Biochemical and Biophysical Research Communications 380 (2009) 22–27

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# The molecular mechanism regulating the autonomous circadian expression of Topoisomerase I in NIH3T3 cells

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ARTICLE INFO

*Article history:*
Received 28 December 2008
Available online 10 January 2009

*Keywords:*
Topoisomerase I
Circadian rhythms
Promoter analysis
Antitumor drugs
Clock genes

ABSTRACT

To identify whether Topoisomerase I (TopoI) has autonomous circadian rhythms regulated by clock genes, we tested mouse TopoI (mTopoI) promoter oscillation in NIH3T3 cells using a real-time monitoring assay and TopoI mRNA oscillations using real-time RT-PCR. Analysis of the mTopoI promoter region with MatInspector software revealed two putative E-box (E1 and E2) and one DBP/E4BP4-binding element (D-box). Luciferase assays indicated that mTopoI gene expression was directly regulated by clock genes. The real-time monitoring assay showed that E-box and D-box response elements participate in the regulation of the circadian expression of mTopoI. Furthermore, a gel-shift assay showed that E2 is a direct target of the BMAL1/CLOCK heterodimer and DBP binds to the putative D-site. These results indicate that TopoI is expressed in an autonomous circadian rhythm in NIH3T3 cells.



## Introduction

Topoisomerase I (TopoI), an essential enzyme in relaxing DNA supercoiling [2], is a nuclear enzyme abundantly expressed throughout the cell cycle and a nuclear target of the antitumor drug camptothecin (CPT) [12]. The circadian rhythm of CPT-11 is dependent on various factors such as mortality, body weight loss, leukopenia, and neutropenia [3,14]. The antitumor activities of CPT and its analogs depend on their binding potencies to the transient, cleavable complex formed between DNA and TopoI [3].

In mammals, circadian rhythms in behavior and physiology are regulated by a central clock located in the suprachiasmatic nucleus of the hypothalamus [16]. Disruption of the circadian rhythm leads to an increased incidence of many diseases, such as cancer and mental illness. Time-dependent drugs have been widely used to improve the effect of treatment and clarify the molecular mechanism of a drug target gene in the treatment of diseases such as CPT-11 for cancer [12] and lithium for bipolar disorder [10]. Furthermore, the circadian clock also controls several cell and organism functions and interacts with the cell division cycle in a 24 h timescale. For example, the cell division cycle gene, *Wee1*, is controlled by circadian clock genes (e.g., *Bmal1*, *Per2*), which negatively control the activity of *CDK1/CyclinB1* in the regulation of the G2-M transition [4]. DNA synthesis is irreversibly inhibited by CPT–TopoI complexes, and S-phase cells cannot progress to the G2-phase of the cell cycle [11]. These studies indicate that TopoI and *Wee1* have similar functions in regulating the cell cycle. Therefore, TopoI may be controlled by circadian clock genes, as in the case of *Wee1*.

The circadian change levels of endogenous glucocorticoid hormones are involved in the circadian regulation of TopoI gene expression, and in tumor-bearing mice, the antitumor efficacy of the TopoI inhibitor, CPT-11, may be enhanced by administering the drug when TopoI activity increases [9,17]. Currently, whether TopoI has a glucocorticoid-independent circadian rhythm regulated by clock genes remains unclear. Furthermore, to establish the molecular basis of a time treatment for antitumor drugs, one must clarify the molecular mechanism of TopoI expression.

Cellular rhythms are generated and maintained through the interconnected transcriptional feedback of clock genes [18]. Moreover, genetic analyses have identified circadian regulation of gene expression through the E-box [5], DBP/E4BP4-binding elements (D-box) [13], and RevErbA/ROR-binding elements (RREs) [21]. Two basic helix–loop–helix–PAS (bHLH–PAS) domain proteins of BMAL1 and CLOCK [5,6] combine to form the BMAL1/CLOCK heterodimer and bind to the E-box to activate several clock genes, including *Per1*, *Per2*, *Cry1*, and *Cry2* [18]. Two bZip-family genes (*DBP*, *E4BP4*) are also thought to function in the circadian regulation of gene expression [13].

