). The primer sets used for quantitative PCR (Q-PCR) were as follows: for *MIE IE72*, 5′-CCTAGTGGATGACCTA-3′ and 5′-GTGACACCAGAGAATCAG-3′; for *β-actin*, 5′-ATGGGTCAGAAGGATTCCTATGT-3′ and 5′-GGTCATCTTCTCGCGGTT-3′; for *GAPDH*, 5′-AGCCAAAAGGGTCATCATCTC-3′ and 5′-GGACTGTGGTCATGAGTCCTTC-3′; for mature *luciferase* (*Luc*), 5′-ACTGCGATTTTAAGTGTTGTTCCAT-3′ (F1) and 5′-GTGCGCCCCCAGAAGCAATTTC-3′ (R1); and for premature *luciferase* (pre*Luc*), 5′-GGGCGGAAAGTCCAAATTGT-3′ (F2) and R2 primer. Q-PCR reactions were performed with FastStart SYBR Green Master (Roche) using Thermal Cycler Dice (Takara).

2.4. Preparation of nuclear extracts and electrophoretic gel mobility shift assay (EMSA)

HeLa cells were treated with or without 1 μM DEX for 24 h at 37 °C. Nuclear extracts were prepared as described previously [13]. Total protein concentration of nuclear extracts was determined by the Bradford method. The 21 bp MIEP probe was obtained by annealing oligonucleotides 5′-GGTTTTGGCAGTACATCAATG-3′ and 5′-CATTGATGTACTGCCAAAACC-3′. Radioactive end-labeling was performed using T4 polynucleotide kinase and [γ-^{32}P]ATP. For competition assays, competitor DNAs were generated by annealing the following oligonuceotides: for SV40 21 bp repeat, 5′-CATTCTCCGCCCCATGGC-3′ and 5′-GCCATGGGGCGGAGAATG-3′; for NF-I, 5′-CGATCTGGCACTGTGCCAAGC-3′ and 5′-GCTTGGCACAGTGCCAGATCG-3′; and for mutated MIEP, 5′-GGTTTAGCGAGTACATCAATG-3′ and 5′-CATTGATGTACTCGCTAAACC-3′. DNA binding reactions were performed in 10 μl reaction mixture containing 20 mM Hepes-NaOH (pH7.9), 1 mM EDTA, 50 mM KCl, 6.25 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 0.2 mg/ml BSA, 10% Glycerol, 4 μg poly (dI-dC). Nuclear extracts (10 μg protein) were incubated with radioactively labeled MIEP oligonucleotide probes (0.01 pmol) in the presence or absence of MIE, SV40, NF-I, or mutated MIE competitor DNAs (0.3 or 1 pmol) at 30 °C for 15 min. Reaction mixtures were subjected to non-denaturing 6% polyacrylamide gel electrophoresis in 0.25 × TBE at 4 °C for 75 min (at 10 mA constant). Gels were dried and analyzed by Phosphoimager (Typhoon FLA 7000).

3. Results

3.1. Transcriptional activation of human cytomegalovirus immediate early promoter with dexamethasone (DEX)

To examine whether the increase of MIE protein synthesis facilitated by DEX is dependent on transcriptional activation of the MIE gene, we first performed quantitative RT-PCR to quantitatively determine the viral MIE gene mRNA level in HCMV-infected cells (Fig. 1A). In cells pre-treated with 1 μM DEX for 24 h prior to infection, we found that the mRNA level of the MIE gene is approximately 2 times higher than that of infected cells without DEX, suggesting that the MIE gene mRNA level is increased by DEX treatment.

