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Clock gene mutation modulates the cellular sensitivity to genotoxic stress through altering the expression of N-methylpurine DNA glycosylase gene

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ABSTRACT

Although *Clock* gene product, a component of the circadian pacemaker, has been suggested to participate in the regulation of cellular sensitivity to genotoxic stress, the underlying mechanism remains to be fully understood. In this study, we showed that *Clock* gene mutation modulates the sensitivity of hepatocytes to alkylating agent-induced genotoxic stress through altering the expression of N-methylpurine DNA glycosylase (MPG), the first enzyme in the base excision repair pathway. Neither wild-type nor *Clock* mutant (*Clock*/*Clock*) mice showed a significant 24-h variation in the hepatic expression of MPG. However, the mRNA and protein levels of MPG in the liver of *Clock*/*Clock* mice were significantly lower than those in wild-type liver. The cytotoxic effect of methyl methanesulfonate (MMS), a methylating agent, on primary cultured hepatocytes prepared from *Clock*/*Clock* mice was more potent than on wild-type hepatocytes, while overexpression of MPG in *Clock*/*Clock* hepatocytes restored their MMS sensitivity to the wild-type level. These findings suggest that the product of the *Clock* gene controls the sensitivity of cells to genotoxic stress through regulating the expression of the *MPG* gene. Our present findings would provide a molecular link between the circadian clock and DNA repair pathway.

1. Introduction

Most living organisms from bacteria to humans exhibit behavioral and physiological rhythms with a period length of about 24 h. Genetic and molecular approaches have identified a basic mechanism of 24 h rhythm that is governed by interlocked transcription-translation feedback loops: the primary loop is composed of the basic helix–loop–helix transcription factors CLOCK and BMAL1, which drive transcription of the *Period* (*Per1*, *Per2*), nad *Cryptochrome* (*Cry1*, *Cry2*) genes [1,2]. Once PER and CRY proteins have reached a critical concentration, they attenuate CLOCK/BMAL1 transactivation, thereby generating circadian oscillation in their own transcription [2–4]. The interlocked loop consists of REV-ERBα and ROR, which modulate function of the *Bmal1* gene by transcriptional repression and activation [5,6]. This mechanism also regulates the 24 h variation in output physiology through the periodic expression of clock-controlled genes [7].

Clock mutant (*Clock*/*Clock*) mice have a point mutation causing the deletion of exon 19 of the *Clock* gene, thus synthesizing mutant CLOCK protein (CLOCKΔ19) deficient in transcriptional activity [8]. The mutant CLOCK protein derives low-amplitude rhythms in the expression of various genes, which leads to abnormality in behavioral and physiological rhythms [7,9]. Studies of *Clock*/*Clock* mice have suggested the role of CLOCK in a wide variety of physiological processes as diverse as obesity [10], reproduction [11], cell proliferation [12], and drug susceptibility [13]. In fact, cyclophosphamide-induced toxicities (body weight loss and myelosuppression) in *Clock*/*Clock* mice are more severe than those in wild-type mice [14]; however, the exact mechanism of CLOCK-dependent toxicity of cyclophosphamide remains to be clarified.

Alkylating agents, including cyclophosphamide, exert their cytotoxic effect by modifying the structure of a base in DNA through oxidation and alkylation reactions [15]. On the other hand, DNA damage, caused by radiation, ultraviolet irradiation, or various chemical compounds, are believed to be repaired by several DNA repair pathways [16]. The base excision repair (BER) pathway involves the recognition and removal of damaged bases by N-methylpurine-DNA glycosylase (MPG) followed by incision of the resulting abasic (AP) site by AP endonuclease 1 (APEX1), DNA synthesis by polymerase beta (POLB) and strand ligation by DNA ligase 1 (LIG1) [17]. O^6-methylguanine, a critical adduct for cell death, is reversed to O^6-methylguanine-DNA methyltransferase

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; APEX1, abasic endonuclease 1; BER, base excision repair; LDH, lactate dehydrogenase; LIG1, DNA ligase 1; MGMT, O^6-methylguanine-DNA methyltransferase; MMS, methyl methansulfonate; MPG, N-methylpurine-DNA glycosylase; MSH2, mutS homolog 2; MSH6, mutS homolog 2; MLH1, mutL homolog 1; PMS2, postmeiotic segregation increased 2; POLB, polymerase beta; UDG, uracil-DNA glycosylase; 8oxoDG, 8-oxoguanine-DNA glycosylase.

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(MGMT), and mismatched base pairs of persistence O^6-methylguanine are recognized by a mismatch repair pathway which runs mutS homolog 2 and 6 (MSH2 and MSH6), mutL homolog 1 (MLH1) and postmeiotic segregation increased 2 (PMS2) [17]. Among the genes involved in DNA repair pathways, MPG is thought to be one of the major determinants of the sensitivity of cells to methyl methanesulfonate (MMS), a potent alkylating agents producing N^7-methylguanine, because a deficient *MPG* gene in murine embryonic stem cells increases the cytotoxicity of this drug [18].

In this study, we found that CLOCK protein acted as a positive regulator for transcription of the *MPG* gene. In consequence, expression levels of *MPG* gene in the liver of *Clock*/*Clock* mice were significantly lower than those of wild-type mice. We therefore investigated how mutation of the *Clock* gene affected alkylating agent-induced cytotoxicity.