Here, we measured the levels of mouse TopoI (mTopoI) promoter activity and mRNA in NIH3T3 cells after dexamethasone

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doi:10.1016/j.bbrc.2008.12.186

treatment. The mTopoI promoter activity and mRNA level oscillated over an approximately 24 h cycle. By analyzing the mTopoI promoter region, we found that the E-boxes (E1 and E2) and the D-box, potential transcription factor-binding motifs, are 100% conserved between mice and humans and that the E2 and D-box are located downstream of the transcriptional start site in exon 1.

## Materials and methods

*Mouse TopoI promoter cloning, mutagenesis, and sequence analysis*. The mTopoI promoter fragment was isolated by PCR amplification of the mouse genomic DNA (Novagen) and subcloned into a customized vector ELuc-PEST-T (Toyobo). The E-box and D-box mutants were created using the QuickChange site-directed mutagenesis kit (Stratagene). Four mutated primers were used in this study: mTopoI-E1mut: 5′-CACGCTTCAATCCAGGGTGTGGGGGGCAGAGGC-3′; mTopoI-Dbox-mut: 5′-GAAATGCGAACTTAGGCTGGACACAACTGCTGGGG-3′; mTopoI-E2mut: 5′-GAACTTAGGCTGTTACATTACTGCTGGGGTCTGTTC-3′; mTopoI-E2/Dmut: 5′-GAACTTAGGCTGGGACATTACTGCTGGGGTCTGTTC-3′. The substituted bases are underlined. Analyses of sequences were performed with an automated 377 DNA Sequencing System (Applied Biosystems).

*Real-time reporting of circadian-regulated gene expression by luciferase bioluminescence in mammalian cells*. NIH3T3 cells were seeded in 35-mm dish, two days before transfection, and 500 ng of the reporter plasmid was transfected by using Lipofectamine PLUS (Invitrogen) according to manufacture's instructions. One days after transfection, the cells were treated with 100 nM of dexamethasone (Nakalai Tesques, Kyoto, Japan) for 2 h, and the medium was replaced with DMEM in the absence of phenol red supplemented with 10% FBS and 100 μM D-luciferin (Toyobo), and overlaid with mineral oil (Sigma–Aldrich) to prevent evaporation. Bioluminescence was measured at 37 °C under 5% $CO_2$ atmosphere and integrated for 1 min at interval of 10 min using a dish-type luminometer AB2500 Kronos (ATTO). Bioluminescence activity was expressed as relative light unit (RLU).

*Analysis of circadian rhythms in bioluminescence*. The cells were cultured in the luminometer for more than 4 days counting their bioluminescence. To compare the phase and amplitude of WT and mutant mTopoI promoters, the obtained crude data (10 min bins) were smoothed by a 10-point moving average method and detredned by subtracting 12 h moving average from smoothed data.

*Quantification of mRNA by real-time RT-PCR*. NIH3T3 cells were harvested at 4-h intervals. Total RNA from NIH3T3 cells was extracted using ISOGEN (Nippongene) and reverse-transcribed. The TopoI and GAPDH transcripts were quantified using an ABI prism 7300 (Applied Biosystems). PCRs were performed using the One Step SYBR® PrimeScript® RT-PCR Kit (Takara) with the following thermal cycling parameters: 94 °C for 5 min followed by 40 cycles at 94 °C for 20 s and 62 °C for 1 min. The GAPDH transcript was used for normalizing the expression of each transcript. Four specific primer pairs were used: GAPDH: 5′-TGCACCACCAACTGCTTAG-3′ and 5′-GGATGCAGGGATGATGTTC-3′; TopoI: 5′-TTCCCAGATCGAAGCGGATTTC-3′ and 5′-GCAGCTCTAATCTTTTCCTCC -3′.

*Confirmation of D and E2 located downstream of the transcription start site*. One pair of primers [TopoI-U: 5′-GAACTTAGGCTGTTACACAACTGCTGGGGTCTGTTC-3′ (+8 to +42); TopoI-D: 5′-CAGCTCTAATCTTTTCCTCC-3′ (+268 to +286)] was designed (Fig. 2B). The cDNA fragments were isolated by PCR amplification of the reverse-transcribed RNA from NIH3T3 cells. The PCR product was analyzed by electrophoresis on a 0.8% agarose gel (Fig. 2D) and subcloned into a TA vector (Invitrogen) and sequenced.

