24-Hour Oscillation of Mouse Methionine Aminopeptidase2, a Regulator of Tumor Progression, Is Regulated by Clock Gene Proteins

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ABSTRACT

Methionine aminopeptidase2 (MetAP2) plays an important role in the growth of endothelial cells during the tumor angiogenesis stage. Recently, we have clarified that mouse methionine aminopeptidases (mMetAPs) show a 24-hour rhythm in implanted tumor masses. In the present study, we investigated the mechanism underlying the 24-hour rhythm of mMetAP2 activity in tumor-bearing mice under a light-dark (lights on from 7 a.m. to 7 p.m.) cycle. The 5′ flanking region of mMetAP2 included eight E-boxes. The transcription of the mMetAP2 promoter was enhanced by the mCLOCK:mBMAL1 heterodimer, and its activation was inhibited by mPER2 or mCRY1. Deletion and mutation of the E-boxes in the regions indicated that the E-box nearest to the initiation start site played an important role in the transcriptional regulation by clock genes. In sarcoma180-bearing mice, the pattern of binding of mCLOCK and mBMAL1 to the E-box and transcription of the mMetAP2 promoter showed a 24-hour rhythm with higher levels from the mid-light to early dark phase. The pattern of m MetAP2 transcription was closely associated with that of mMetAP2 mRNA expression in three types of tumor-bearing mice. mMetAP2 protein expression varied with higher levels from the late-dark to early light phase. The rhythmicity of the protein expression was synchronous with that of the activity of mMetAPs but out of phase with that of the mMetAP2 mRNA expression. These results suggest that the 24-hour rhythm of mMetAP2 activity is regulated by the transcription of clock genes within the clock feedback loops.

INTRODUCTION

The methionine aminopeptidases (MetAPs) are a class of proteases that selectively remove methionine from the NH2 terminus of newly synthesized proteins (1). Protein synthesis is initiated with methionine in eukaryotes. MetAPs remove the initiator methionine of nascent polypeptide chains and play an important role in cell cycle initiation in eukaryotes (2, 3). MetAPs have been identified in all mammalian tissues and cells examined (4, 5). Down-regulation of human MetAP2 expression by an antisense oligonucleotide leads to the inhibition of endothelial cell proliferation (6). Antisense of MetAP2 also induces apoptosis in human mesothelioma cells and rat hepatoma cells (7, 8). These results suggest that MetAP2 plays an important role in the growth of not only endothelial cells during the tumor angiogenesis stage but of tumor cells in mammals.

One approach to increasing the efficacy of pharmacotherapy is to administer the drugs at the time of day when they are most effective and/or best tolerated. A chronopharmacological strategy can enhance the effects of drugs and/or attenuate their toxicity (9–11). Daily variations in biological functions, such as gene expression and protein synthesis, are thought to be important factors affecting the efficacy of drugs. MetAP2 has been examined as a physiologic target for the potent angiogenesis inhibitor TNP-470, a synthetic analogue of fumagillin, which acts by directly inhibiting endothelial cell proliferation (12). Recently, we clarified that the antitumor effect of TNP-470 is more potent in mice injected with the drug during the early light phase than in those injected with the drug during the early dark phase (9). In a nondrugged state, mouse methionine aminopeptidase (mMetAP) activity in tumor masses showed a significant 24-hour rhythm. The dosing time-dependent antitumor effect of TNP-470 is closely related to the 24-hour rhythm of mMetAP activity in tumor masses. However, the mechanism underlying the 24-hour rhythm of the enzyme activity has not been clarified.

The central circadian pacemaker in mammals is located in the hypothalamic suprachiasmatic nucleus (13). The core circadian oscillator is composed of interacting positive and negative transcription–translation feedback loops. CLOCK and BMAL1 induce Period (Per) and Cryptochrome (Cry) gene expression (14). PER and CRY proteins in turn act as negative components of feedback loops by suppressing CLOCK:BMAL1 heterodimer-mediated transcription through CACGTG E-box enhancer elements (15, 16). Recent studies have discovered circadian oscillations in the transcription of various genes in both peripheral tissues and cultured cells (17–19). The transcriptional machinery of the core circadian clockwork also regulates circadian output functions. CLOCK and BMAL1 heterodimer acts through an E-box enhancer to activate the transcription of vasoressin, albumin D-element-binding protein, and prokineticin2 mRNAs, thereby showing a specific circadian output function (20–22). Several factors can cause alterations to the clock function leading to illness and altered homeostatic regulation (10, 11). The functional significance of the mammalian peripheral clocks is still unknown.

Taking the findings described above into consideration, we hypothesized that the transcription of mMetAP2 mRNA may be regulated by clock gene proteins in tumor masses. The purpose of the present study was to investigate the mechanism underlying the 24-hour rhythm of mMetAP2 activity in implanted tumor masses: (1) we cloned the 5′ flanking region of the mMetAP2 gene and identified an E-box in the arrangement, (2) we investigated whether the transcription of mMetAP2 mRNA was enhanced by the mCLOCK::mBMAL1 heterodimer and the activation was inhibited by mPER2 or mCRY1 in sarcoma180 tumor masses using the luciferase reporter assay, (3) we identified the E-boxes that were enhanced by the clock gene heterodimer after deletion and mutation of the E-box in the 5′ flanking region of mMetAP2, and (4) we observed when the mCLOCK::mBMAL1 heterodimer bound to the 5′ flanking region and the transcription of mMetAP2 mRNA occurred in Sarcoma180-bearing mice, and (5) we determined the 24-hour rhythm of mMetAP2 mRNA and protein
expression in three types of tumor (Sarcoma180, Lewis lung carcinoma, and B16 melanoma)-bearing mice.