2. Materials and methods

2.1. Animals and cells

A breeding colony of *Clock* mutant mice (C57BL/6J-*Clock*$^{m1Jt/J}$) were purchased from The Jackson Laboratory (Bar Harbor, USA). Animals were kept under standard laboratory conditions (food and water *ad libitum*, room temperature, 24 ± 1 °C; relative humidity, $60 \pm 10\%$; 12:12-h light/dark cycle, lights on 7:00–19:00). Genotypes were determined by PCR according to the protocol recommended by The Jackson Laboratory. The animals were treated in accordance with the guidelines stipulated by the animal care and use committee of Kyushu University. NIH3T3 mouse embryonic fibroblasts were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). The cells were maintained in Dulbecco's modified Eagle's medium (Sigma–Aldrich Japan K.K., Tokyo, Japan) supplemented with 10% fetal bovine serum (AFC Biosciences, Lenexa, USA) at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Experimental design

To investigate the mRNA levels of gene involved in DNA repair pathway, total RNA was extracted from liver of male wild-type and *Clock*/*Clock* mice at 13:00. In order to investigate the temporal expression profile of MPG, total RNA and nuclear proteins from the liver of wild-type and *Clock*/*Clock* mice were extracted at 09:00, 13:00, 17:00, 21:00, 01:00, or 05:00. The levels of MPG mRNA and its protein were analyzed by quantitative RT-PCR and Western blotting, respectively. MPG activity in the liver of both genotype mice was also determined using the 1-N^6-ethenoadenine (εA) containing oligonucleotide, as reported previously [19]. To explore the role of *Clock* gene on the regulation of MPG expression, endogenous CLOCK in primary cultures of hepatocytes prepared from wild-type mice were down-regulated by small interfering RNA (siRNA) methods. To explore whether molecular components of the circadian clock regulate the expression of the *MPG* gene, the influence of clock gene products on transcriptional activity of the *MPG* gene was assessed using luciferase reporter constructs containing various lengths of the 5′-flanking region of the *MPG* gene. To analyze the binding of endogenous CLOCK and BMAL1 on the *MPG* promoter in the liver of wild-type mice, chromatin immunoprecipitation (ChIP) assay was performed. To investigate alkylating agent-induced cytotoxicity, primary cultures of hepatocytes were prepared from wild-type and *Clock*/*Clock* mice. Confluent cultures of hepatocytes were treated with medium containing various concentrations of MMS (Sigma–Aldrich) for 24 h. After treatment, cell viability was determined by measuring ATP levels in hepatocytes.

2.3. Quantitative RT-PCR analysis

The cDNA of mouse *MPG* (GenBank accession no. NM_010822), *8-oxoguanine-DNA glycosylase* (*8oxoDG*; BC138736), *Uracil–DNA glycosylase* (*UDG*; NM_011677), *APEX1* (NM_009687), *POLB* (NM_011130), *LIG1* (NM_001083188), *MGMT* (NM_008598), *MSH2* (NM_008628), *MSH6* (NM_010830), *MLH1* (NM_026810), *PMS2* (NM_008886), and *18srRNA* (X00686) were synthesized and amplified with a SuperScript One-Step RT-PCR System (Invitrogen). The mRNA levels of albumin D-site binding protein (*DBP*; NM_016974) and Rev-erbα (AF291821), known as components of the CLOCK-regulated gene, were also evaluated as a positive control. To evaluate the quantitative reliability of RT-PCR, kinetic analysis of amplified products was performed to ensure that signals were derived only from the exponential phase of amplification, as previously described [20].

2.4. Small interfering RNA (siRNA)

The siRNA sequence of *Clock* gene was designed for targeting the sequence CAGTGTATCAACTTCAACA and synthesized by the using BLOCK-iTTM RNAi Designer (Invitrogen, Carlsbad, USA). The scrambled sequence of CLOCK siRNA was used as a control. The oligonucleotides were transfected into primary culture of hepatocytes at a final concentration of 20 nM using Lipofectamine 2000 (Invitrogen).

2.5. Western blotting

Nuclear fractions containing 20 μg of total protein were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane that was reacted with antibodies against MPG (Imgenex Corporation, San Diego, USA), or ACTIN (Santa Cruz Biotechnology, Santa Cruz, USA). Specific antigen–antibody complexes were visualized using horseradish peroxidase-conjugated secondary antibodies and Chemi-Lumi One (Nacalai Tesque Inc., Kyoto, Japan).

2.6. Determination of MPG activity

MPG activity was measured using a double-stranded oligonucleotide (25 bp) substrate containing a single etheno-adenine (εA) lesion: 5′-GGATCATCGTTTTT(εA)GCTACATCGC-3′ (Hokkaido system sciences, Sapporo, Japan). Protein extracts were prepared from the liver of wild-type and *Clock*/*Clock* mice. 20 μg of protein extracts were resuspended in assay buffer (100 mM Tris–HCl, pH 7.2/100 mM KCl/5 mM EDTA/1 mM EGTA/5 mM 2-mercaptoethanol) and incubated with ^{32}P-5′-labeled oligonucleotide substrate (0.5 pmol) for 60 min at 37 °C. The cleaved oligonucleotide product was analyzed by electrophoretic separation on a 20% polyacrylamide gel (7 mol/L urea, Tris-borate EDTA). The reaction product was visualized by autoradiography and quantified by NIH image software.