*Cell transfection and transient luciferase assay*. Luciferase assay was examined as described previously [23]. NIH3T3 cells were transfected using lipofectamine (Invitrogen). The luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega) and a Fluoroskan Ascent FL luminometer (Labsystems).

*Gel-shift assay*. Gel shifts were examined as previously described [15]. BMAL1, CLOCK, and DBP proteins were synthesized using the TNT T7 coupled Reticulocyte Lysate System (Promega). The oligonucleotide probe sequences used for the analysis were as follows: mTopoI-E1: 5′-GCCTTTAAGGCCTACCACGTGGCCGCCCGGGTG-3′; mTopoI-E1-mut: 5′-GCCTTTAAGGCCTACGGTGTGGCCGCCCGGGTG-3′; mTopoI-E2: 5′-GAACTTAGGCTGTTACACAACTGCTGGGGTCTGTTC-3′; mTopoI-E2-mut: 5′-GAACTTAGGCTGTTACATTACTGCTGGGGTCTG TTC-3′; mTopoI-D: 5′-GAAATGCGAACTTAGGCTGTTACACAACTGCTGGGG-3′; mTopoI–D-mut: 5′-GAAATGCGAACTTAGGCTGGGACACAACTGCTGGGG-3′. The underlined portion indicates the WT- and mutant-binding sequences, respectively.

## Results and discussion

### *Circadian expression of mTopoI gene in NIH3T3 cells*

Kuramoto et al. [9] found that the oscillation in TopoI mRNA levels was caused by circadian changes in circulating glucocorticoid levels. However, whether mTopoI has glucocorticoid-independent circadian rhythms regulated by clock genes is unclear. We examined the rhythmicity of TopoI mRNA expression in NIH3T3 cells using an ABI prism 7300 (Applied Biosystems). After 24 h of dexamethasone treatment, mTopoI mRNA levels displayed an overt rhythm (Fig. 1A). To test the transcriptional oscillation of mTopoI, 646 bp of the mTopoI promoter sequence containing exon1 were cloned (Fig. 2A), and a real-time monitoring assay was performed using Kronos (AB-2500, ATTO). A luciferase-bound promoter displayed circadian rhythms after 2 h of dexamethasone treatment (Fig. 1B). These results suggest that in NIH3T3 cells, TopoI has a circadian rhythm independent of glucocorticoid hormones.

### *Analysis of the region of the mTopoI promoter*

Kunze et al. [8] and Baumgartner et al. [1] analyzed the topoI promoter region sequences from humans and mice, and they found that the regulatory elements of the promoter in these two species are conserved. We confirmed these results in our study. We focused on transcription factors participating in the circadian regulation of gene expression by analyzing the 646-bp region upstream of exon 1 for transcription factor-binding motifs using MatInspector (Genomatix). Sequence analysis revealed one E-box site (CACGTG) (named E1), one Dbox site (named D), and an E'-box, similar to the consensus E-box site (CAACTG) (named E2), with D and E2 located downstream of the transcription start site (TSS) (Fig. 2A and B). The sequences of E1, D, and E2 are 100% conserved between mice and humans (Fig. 2C). A real-time monitoring assay (Fig. 1B) indicated that the promoter region we cloned is sufficient for producing the circadian transcriptional oscillation. We also confirmed that D and E2 located downstream of the transcription start site because there is no general acknowledgement that transcription factor binding sites are located in 5'UTR (Fig. 2D).