MATERIALS AND METHODS

Animals and Cells. Male ICR mice (5 weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). They were housed in a light-controlled room (lights on from 7 a.m. to 7 p.m.) at room temperature (24°C ± 1°C) and a relative humidity of 60% ± 10% with food and water ad libitum. All mice were adapted to the light/dark cycle for 2 weeks before the experiments began. During the dark period, a dim red light was used to aid treatment of the mice. Two marine tumor cell lines (Sarcoma180 and B16 melanoma) were commercially obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). Lewis lung carcinoma cells were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). The tumor cells were maintained in Dulbecco’s Modified Eagle’s Medium, supplemented with 10% heat-inactivated fetal bovine serum, 0.5% penicillin, and 0.5% streptomycin at 37°C in a humidified 5% CO2 atmosphere. A 50-μL volume of 1.5 ± 106 viable tumor cells was inoculated into the right hind footpads of each mouse.

Experimental Design. To observe the existence of E-boxes in the 5’ flanking region of the mMetAP2 gene, the unknown arrangement was amplified by PCR from Sarcoma180 genomic DNA. To investigate how the rhythm of mMetAP2 mRNA expression occurs in tumor masses, the transcriptional activity of clock gene proteins in the cloning of the 5’ flanking region of mMetAP2 was measured using a luciferase reporter assay system. To identify which E-box in the mMetAP2 promoter region is enhanced by the clock gene deletion, the deletion of the promoter region and mutation of the E-box were performed. To observe when the mClock:mBmal1 heterodimer binds the mMetAP2 promoter region and transcription occurs in the implanted Sarcoma180 masses, formaldehyde-cross–linked chromatin immunoprecipitation and semi quantitative PCR analysis were performed. The tumor masses were removed at the zeitgeber times ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22 on day 14 after the implantation of each mass of tumor cells. To determine the 24-hour rhythm of mMetAP2 mRNA and protein expression, tumor masses (Sarcoma180, Lewis lung carcinoma, and B16 melanoma) were removed from six tumor-bearing mice at the zeitgeber times described above. mMetAP2 mRNA or its protein levels were measured by reverse transcription–PCR (RT-PCR) or Western blot analysis.

Cloning of the 5’ Flanking Region of mMetAP2 and Construction of mMetAP2 Promoter Reporter. The 5’ flanking region of the mMetAP2 gene was amplified by PCR from mouse tumor cell Sarcoma180 DNA (DNA Databank of Japan accession no. AB181293). PCR was performed using the forward primer: 5’-GAT CAA TAA TGA ATG CTT-3’ and reverse primer: 5’-AGG GCC GCG TTC TTC TTC A-3’. These primers were designed based on the arrangement of the 5’ flanking region of rat MetAP2 (GenBank accession no. U377110). The PCR products were purified and ligated into the TOPO-XL vector using TOPO-XL vector (Invitrogen, Carlsbad, CA). A 1212-bp (−1272 to −60) 5’ flanking region of mMetAP2 was isolated from the TOPO-XL vector using HindIII and XhoI, and this region was ligated into the Luciferase pGL3 basic vector (Promega, Madison, WI).

Deletion of mMetAP2 Promoter Region. The deletion was used to clone the mMetAP2 promoter. A 782-bp (−842 to −60) and 422-bp (−60 to −60) 5’ flanking region were produced using the forward primers: 5’-CTA GGT ACC-3’ and reverse primer: 5’-GAC GGG GCC TTC TTC TTC TTC A-3’. These primers were designed based on the arrangement of the 5’ flanking region of mMetAP2 (Promega, Madison, WI).

Deletion of mMetAP2 Promoter Region. The deletion was used to clone the mMetAP2 promoter. A 782-bp (−842 to −60) and 422-bp (−60 to −60) 5’ flanking region were produced using the forward primers: 5’-CTA GGT ACC-3’ and reverse primer: 5’-GAC GGG GCC TTC TTC TTC TTC A-3’. These primers were designed based on the arrangement of the 5’ flanking region of mMetAP2 (Promega, Madison, WI).

Mutation of E-Boxes in the mMetAP2 Promoter Region. Two E-boxes in the 422-bp (−60 to −60) 5’ flanking region of the mMetAP2 gene were mutated at bp −163 to −168 (CAAGTTG to GAATTC), −200 to −205 (CAGTGG to GAAATT), or both using a QuickChange Site-directed Mutagenesis kit (Stratagene, La, Jolla, CA).