2.7. Luciferase reporter assay

Transcription assays were performed using Lipofectamine reagent (Invitrogen). NIH3T3 cells were seeded at 1×10^5 cells/well in 6-well culture plates (BD Biosciences, San Jose, USA). After 18-h culture, cells were transfected with 100 ng/well of reporter vectors and 1–2 μg/well (total) of expression vectors, using Lipofectamine LTX reagent (Invitrogen). A 0.5 ng/well sample of phRL-TK vector (Promega, San Luis Obispo, USA) was also co-transfected as an internal control reporter. After 34-h transfection, cells were harvested and the cell lysate was analyzed using a dual-luciferase reporter assay system (Promega). The ratio of firefly

luciferase activity (expressed from reporter construct) to *Renilla* luciferase activity (expressed from pRL-TK) in each sample served as a measure of normalized luciferase activity.

2.8. Construction of reporter and expression vectors

The mouse *MPG* promoter region, a fragment spanning −939 to +239 (number is distance in base pairs from the putative transcription start site, +1; GenBank accession no. S81120) was amplified by PCR, and the product was ligated into the pGL3-Basic luciferase reporter vector (Promega). Forward deletions of *MPG* (−939)-Luc were constructed in a similar manner, using specific primers for *MPG* (−428)-Luc and *MPG* (−166)-Luc, as shown in Fig. 3C. Three proximal E-boxes on *MPG* (−428)-Luc were mutated alone or in combination with others, using a QuickChange Site-Directed mutagenesis kit (Stratagene, San Diego, USA). The changed sequences were as follows: base pairs (bp) of −394 to −389 were changed from CATCTG to CACGCG, bp of −365 to −360 were changed from CAGATG to AAGCTT, and bp of −348 to −343 were changed from CACTTG to CTCGAG. Expression vectors for mouse CLOCK, CLOCK-Δ19, BMAL1, DBP, RORα, Rev-erbα, and MPG were constructed using cDNA obtained from RT-PCR of mouse liver RNA. All coding regions were ligated into the pcDNA3.1 (+) vector (Invitrogen), as previously described [21].

2.9. ChIP assay

Cross-linked chromatins from livers were sonicated on ice, and nuclear fractions were obtained by centrifugation at 10,000 × *g* for 5 min. Supernatants were incubated with antibodies against CLOCK (Alpha Diagnostic, San Antonio, USA), Acetylated histone H3 (Upstate, Lake Placid, NY) and rabbit-IgG (Santa Cruz Biotechnology). Chromatin/antibody complexes were extracted using a protein G agarose kit (Roche Diagnostics, Mannheim, Germany). DNA was isolated using the GeneElute Mammalian Genomic DNA kit (Sigma–Aldrich) and subjected to PCR using the following primer pairs: for the surrounding E-boxes in *MPG* promoter (from bp −481 to −311), 5′-CTC-TTCGTAGCAATTCCAGGC-3′ and 5′-CCCAACCTTATCTCTGAATG-GTG-3′; for *MPG* promoter that does not contain E-boxes (from bp −2199 to −2068), 5′-TGGATCCTGGACACTACCTA-3′ and 5′-CTGAGTCCAATACCATGAGG-3′; for the surrounding E-boxes in *Cry1* promoter, 5′-GCACGCGGGGGTCTGAGCCA-3′ and 5′-CCGGTCCCGAGGCTGCCCG-3′. The quantitative reliability of PCR was evaluated by the same method as described in the above section. As negative controls, chromatin immunoprecipitations were performed in the absence of antibody or in the presence of rabbit IgG. PCR products from these samples were not detectable by ethidium bromide staining.

2.10. Preparation of primary hepatocyte cultures

Wild-type and *Clock*/*Clock* mice were anesthetized with urethane, and liver cells were isolated by sequential perfusion with collagenase, and purified by density gradient separation. Cells were resuspended in hepatocyte maintenance medium (Lonza, Walkersville, USA), and seeded at 2×10^5 cells/well in 24-well tissue culture plates (Nalge Nunc International KK, Tokyo, Japan). After an attachment period of 4 h, media were changed to Dulbecco's modified Eagle's medium (Sigma–Aldrich) supplemented with insulin and dexamethasone using an HMM SingleQuots kit (Walkersville, USA). The primary cultures of hepatocyte were also transfected with MPG expression constructs. The MPG-overexpressing cells were used for investigating the cytotoxic effect of MMS.

2.11. Determination of cell viability

Cell viability was determined by measuring the level of ATP in cells at 24 h after treatment with MMS using the CellTiter-Glo luminescence cell viability assay system (Promega), according to the manufacturer's instructions.

2.12. Statistical analysis

Significance of differences between two groups was analyzed by Student's *t*-test. The statistical significance of differences among groups was analyzed by ANOVA and post hoc Bonferroni comparisons. A 5% level of probability was considered significant.