### *E-box and D-box response elements participate in the regulation of the circadian expression of mTopoI*

To test the importance of the E1/E2/D sites in the transcriptional oscillation of the mTopoI promoter, we prepared several mutant mTopoI promoters, including wild type (WT) and E1, E2, Dbox, E1/Dbox, E1/E2, and E1/E2/Dbox mutant types, and measured bio-

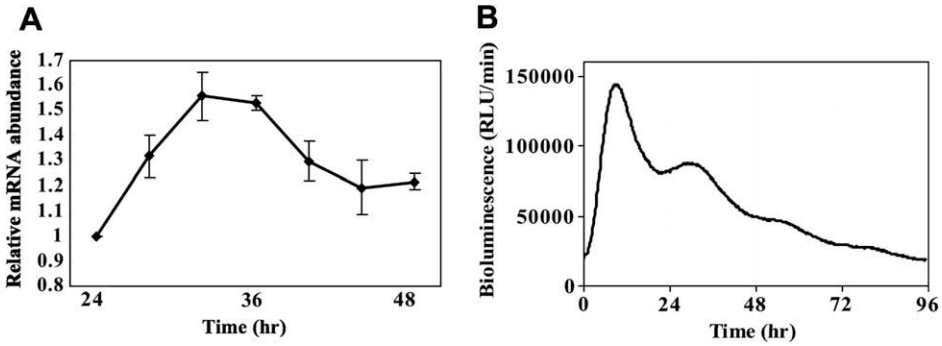


**Fig. 1.** Gene expression in NIH3T3 cells. (A) Transcript levels of TopoI were determined by real-time PCR for three plates at each time point. Total RNA was extracted every 4 h, beginning 24 h after treatment with dexamethasone for one 24 h cycle, and TopoI transcripts were quantified. Error bars indicate the standard deviations of mean values ($n = 3$). Time series data were analyzed by one-way ANOVA for rhythmicity ($P < 0.05$) in the cells. (B) Real-time monitoring of the luciferase activity of the mTopoI promoter showed that activity oscillated over an approximately 24 h cycle. The luciferase activities are shown on the *y*-axis and the measurement of time after starting bioluminescence is shown on the *x*-axis. These cultures clearly had circadian rhythms.