Next, we constructed a *luciferase* reporter plasmid by cloning the HCMV genomic DNA fragment between nucleotide positions of 1994 bp upstream and 121 bp downstream of the transcription start site including promoter, enhancer, and exon 1 (pMIEP-Luc-1994/+121) of the MIE gene (Fig. 1B). The MIE gene exon 1 corresponding to 5′ UTR is common among alternative MIE transcripts [14]. To address whether the exon 1 of the MIE gene is involved in DEX-mediated increase of the MIE gene expression, we also constructed *a luciferase* reporter plasmid lacking almost all region of exon 1 (pMIEP-Luc-1994/+10) (Fig. 1C). In the presence of every concentration of DEX, the relative luciferase unit was increased approximately 1.5–2 times higher than that of non-treated cells transfected with pMIEP-Luc-1994/+10 similarly to the observation when pMIEP-Luc-1994/+121 was used (Fig. 1D). These results suggest that the upstream region of the MIE gene transcription start site, but not the MIE gene exon 1 region, is involved in the DEX-mediated transcriptional activation.

To determine which region within the 2 kbp-long MIE regulatory region is involved in DEX-mediated transcriptional activation,

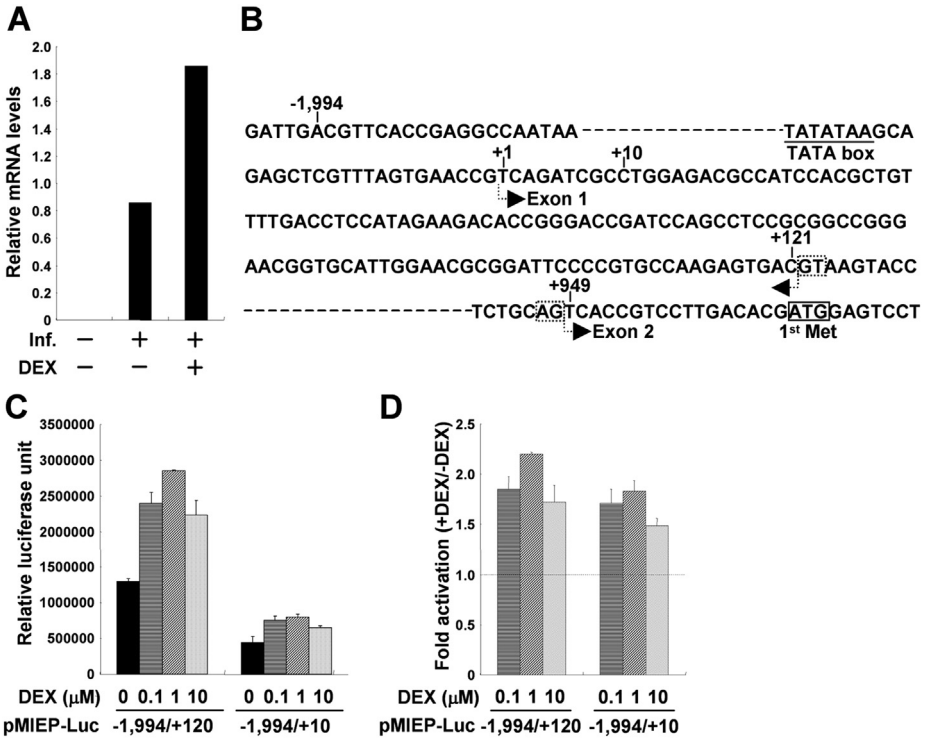

Fig. 1. DEX facilitated the gene expression from the HCMV MIE promoter. (A) Transcription of the MIE gene was activated by DEX in HCMV-infected cells. Total RNA were isolated from mock-infected BJ-TERT cells and BJ-TERT cells infected with HCMV at infectious unit of 0.1 with or without 1 μM DEX pre-incubation, and were subjected to quantitative RT-PCR analyses. *β-actin* was used for normalization as an internal control. (B) DNA structure around the HCMV MIE gene transcription start site and its downstream. The transcription start site of MIE gene is indicated as the nucleotide position +1. The MIE gene exon 1 encompasses the DNA region from transcription start site to the nucleotide position +120, and splicing donor and acceptor nucleotides of intron1 are shown by dashed box. The ATG nucleotides corresponding to first methionine of the IE gene open reading frame is shown by box in exon 2 region. (C and D) Luciferase assays. HeLa cells transfected with pMIEP-Luc-1994/+120 or pMIEP-Luc-1994/+10 were treated with desired concentration of DEX. The luciferase activity was normalized by the SEAP activity in panel (C). Fold activations when the luciferase activity of cells without DEX was set to be 1.0 as represented in panel (D). Results are represented as mean values ± SD from three independent experiments.