3. Results

3.1. CLOCK protein acts as a positive regulator of the expression of MPG gene

To explore whether the expression of genes involved in DNA repair pathways is altered by *Clock* mutation, we investigated the mRNA levels of *MPG*, *8oxo DG*, *UDG*, *APEX1*, *POLB*, *LIG1*, *MGMT*, *MSH2*, *MSH6*, *PMS2*, and *MLH1* in the liver of male wild-type and *Clock*/*Clock* mice. Among the genes, mRNA levels of *MPG* in the liver

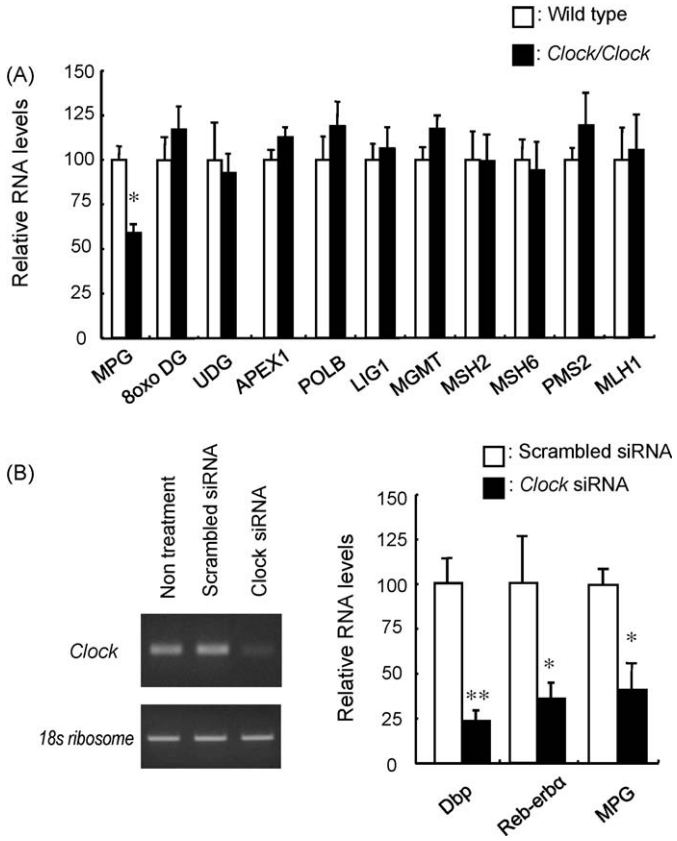

Fig. 1. Influence of *Clock* mutation on hepatic expression of genes involved in DNA repair pathway. (A) Comparison of mRNA levels of the gene involved in DNA repair pathway in liver of *Clock*/*Clock* mice with those of wild-type mice. Mean values of wild-type mice are set at 100. Each value represents the mean ± S.E. ($n = 6$). $^{*}p < 0.05$ when compared between two genotypes. (B) Influence of down-regulation of *Clock* gene on the hepatic expression of Dbp, Rev-erbα, and MPG. Left panel shows representative photographs of down-regulation of *Clock* gene by siRNA. Primary cultures of wild-type hepatocytes were transfected with scrambled siRNA (Control siRNA; 20 nM) or specific siRNA for CLOCK (CLOCK siRNA; 20 nM). Non-transfected cells were served as non-treated control cells. Mean values of random oligo (control) transfected cells are set at 100. Each value represents the mean ± S.E. ($n = 4$). $^{**}P < 0.01$; $^{*}p < 0.05$ when compared with random oligo (control) transfected cells.

of *Clock*/*Clock* mice was significantly lower than that in wild-type liver ($p < 0.05$; Fig. 1A), whereas the mRNA levels of other genes did not differ significantly between the two genotypes. A similar genotype-dependent difference in the mRNA levels of MPG was also observed in female mice (Supplemental data 1). As expected, a significant decrease in the mRNA levels of CLOCK-regulated gene, *Dbp* and *Rev-erbα*, was observed when *Clock* gene was down-regulated in primary culture of wild-type hepatocytes (Fig. 1B). The *Clock* gene down-regulation also caused a significant reduction of MPG mRNA levels (Fig. 1B). These results suggest that hepatic expression of the *MPG* gene, at least in part, depends on the function of CLOCK protein.

CLOCK protein acts as a regulator of circadian gene expression [7,9]. We thus investigated how the mutation of *Clock* gene affected the temporal expression of *MPG* gene. As reported previously [5,22], the amplitude of the oscillation in the mRNA levels for CLOCK-target genes, *Dbp* and *Rev-erbα*, was decreased significantly in the liver of *Clock*/*Clock* mice (Fig. 2A). However, no obvious fluctuation in the expression of MPG mRNA was found in the liver of wild-type mice (Fig. 2B). The expression of MPG mRNA in the liver of *Clock*/*Clock* mice also failed to show a significant circadian oscillation, but the mRNA levels were reduced throughout the day (Fig. 2B). Consequently, the protein levels of MPG and its enzymatic activity in the liver of *Clock*/*Clock* mice were also lower than those in wild-type mice (Fig. 2C and D). Taken together, these results suggest that CLOCK protein acts as a positive regulator of the expressions of the *MPG* gene but does not derive its rhythmic expressions.