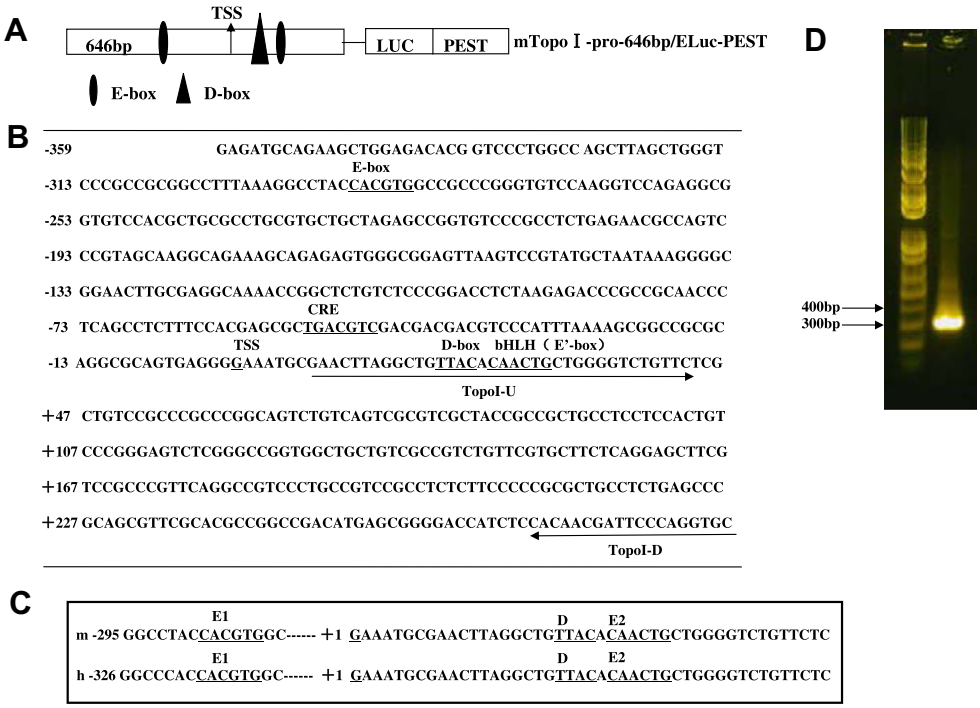


**Fig. 2.** The structure of the TopoI promoter and an analysis of the potential transcription factor-binding motifs in this region. (A) The mTopoI upstream fragment was isolated by PCR amplification from mouse genomic DNA (Novagen), and subcloned into an ELuc-PEST-T-vector (Toyobo) using the TA cloning method. The 646-bp region contains two putative E-boxes [an E-box (CACGTG): named E1, an E′-box (CAACTG): named E2] and one D-box (named D) response element. (B) We identified several potential transcription factor-binding motifs in this promoter region, including the E-box, CRE, D-BOX, and E′-box, using a computer-based analysis of the mTopoI promoter region with MatInspector software. The D-box and E′-box were located downstream of the TSS. (C) Sequence comparisons: upper line, mouse sequence; lower line, human sequence. The nucleotide sequence of potential transcription factor-binding motifs for E1, E2, and D are 100% conserved between mice and humans. (D) D and E2 located downstream of the transcription start site (TSS) were confirmed. The cDNA fragments were isolated by PCR amplification of the reverse-transcribed RNA from NIH3T3 cells. The PCR product (about 300 bp) was analyzed by electrophoresis on a 0.8% agarose gel. One pair of primers: TopoI-U and TopoI-D were used. lane 1: molecular marker, lane 2: PCR product.

luminescence using a real-time monitoring assay (Fig. 3). Superimposing the oscillation profiles of the mutant constructs on that of the WT showed that the amplitude of oscillation in the E1 and D box mutants was lower than that in the WT (Fig. 3A and C), whereas the amplitude of oscillation of the E2 mutant was almost lost (Fig. 3B). Note that the circadian rhythm of the mTopoI promoter in the E1/D box, E1/E2, E1/E2/D box and E2 mutants was almost similar (Fig. 3B, D–F). No change was observed in the phase of oscillation in the all of the mutants (Fig. 3A–F). These results suggest that the E-box and D-box response elements are very important for the circadian expression of mTopoI and have no influence on the phase of oscillation.

### *mTopoI transcription is directly regulated by BMAL1/CLOCK heterodimer and DBP though E-box/D-box*

To clarify whether mTopoI transcription was regulated by bHLH-PAS and PAR bZip transcription factors through E-box/D-box, we tested the effects of BMAL1, CLOCK, and DBP on mTopoI expression using the luciferase assay. The data indicate that BMAL1 or CLOCK alone had no apparent effect on mTopoI reporter activity, but a combination of BMAL1 and CLOCK increased mTopoI transcriptional activity when compared to the control and the transcriptional enhancements were dose-dependent. In addition, DBP upregulated mTopoI transcription (Fig. 4A).