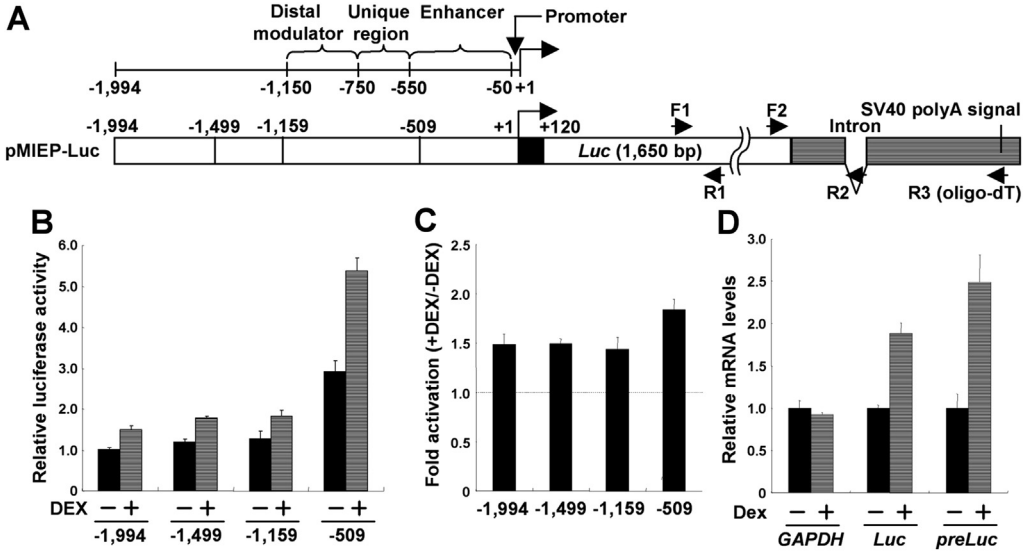

Fig. 2. DEX-treatment facilitated the transcription from pMIEP-Luc reporter plasmid in the MIE promoter and enhancer dependent manner. (A) Schematic diagrams of HCMV MIE regulatory region and pMIEP-Luc serial deletion mutants. The upstream nucleotide positions of each pMIEP-Luc mutant are shown in lower panel. Primers used for reverse transcription and quantitative PCR in panel (D) are shown by arrows (F1~R2). (B and C) Luciferase assays. HeLa cells transfected with each pMIEP-Luc were treated with or without 1 μM DEX. The relative luciferase activity was normalized by the SEAP activity (panel B). Fold activation when the luciferase activity of cells treated without DEX was set to be 1.0 were represented (panel C). Results are represented as mean values ± SD from three independent experiments. (D) Reverse transcription and quantitative PCR analyses. HeLa cells transfected with pMIEP-Luc-509 were treated with or without 1 μM DEX. Cells were harvested, and total RNA was extracted and then quantitative RT-PCR analyses were performed. Relative mRNA levels when that of cells treated without DEX was set to be 1.0 were represented. Results are represented as mean values ± SD from three independent experiments.

we constructed plasmids containing serial deletion of the MIE regulatory region (Fig. 2A). In the presence of DEX, all constructs showed approximately 1.5–2 times higher activity compared with that in the absence of DEX (Fig. 2B and C). .These results suggest that the enhancer and promoter regions are responsible for DEX-mediated transcriptional activation.