3.2. Transcriptional regulation of MPG gene by CLOCK gene products

To investigate whether CLOCK regulates the expression of MPG at the transcriptional level, we performed a transient transcriptional assay using luciferase reporter construct containing the 5′-flanking region of the *MPG* gene. The fragment-spanning from bp −939 to +239 of mouse *MPG* gene contains five E-box elements (CANNTG), but none of these E-boxes corresponds to the CACGTG sequence, the strict consensus-binding site for CLOCK/BMAL1 heterodimer [23]. We thus explored whether these E-boxes on the *MPG* promoter are functional in response to CLOCK/BMAL1. As shown in Fig. 3A, co-transfection of *MPG* (−939)-Luc with CLOCK and BMAL1 resulted in a 2.3-fold increase in transcriptional activity; however, other components of circadian clock, DBP, RORα, and REV-ERBα, had little effect on the MPG promoter activity. No significant increase in the transcriptional activity of *MPG* (−939)-Luc was also observed when cells were transfected with CLOCK-Δ19 and BMAL1 (Fig. 3B). As CLOCK-Δ19 is deficient in transactivation function despite still having DNA binding ability [9], the mutated protein might have a subtle effect on the activity of *MPG* (−939)-Luc reporter. These findings suggest that CLOCK protein is functionally important for transcription of the *MPG* gene.

To identify the critical regions responsible for CLOCK/BMAL1-mediated transactivation of the *MPG* gene, we carried out a transient transcriptional assay using *MPG* luciferase reporter constructs containing various lengths of the 5′-flanking region

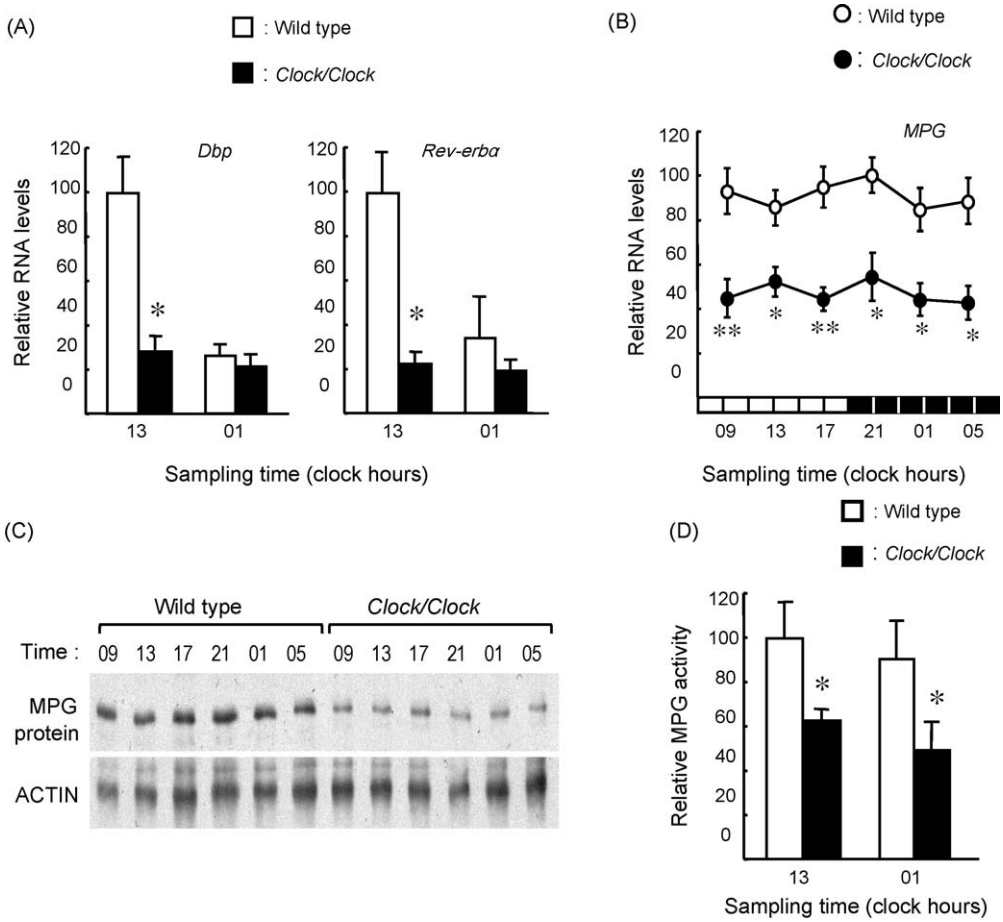

Fig. 2. Influence of *Clock* mutation on hepatic expression of MPG. (A) Temporal expression profiles of Dbp and Rev-erbα in liver of wild-type and *Clock*/*Clock* mice. The mean value of wild-type mice at 13:00 is set at 100. Each value represents the mean ± S.E. ($n = 3$). $^{*}p < 0.05$ when compared with wild-type mice. (B) Temporal expression profiles of MPG mRNA in liver of wild-type and *Clock*/*Clock* mice. The mean peak value of wild-type mice is set at 100. Each value represents the mean ± S.E. ($n = 3$). (C) Temporal expression profiles of MPG protein in liver of wild-type and *Clock*/*Clock* mice. (D) Temporal profiles of MPG activity in liver of wild-type and *Clock*/*Clock* mice. The mean value of wild-type mice at 13:00 is set at 100. Each value represents the mean ± S.E. ($n = 3$). $^{*}p < 0.05$ when compared with wild-type mice.