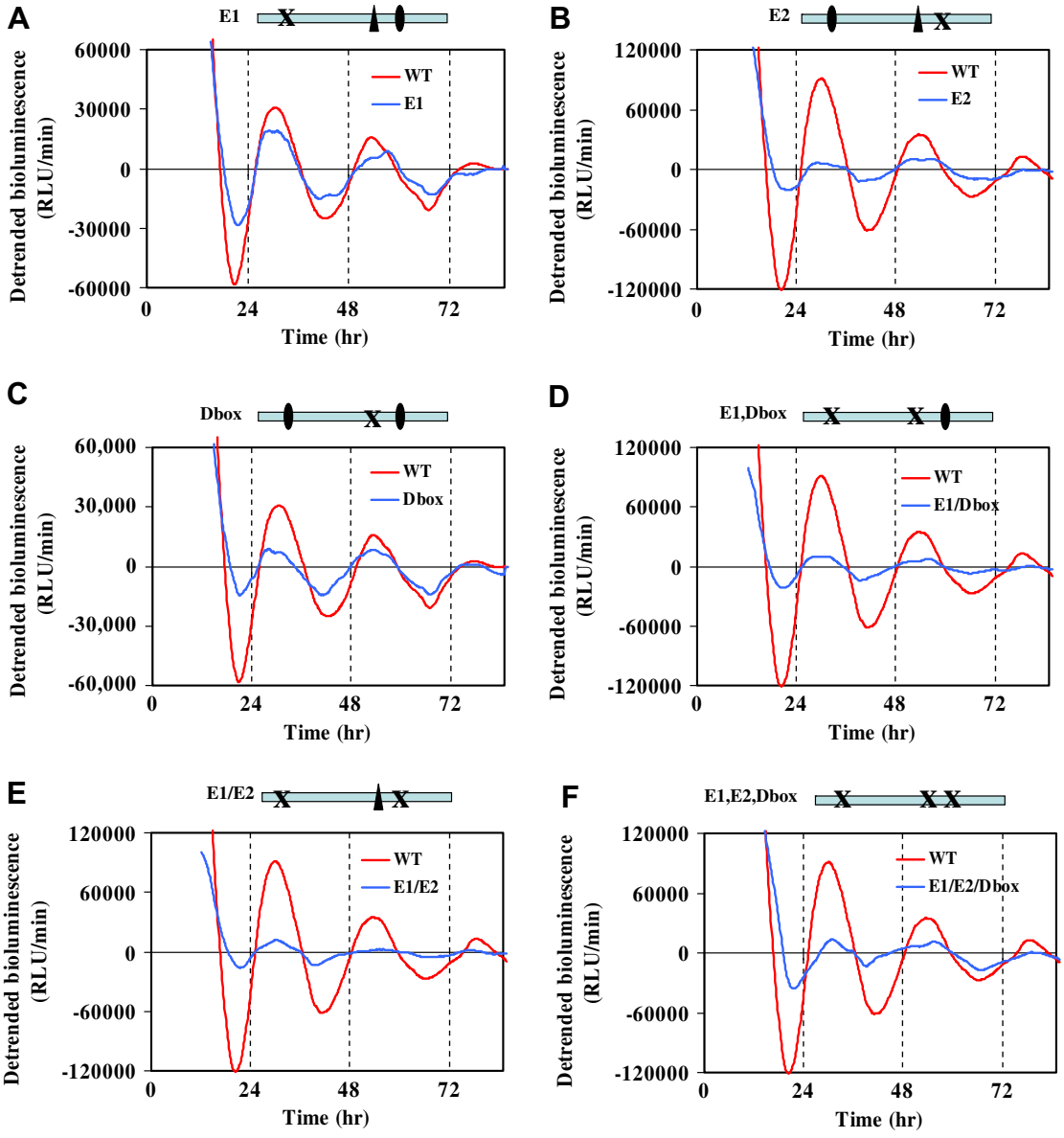


**Fig. 3.** E-box and D-box response elements control the circadian expression of mTopoI. (A-F) NIH3T3 cells were transfected with the indicated mutant constructs and treated with dexamethasone; the bioluminescence was then measured using a real-time monitoring assay (AB-2500 Kronos; ATTO). Results are representative of three independent experiments that generated similar results. WT, wild-type mTopoI promoter (red line); E1, mutant E1-site; E2, mutant E2-site; Dbox, mutant D-site (blue line). The relative activities are shown on the *y*-axis and the measurement of time after starting bioluminescence is shown on the *x*-axis. The method of analyzing circadian rhythms via bioluminescence is described in the experimental procedures.

Furthermore, to test whether the E1/E2 sites and the putative D-sites are actually interacting with the BMAL1/CLOCK heterodimer and DBP in the transcriptional regulation of the mTopoI promoter, we examined DNA–protein binding using a gel-shift assay (Fig. 4B). For the E1/E2 sites, only the shifted band for the E2 oligonucleotide was specifically detected in the presence of BMAL1 and CLOCK. To ascertain the specificity of the E2-box, we prepared unlabeled E2 probes and unlabeled E2 mutant probes. Excess unlabeled E2 probes inhibited the band shift, whereas unlabeled E2 mutant probes did not. These results suggested that the E2 element is a direct target of the BMAL1/CLOCK heterodimer. For the D-sites, we used labeled D-box and unlabeled D-box or unlabeled D-box mutant probes. The shifted band for the D-box oligonucleotide was specifically detected in the presence of DBP and all bands disappeared in the presence of unlabeled D-box probe competitors but not in the presence of unlabeled D-box mutant probes. These results indicate that DBP binds to the putative D-site. Additionally, to determine which boxes play a primary role in the regulation of the circadian expression of mTopoI, we overexpressed BMAL1/CLOCK or DBP in NIH3T3 cells and analyzed their effect using a real-time monitoring assay. We found that overexpression of BMAL1/CLOCK heterodimer reduced the amplitude of oscillation of TopoI without affecting on the circadian rhythmicity, whereas the circadian rhythmicity of the mTopoI promoter disappeared under the influence of DBP (Fig. 4C, $n = 2$). These results indicate that the D-box response element is more important than the E-box response element in the circadian expression of mTopoI.