To confirm that the luciferase activity is correlated with the expression level of its mRNA, we performed quantitative RT-PCR analyses using total RNA prepared from DEX-treated or mock-treated cells transfected with pMIEP-Luc-509 plasmid (Fig. 2D). The expression level of both mature and premature *luciferase* mRNAs were increased approximately 2 times higher than that of mock-treated cells, suggesting that the luciferase activity is correlated with the amount of *luciferase* mRNA. Taken together, these results indicate that DEX treatment facilitates the transcriptional activity of the MIE promoter through its enhancer and promoter region.

3.2. Identification of the one 17 bp-repeat within MIE enhancer as a responsible element for DEX- and GR-dependent transcriptional activation

Any GREs have never been identified in the MIE enhancer so far. To identify which DNA sequence within the MIE enhancer and promoter is responsible for the DEX-mediated transcriptional activation, we further constructed serial deletion mutant plasmids within the enhancer region (Fig. 3A). In the presence of DEX, cells transfected with pMIEP-Luc-509, -399, and -249 showed approximately 1.5–2 times higher luciferase activity compared with those of mock-treated cells (Fig. 3B). In sharp contrast, cells transfected with pMIEP-Luc-130 did not show any increase DEX (Fig. 3B). Further to narrow down a DEX-responsible DNA region, we constructed pMIEP-Luc-219 and pMIEP-Luc-167 (Supplemental Fig. 1A). We found that cells transfected with pMIEP-Luc-167 do not show any increase of the luciferase activity by DEX treatment, while cells transfected with pMIEP-Luc-219 show approximately 1.6 times increase (Supplemental Fig. 1B), suggesting that the DNA region responsible for DEX-mediated transcriptional activation is located between nucleotide positions -219 and -168 from the transcription start site of the MIE promoter. It was previously predicted that there are an AP1 binding site and a 17 bp repeat between nucleotide positions -219 and -167 (Fig. 3C) [15,16]. We constructed a series of deletion mutants, pMIEP-Luc-210, -199, and -192 (Fig. 3D). We found that both cells transfected with pMIEP-Luc-199 and -192 do not show any increase of the luciferase activity, while cells transfected with pMIEP-Luc-219 and -210 show approximately 1.6 times increase by DEX (Fig. 3E). These results strongly suggest that the 17 bp repeat within the MIE enhancer region is involved in the DEX-mediated transcriptional activation.

Glucocorticoid not only regulates the gene expression through GR activation but also affects cellular signal transduction through a GR-independent pathway [9]. Next to clarify whether the DEX-mediated transcriptional activation of the MIE promoter is dependent on GR, we examined the effect of RU486, a GR antagonist, on DEX-mediated transcriptional activation (Fig. 3F) [11]. In cells transfected with pMIEP-Luc-210, the DEX-mediated increase of the luciferase activity was suppressed by RU486. In contrast, both RU486 and DEX did not have significant effects on the luciferase activity in cells transfected with pMIEP-Luc-199 and -192. These results indicate that the 17 bp repeat within the MIE enhancer located between nucleotide positions -210 and -193 is involved in the DEX-mediated transcriptional activation from the MIE promoter in a GR-dependent manner.

3.3. Involvement of 17 bp repeat binding NF-I like protein in DEX-mediated transcriptional activation of the MIE promoter

Five NF-I binding sites have been identified within the upstream region of the enhancer between nucleotide positions -765 and -620