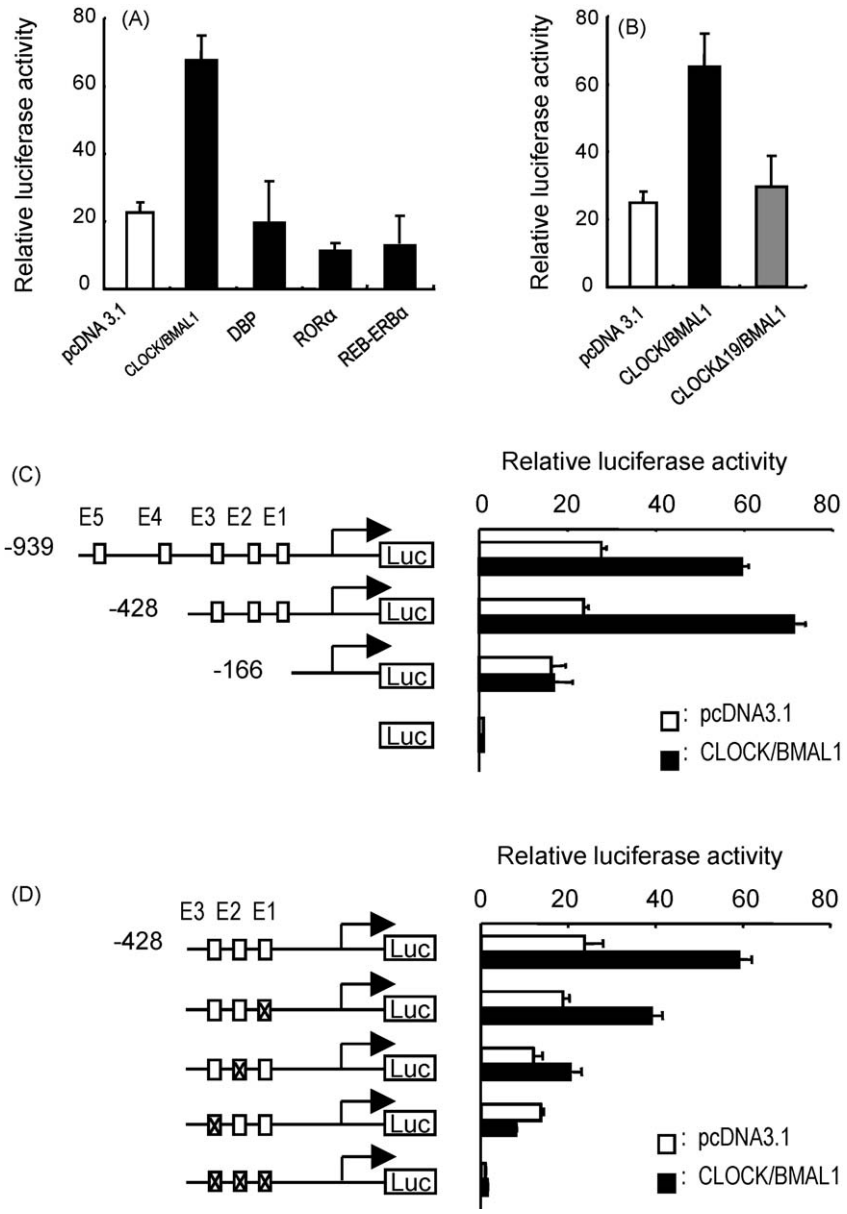

Fig. 3. Transcriptional regulation of *MPG* promoter activity by clock gene products. (A) NIH3T3 cells were transfected with MPG (−939)-Luc reporter constructs in the presence or absence of expression plasmids, encoding CLOCK, BMAL1 (0.5 μg each), DBP, RORα or REV-ERBα (1.0 μg each). Each value represents the mean ± S.E. (n = 3). (B) Cells were transfected with MPG (−939)-Luc reporter constructs in the presence or absence of expression plasmids, encoding CLOCK, BMAL1 or CLOCK-Δ19 (0.5 μg each). Each value represents the mean ± S.E. (n = 3). (C) Cells were transfected with luciferase reporter constructs containing various lengths of the 5′-flanking region of MPG gene in the presence or absence of CLOCK/BMAL1 expression plasmids (0.5 μg each). Each value represents the mean ± S.E. (n = 6). (D) Schematic representation of wild-type or E-boxes mutated MPG promoter reporters is shown on the left, and their corresponding luciferase activities are shown on the right. Each value represents the mean ± S.E. (n = 3–6).

of the *MPG* gene. As shown in Fig. 3C, the transactivation effect of CLOCK/BMAL1 on the *MPG* promoter was markedly attenuated by elimination of the sequences up to −166; *MPG* (−166)-Luc, suggesting that the CLOCK/BMAL1 response region in the *MPG* gene is located between bp −428 and −166 with respect to the transcriptional start site. The region between bp −428 and −166 of the *MPG* gene contains three E-boxes. To identify the regulatory E-box element, three proximal E-boxes on the *MPG* (−428)-Luc were mutated alone or in combination with others, as shown in Fig. 3D. While the introduction of mutation in the E1- or E2-box of the *MPG* (−428)-Luc attenuated CLOCK/BMAL1-mediated transactivation, the reporter constructs, including mutation in the E3-box, showed no significant response to CLOCK/BMAL1. Furthermore, mutation of all three E-boxes (E1, E2, and E3) on *MPG* (−428)-Luc not only decreased basal transcriptional activity, but also abrogated the responsiveness of the promoter to CLOCK/BMAL1, suggesting that the E-boxes were responsible for CLOCK/BMAL1-mediated transactivation of the *MPG* gene.