*The oscillation phases of both TopoI and Per2 were almost in phase*

The circadian phases of *Bmal1* and *Per2* are almost opposite [7]. So we used *Bmal1* and *Per2* as references to test the circadian phase of TopoI. NIH3T3 cells were transfected with TopoI, *Bmal1* [23], and *Per2* promoters and incubated with dexamethasone. The biolumi-

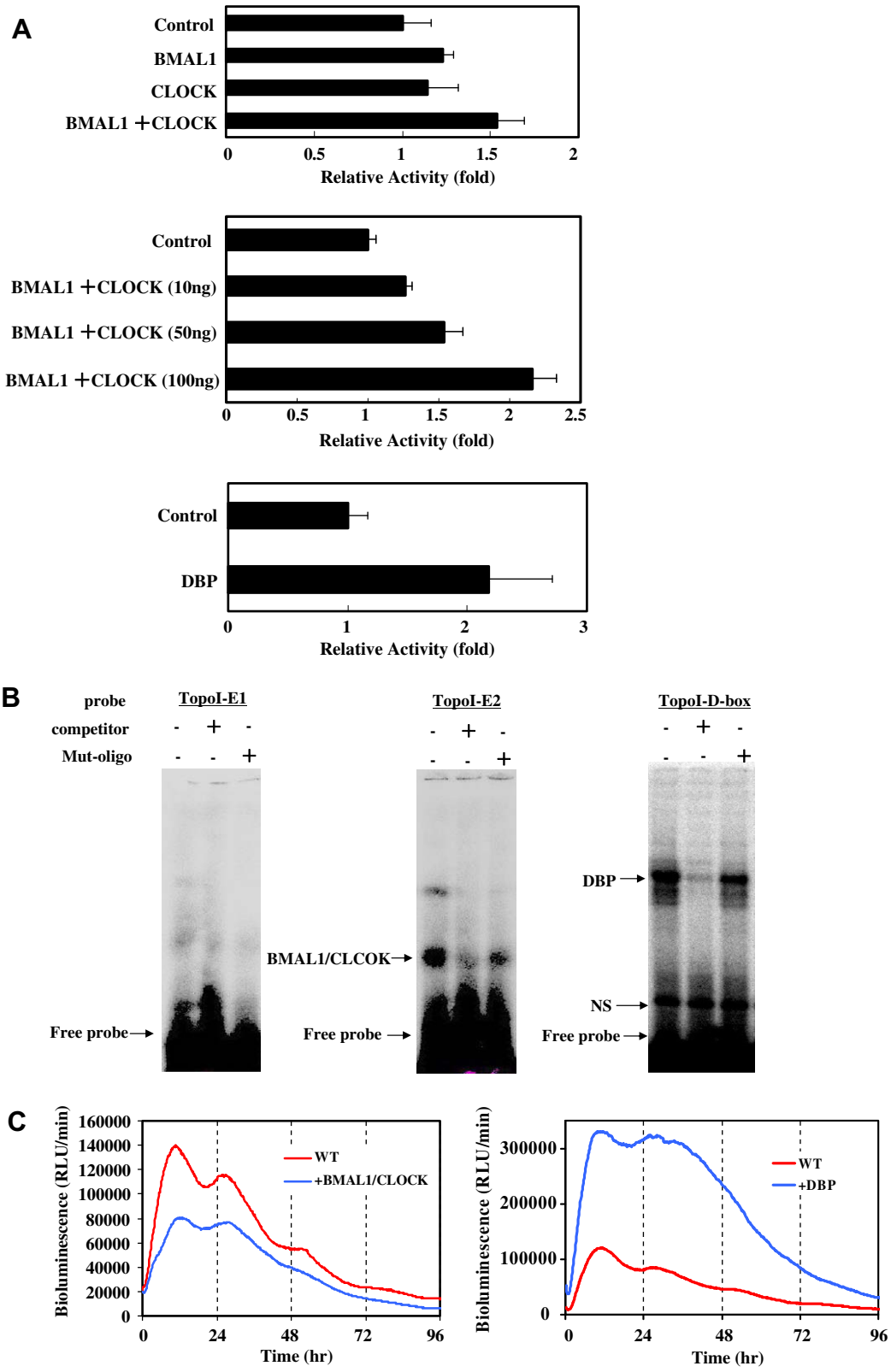


**Fig. 4.** mTopoI transcription is directly regulated by BMAL1/CLOCK heterodimer and DBP. (A) BMAL1, CLOCK, and BMAL1/CLOCK heterodimer potently induced mTopoI and the transcriptional enhancement was dose-dependent; DBP strongly induced mTopoI transcription. The mTopoI promoter-driven reporter (60 ng) was co-transfected with the indicated expression plasmids (+; 50 ng) or (+; 10 ng; 50 ng; 100 ng) (The experiment of dose-dependent). All values are shown as fold increases (mean ± S.E.M, $n = 5$) compared to the control (without the expression plasmid). (B) Gel-shift assay showing the E2-box as a direct target of the CLOCK/BMAL1 heterodimer and DBP binding to the putative D-site. The gel-shift assay was performed with E1 or E2 or D-box radiolabeled oligonucleotides and E1 or E2 or D-box unlabeled probes or unlabeled mutant probes. "NS" means nonspecific. (C) A primary role of the D-box response element is the regulation of circadian expression in mTopoI. NIH3T3 cells were co-transfected with the mTopoI promoter reporter (500 ng) and the indicated expression plasmids (250 ng) of BMAL1/CLOCK and DBP. The bioluminescence was then measured using a real-time monitoring assay ($n = 3$). WT, wild-type mTopoI promoter (red line); +BMAL1/CLOCK and +DBP (blue line).

nescence was then measured using a real-time measuring assay for testing the phase of oscillation. The result of oscillation phases of TopoI and *Per2* were similar (Supplementary Fig. S). In other words, the circadian phase of TopoI and *Bmal1* is opposite. These results can offer some evidences for the research of TopoI such as on the relationship between cell cycle and circadian system and the chronotherapy for cancer in the future.