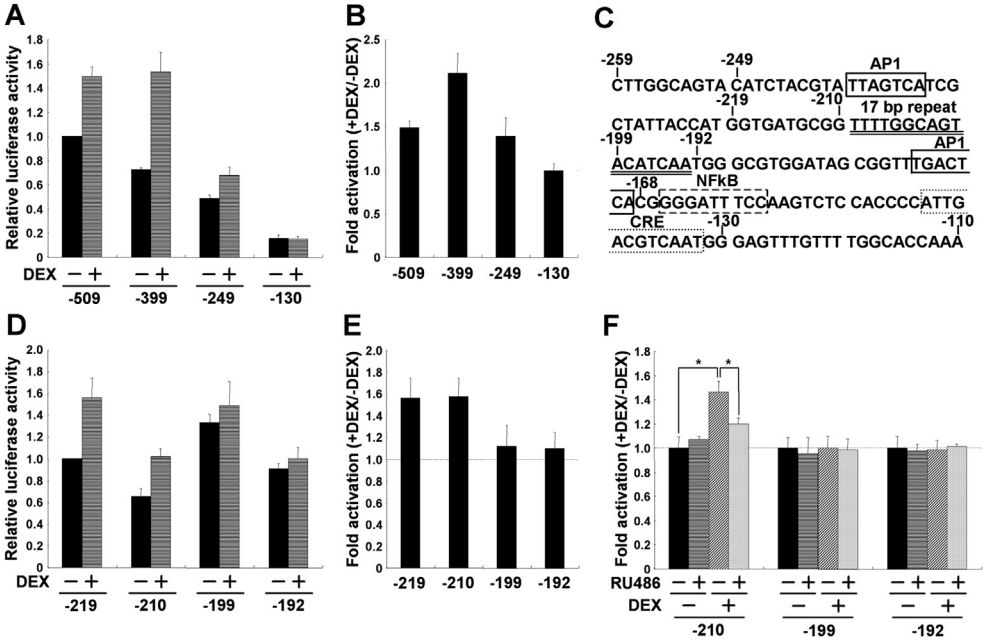

Fig. 3. The 17 bp repeat within the MIE enhancer was responsible for DEX- and GR-dependent transcriptional activation. (A, B, D and E) Luciferase assays with cells transfected with various pMIEP-Luc deletion mutants. The luciferase activity was normalized by the SEAP activity (panel A). Fold activation when the luciferase activity of cells treated without DEX was set to be 1.0 were represented (panel B). Results are represented as mean values ± SD from three independent experiments. (C) The DNA structure around nucleotide positions -259 and -110 within the MIE enhancer. The 5′ terminal nucleotide positions of pMIEP-Luc mutant series are shown. (F) GR dependency was examined with RU486 on transcriptional activation of MIE promoter. Cells transfected with pMIEP-Luc mutants were treated with or without 1 μM DEX and/or 1 μM RU486 for 24 h, then subjected to luciferase assays. Fold activation when the luciferase activity of cells treated without DEX was set to be 1.0 were represented. Results are represented as mean values ± SD from four independent experiments (*$P < 0.05$).

and the first intron of the MIE gene [16]. However, the function of the 17 bp repeat located between nucleotide positions -210 and -193 within the MIE enhancer (the 17 bp DEX-responsible repeat) is not clarified. To characterize the binding properties of a protein(s) to the 17 bp DEX-responsible repeat, we performed the electrophoretic gel mobility shift assay (EMSA) using a 21 bp oligonucleotide containing the 17 bp DEX-responsible repeat as a radiolabeled probe. We prepared nuclear extracts (NE) from HeLa cells treated with or without 1 μM DEX. First, we examined whether there is a protein(s) binding to the 17 bp DEX-responsible repeat and whether there is a difference in band shift pattern between NE prepared from mock-treated cells and that prepared from DEX-treated cells. In the presence of mock-treated NE, we observed the increase of shifted bands in a dose-dependent manner, suggesting that there is a nuclear protein(s) that binds to the 17 bp DEX-responsible repeat (Fig. 4A, lanes 1–5). In the presence of DEX-treated NE, we found the increase of shifted band in a dose-dependent manner, but the band shift pattern was similar to that of mock-treated NE (Fig. 4A, compare lanes 2–5 and lanes 6–9). These results suggest that a factor that binds to the 17 bp DEX-responsible repeat is present equally in both mock-treated NE and DEX-treated NE. Next to examine whether a protein(s) binding to the 17 bp DEX-responsible repeat have the sequence specificity, we performed the competitive EMSA using several kinds of competitor DNAs. In the presence of mock-treated NE, shifted bands disappeared by a competitor DNA containing 17 bp DEX-responsible repeat (MIE), but not by one containing SV40 21 bp repeat (SV40), suggesting that a protein(s) binds to the 17 bp DEX-responsible repeat in a sequence-specific manner (Fig. 4B, lanes 2–6). We further performed competitive EMSA using a non-radiolabeled double stranded oligonucleotide containing consensus NF-I binding sequence motif (NF-I) as competitor DNA [16]. The shifted bands disappeared by the addition of an NF-I oligonucleotide (Fig. 4B, compare lanes 3–4 and lanes 7–8), suggesting that a protein(s) binding to the 17 bp DEX-responsible repeat has binding affinity to the consensus NF-I binding sequence motif. In the presence of DEX-treated NE, almost all band shift pattern were comparable to that of mock-treated NE, indicating that DEX treatment does not change the sequence specificity of a protein(s) binding to the 17 bp DEX-responsible repeat.