3.3. Binding of CLOCK protein to proximal E-box region of MPG gene

We next examined the binding of endogenous CLOCK protein in the liver to proximal E-boxes containing the *MPG* promoter by ChIP assay. By means of gene-specific primers, E-boxes contained in the *MPG* (−481 to −311) promoter were detected in both input-DNA as well as in immunoprecipitated DNA (Fig. 4). Taken together, these results suggest that CLOCK protein directly bind to E-boxes in the *MPG* promoter and induce its transactivation.

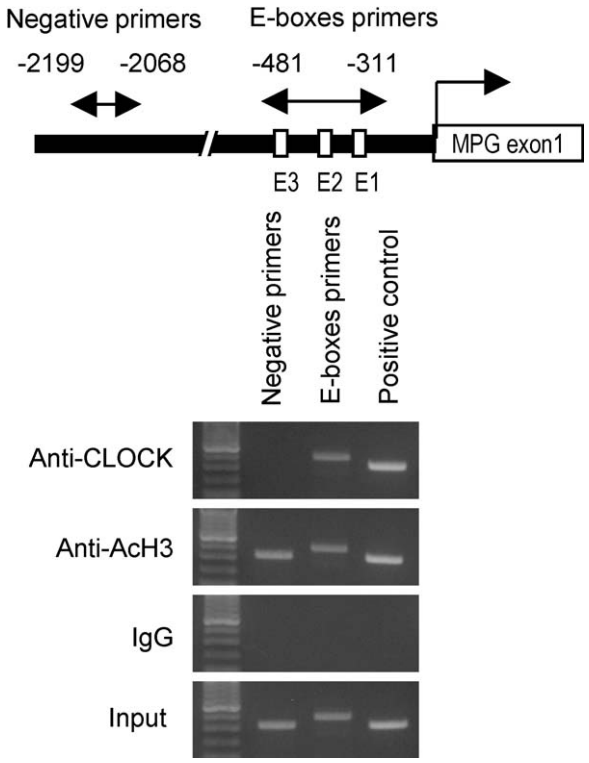

Fig. 4. CLCOK and BMAL1 binding to E-boxes on the *MPG* promoter. Schematic structures of the sequence of 5′-flanking region of *MPG* gene is illustrated in upper panels. Solid line arrows indicate the primer sets (A and B) for amplification area by PCR. Primer sets for *Cry1* E-box indicates the amplification of the functional E box-containing region on the *Cryptochorme 1* gene promoter [30]. Cross-linked chromatins from livers collected were immunoprecipitated with antibodies against CLOCK, Acetylated histone H3 (AcH3), or IgG. Representative electrophoretic image of PCR products are shown in lower panel.

3.4. Modulation of MMS-induced cytotoxicity by Clock gene mutation

Because the hepatic expression of *MPG* was decreased by *Clock* gene mutation, we explored whether CLOCK protein associated with the sensitivity of hepatocytes to alkylating agents. MPG repairs 3-alkyladenine, the major cytotoxic lesion resulting from alkylating agents and functions to cleave N^7-alkylguanine, the principal product of alkylation in DNA [17]. As N^7-methylguanine and 3-methyladenine are the primary lesions induced by MMS treatment [24], we investigated the effects of MMS on primary hepatocytes prepared from wild-type and *Clock*/*Clock* mice. As shown in Fig. 5A, the viabilities of hepatocytes prepared from both genotypes were decreased in an MMS concentration-dependent manner ($p < 0.01$); however, the cytotoxic effect of MMS on *Clock*/*Clock* hepatocytes was more severe than on wild-type hepatocytes. LD_{50} values of *Clock*/*Clock* hepatocytes (0.13 mM) were approximately 2-fold greater than those of the wild-type (0.24 mM). MMS-induced cytotoxicity of *Clock*/*Clock* hepatocytes was attenuated by transfecting cells with MPG expression constructs (Fig. 5B), suggesting that modulation of MMS sensitivity induced by *Clock* gene mutation is attributable to the decrease in MPG expression levels. *Clock* gene acts as a determinant for cellular sensitivity to MMS-induced genotoxic stress.

4. Discussion

Although there was no obvious circadian oscillation in the expression of *MPG* mRNA and its protein in the liver of wild-type mice, their expression levels in the liver of *Clock*/*Clock* mice were