In the recently study, the various clock genes of the mammalian molecular clock system and their protein products are involved in interlocked feedback loops of transcriptional and translational regulation through E-box, D-box, and RREs binding elements in the promoter/enhancer regions [19,21,22]. Our present study indicates that the E-box and D-box response elements are very important for the circadian expression of mTopoI but have no influence on the phase of oscillation (Fig. 3). These results suggest the possibility that the E-box alone and/or D-box response elements could not completely determine the phase of the clock genes. In addition, whether the mTopoI promoter includes other response elements regulating the phase of oscillation, besides E-box and D-box, remains unclear.

The circadian timing system and the cell division cycle are frequently deregulated in cancer [20]. A central role of *Per2*, *Bmal1*, and *Rev-erbβ* is the regulation of 24 h expression patterns in tumor mRNAs and the clock-controlled cell cycle genes, including *Wee1*, *c-Myc*, and *cyclinB1*[4]. CPT–TopoI complexes inhibited cell progress from the S-phase to the G2-phase [11]. The DNA synthesis and TopoI activity in bone marrow cells showed prominent circadian stage-dependent changes [14]. Results indicate that TopoI is very important for the antitumor efficacy of many drugs, through the regulating the circadian clock genes and cell cycle control genes. More studies are needed to analyze the molecular mechanisms of TopoI in the link between the molecular clock and the cycling of cell division to establish the molecular basis of the time treatment in antitumor drugs. TopoI is a cell cycle control gene, and our current results suggest a way to achieve better antitumor efficacy of CPT-11 via the regulation of TopoI gene expression through clock genes.

## Acknowledgements

We thank Dr. M. Nomura for helpful discussions and Dr. M. Noshiro (Hiroshima University) for the generous gift of the mouse DBP expression construct. We also thank the Research Center for Genomic Medicine, Saitama Medical University, for the use of their facilities. F.Y. was supported by the Uehara Memorial Foundation. This research was supported by the Japan Private School Promotion Foundation and Saitama Medical University Internal Grant.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.12.186.

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