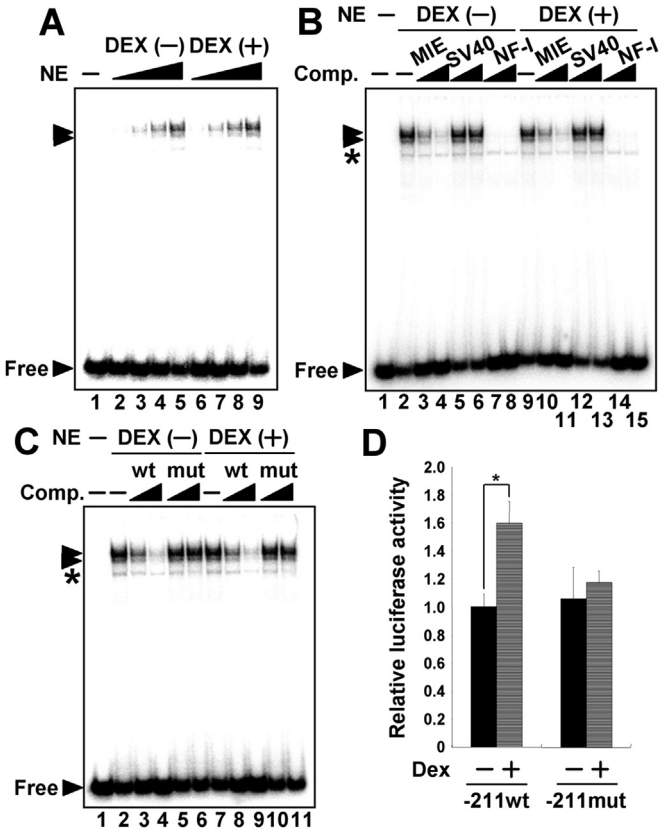

Fig. 4. Involvement of NF-I like protein that binds to the 17 bp DEX-responsible repeat in DEX-mediated transcriptional activation of the MIE promoter. (A) NE dose-dependent protein-DNA complex formation on 17 bp DEX-responsible repeat was examined by EMSA. The positions of 21 bp radiolabeled probe and protein-bound shifted DNA bands (triangles) are indicated. (B) Competitive EMSA. The radiolabeled MIE probe was incubated with each NE under the presence or absence of MIE, SV40, or NF-I competitor DNA. The asterisk (*) indicates the non-specific band. (C) The effect of point mutations on sequence motif TGGCA within the 17 bp DEX-responsible repeat was examined by competitive EMSA. The radiolabeled MIE probe was incubated with each NE under the presence or absence of MIE wild type (wt) or mutant (mut) competitor DNA. (D) Luciferase assays in cells transfected with pMIEP-Luc-210 point mutant. The luciferase activity was normalized by the SEAP activity. Results are represented as mean values ± SD from four independent experiments (*$P < 0.05$).