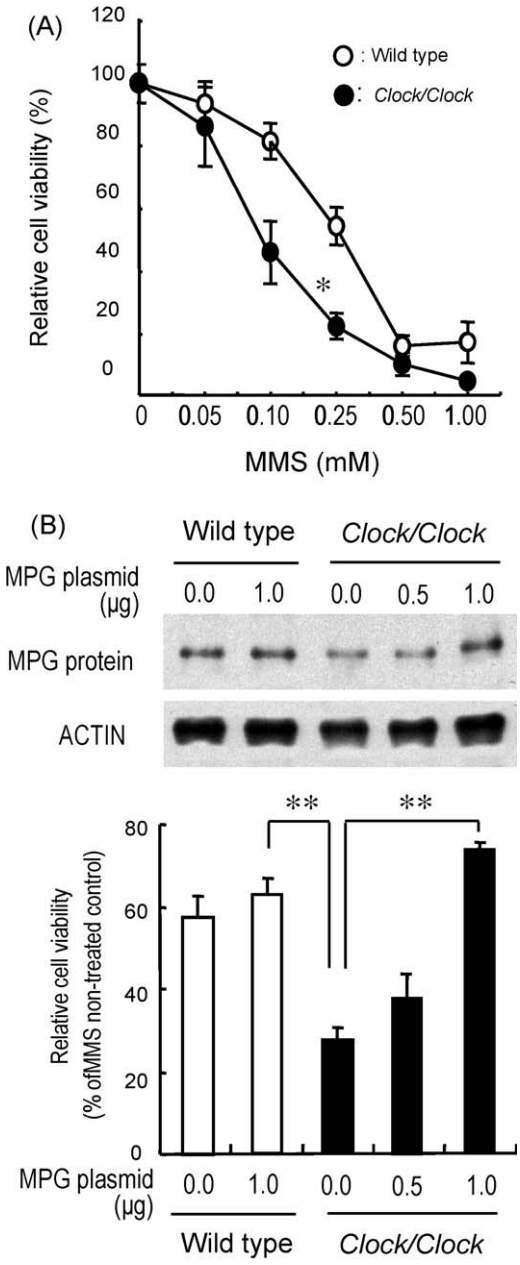

Fig. 5. Cytotoxic effect of MMS on primary cultured hepatocytes prepared from wild-type and *Clock*/*Clock* mice. (A) Cells were treated with MMS at the indicated concentration for 24 h, and cell viability was determined by assessing ATP levels. The cellular ATP level of non-treated group is set at 100. Each value represents the mean ± S.E. ($n = 4$). $^*p < 0.05$, for compared between two genotypes at corresponding concentrations. (B) Overexpression of MPG attenuates MMS-induced cytotoxicity in *Clock*/*Clock* hepatocytes. MPG expression vector- or empty vector (0 control)-transfected hepatocytes were treated with 0.25 mM MMS for 24 h. Each value represents the mean ± S.E. ($n = 4$). $^*P < 0.05$, when compared between two groups.

40–60% lower than those of wild-type mice. The decreased *MPG* expression in *Clock*/*Clock* hepatocytes was likely to diminish its enzymatic activity. The results of luciferase reporter assays and ChIP analysis revealed that CLOCK formed heterodimer with BMAL1 and transactivated the *MPG* gene through proximal E-boxes located between −428 and −166 from the transcriptional start site; however, these proximal E-boxes did not correspond to the consensus sequence (CACGTG) for the CLOCK/BMAL1 binding [7]. In addition to disrupting the rhythmicity of circadian genes [8,9], *Clock* mutation has been shown to affect the expression of many non-rhythmic genes [9,25], suggesting

that CLOCK protein is also essential for the control of non-circadian functions of cell physiology. It should be noted that genes showing circadian expression have short half-lives [20]. However, half-life of *MPG* mRNA was much longer than that of *Dbp* and *Rev-erbα* (Supplemental data 2). It is thus possible that the stability of *MPG* mRNA prevents its rhythmic expression.

Since CLOCK protein positively and negatively regulates the expression of a variety of genes [7,8,25], mutation of this protein is likely to influence many aspects of cell physiology. The cytotoxicity of MMS on hepatocytes derived from *Clock*/*Clock* mice was more severe than that on wild-type hepatocytes. These findings suggest that mutation of the *Clock* gene caused an increase in the sensitivity of hepatocytes to MMS. MPG is thought to be one of the major determinants of the sensitivity of cells to MMS, because a deficient *MPG* gene in murine embryonic stem cells results in an increase in the cytotoxicity of MMS [18]. In addition, suppression of the *MPG* gene in human epithelial carcinoma by siRNA also increases the sensitivity of the cells to another types of alkylating agents [26]. In fact, overexpression of MPG in *Clock*/*Clock* hepatocytes restored their MMS sensitivity to the wild-type level. It is thus conceivable that low-level expression of the *MPG* gene in *Clock*/*Clock* hepatocytes is associated with the enhancement of MMS-induced cytotoxicity.

It has, however, been reported that MPG knockout mice show a mild phenotype when they are treated with MMS [27]. We are thus unable to rule out the possibility that another mechanism, except for decrease in MPG activity, is also involved in the enhancement effect of *Clock* gene mutation on MMS-induced cytotoxicity. In fact, recent study has suggested that the DNA repair capacity in the mouse cortex varied depending on the circadian oscilation of xeroderma pigmentosum A DNA damage recognition protein [28]. On the other hand, the effectiveness and/or toxicity of many drugs depend not only on the sensitivity of living organisms to the drug, but also on its pharmacokinetics [29]. DNA microarray analysis by using *Clock*/*Clock* mice have revealed that mutation of the *Clock* gene causes an alteration in the hepatic expression of many genes involved in xenobiotic detoxification [7]. The alteration may also contribute to genotype-dependent difference in MMS-induced hepatotoxicity. Further study will be required to investigate whether the disposition of MMS in *Clock*/*Clock* mice differs from that in wild-type mice.

The present findings in this animal model suggest that CLOCK protein acts as a positive regulator for expression of the *MPG* gene and is a critical determinant of cellular sensitivity to the alkylating agent MMS. Our present findings will provide a clue to clarify the mechanism for transcriptional regulation of the *MPG* gene by CLOCK protein and will help to understand how mutation of the *Clock* gene causes an alteration in cellular sensitivity to the alkylating agent-induced genotoxic stress.

Disclosure statement

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.06.013.

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