It was previously reported that an NF-I binding site indirectly contributes to the glucocorticoid-dependent transcriptional regulation of the cytosolic aspartate aminotransferase (cAspT) gene promoter [17]. To test such the indirect effect, we made a point mutant DNA in which an NF-I-like protein binding site was mutated. We changed the sequence motif 'TGGCA' within the 17 bp DEX-responsible repeat into 'AGCGA', and performed a competitive EMSA using this mutant oligonucleotide as competitor DNA. The mutated oligonucleotide did not show competitive property using both mock-treated and DEX-treated NE (Fig. 4C), indicating that the sequence motif 'TGGCA' is essential for an NF-I like protein(s) that binds to the 17 bp DEX-responsible repeat. Finally to address whether this NF-I-like protein is involved in the DEX-mediated transcriptional activation of the MIE promoter, we changed the sequence motif 'TGGCA' within the 17 bp DEX-responsible repeat into 'AGCGA' in pMIEP-Luc-210 (pMIEP-Luc-210 mut) (Fig. 4D). In the presence of DEX, we found that cells transfected with pMIEP-Luc-210 mut do not show any increase of the luciferase activity compared with that in the absence of DEX, while cells transfected with pMIEP-Luc-210 show approximately 1.6 times increase. These results indicate that an NF-I-like protein(s) is involved in DEX-mediated transcriptional activation of the MIE promoter through the 17 bp repeat.

4. Discussion

HCMV progeny virus production from its latent state requires MIE gene products, and it has been considered that glucocorticoid might be a causative agent. Thus, we have examined whether DEX facilitates the transcription of the HCMV MIE genes through its enhancer and promoter regions. We have demonstrated that DEX facilitates the transcription from the HCMV MIE promoter in GR- and NF-I-like protein-dependent manner. This is a first report revealing the mechanism of DEX-mediated transcriptional activation of the HCMV MIE promoter.

We first hypothesized that DEX-bound GR indirectly increases the expression level of an NF-I-like protein(s), and thereby activates the transcription of MIE genes. However, we could not observe any differences between DEX-treated and mock-treated NE as protein sources in the amount of the DNA-protein complexes (Fig. 4), suggesting that the expression level of an NF-I-like protein(s) do not change by DEX treatment. GR affects transcription through protein complex formation by direct binding to some transcription factors such as AP-1 and NFkB [9]. Thus, it is possible that DEX-bound GR directly binds to NF-I and modulates the transcriptional activity. However, it is unlikely because we could not observe any differences between DEX-treated and mock-treated NEs in the

pattern of protein-DNA complex formation (Fig. 4). It was previously reported that the inactivation of the NF-I binding site located at a site 80 base pairs upstream of a functional GRE in the cAspT promoter results in attenuation of the glucocorticoid-mediated transcriptional activation [17]. Although the mechanism how NF-I contributes to GR-mediated transcriptional activation on the cAspAT promoter is unclear, it is possible that these two factors bind to cognate binding sites close together by positioned nucleosome and cooperatively facilitates the transcription. If an unrevealed GRE exists around the MIE promoter, a similar mechanism as the cAspAT promoter may operate.

Recently, it was reported that glucocorticoid plays a role in the HCMV kinetics of latency and reactivation, and that treatment with these compounds may increase the probability of HCMV-related complications post transplantation [11]. Our findings strongly suggest that glucocorticoid metabolism is deeply involved in HCMV life cycle and its related diseases in human body. Clinically, the virus is reactivated more often in the third trimester than in the second or the first of pregnant women [2], so that the babies are exposed to HCMV at delivery and acquire immunity against HCMV with subclinical infection. Furthermore, it is suggested that HCMV infection may be due to elevation of plasma hydroxycorticoid levels during pregnancy [12]. It has never been discussed how the HCMV was reactivated in the third trimester, and this study supports a notion that increased glucocorticoid in the third trimester may reactivates HCMV virus from latency.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrc.2015.01.091.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.091.

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