Cell Transfection and Luciferase Assay. On the day before transfection, the cells were seeded (1 × 105/well) into six-well plates containing Dulbecco’s Modified Eagle’s Medium, supplemented with 10% fetal bovine serum. Cells were transfected with 100 ng of reporter construct and 1 μg (total) of expression construct using Lipofectamine Plus (Invitrogen), according to the manufacturer’s instructions. To correct for variations in transfection efficiency, 0.5 ng of pRL-SV40 (Promega) was cotransfected in all experiments. The total amount of DNA per well was adjusted by adding pcDNA3.1 vector (Invitrogen). At 48 hours post-transfection, the cells were disrupted with 200 μL of passive lysis buffer (Promega). Luciferase activity was determined using a Dual Luciferase Reporter Assay System (Promega). For each sample, the measured luciferase activity was corrected for transfection efficiency by dividing the firefly luciferase activity (expressed from the reporter construct) by the Renilla luciferase activity (expressed from pRL-SV40). The level of transcription activity in individual experiments was normalized to the corresponding control (set at 1-fold). Expression constructs were made as follows: the coding regions of mClock, mBmal1, mPer2, and mCry1 were obtained by RT-PCR and used after conformation of their sequences. All coding regions were ligated into the pcDNA3.1 vector.

Formaldehyde-Cross–Linked Chromatin Immunoprecipitation Assays. Tumor masses removed from Sarcoma180-bearing mice were cross-linked with formaldehyde for 20 minutes. Cross-linked samples were sonicated on ice, and nuclear fractions were obtained by centrifugation at 10,000 × g for 10 minutes. Supernatants were diluted with 10 volumes of lysis buffer [50 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl1 mmol/L EDTA/10% glycerol/0.5% NP40, and 0.25% Triton X-100] and incubated with antibodies against mClock, mBmal1, acetyl histone H3 (Upstate, Lake Placid, NY), and RNA polymerase II (Santa Cruz Biotechnology, Santa Cruz, CA). Chromatin/anti-body complexes were extracted using a protein G agarose kit (Roche Diagnostics, Mannheim, Germany). DNA was isolated using the GeneElute Mammalian Genomic DNA kit (Sigma-Chemical Co., Steinheim, Germany) and subjected to PCR using the following primers for the E-box: forward: 5’-TTA GAT GTC CCT CAA CAG AA-3’ and reverse: 5’-AGT GCT CAC TTC TTC A-3’ and for acetyl histone H3 or polymerase II: forward: 5’-TGA GAT GTC CCT CAA CAG AA-3’ and reverse: 5’-GGA AGC TTC ATT GGC A-3’.

Quantitative Reverse Transcription-PCR Analysis of mMetAP2 mRNA. Total RNA was extracted from tumor-bearing mice using TRIzol reagent (Invitrogen). The cDNA of mouse mMetAP2 (GenBank accession no. AF343712) and mouse glyceraldehyde-3-phosphate dehydrogenase (mGAPDH; M35299) were synthesized and amplified with a superscript one-step RT-PCR system (Invitrogen). PCRs were performed for 30 cycles with mMetAP2 and mGAPDH in a single tube. The mMetAP2 primers used were forward: 5’-CAG TAT GAT GAC ATG TC-3’ and reverse: 5’-CTT GGA AGC CCC ATT GGC A-3’, yielding a 467-bp product. The mGAPDH primers used were forward: 5’-GAC CTC AAC TAC ATG GTC TAC A-3’ and reverse: 5’-ACT CCA CGA CAT ACG CAC A-3’, yielding a 178-bp product. To evaluate the quantitative reliability of RT-PCR, we performed a kinetic analysis of the amplified products to ensure that signals were derived only from the exponential phase of the amplification. From each sample after the first 27 cycles of amplification, we drew a 5-μL aliquot for electrophoresis and submitted the tubes for one more cycle of PCR. This procedure was repeated a total of 32 cycles. The PCR products were run on 5% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were reacted with antibody against mMetAP2 (Zymed Lab-
RESULTS

Cloning of the 5′ Flanking Region of the mMetAP2 Gene. This is the first demonstration of cloning of the 5′ flanking region of MetAP2 in the mouse (Fig. 1). The sequence for the 5′ flanking region of the gene was analyzed. Consequently, although an E-box with the sequence CACGTG and an especially high affinity for clock gene proteins was not observed within 1272 bp of the mMetAP2 promoter from the transcription start site, eight E-boxes with a CANNTG sequence were observed.

Activation of the mMetAP2 Promoter Region by Clock Gene Proteins. We performed a transient transcription assay using the Bp 1212-LUC (−1272 to −60) 5′ flanking region of the mMetAP2 gene luciferase reporter plasmids to elucidate whether mMetAP2 mRNA levels are controlled by clock gene proteins (Fig. 2). Cotransfection of mCLOCK+mBMAL1 with 1 μg of total plasmid increased mPer1 promoter activity, a positive control, by 4.7-fold (Fig. 2A). Cotransfection of the mCLOCK:mBMAL1 heterodimer increased mMetAP2 promoter activity by 3-fold (Fig. 2B). Coexpression of the mMetAP2 promoter with mBMAL1 or mCLOCK had little effect on promoter activity. Next, the suppressive effect of mPER2 or mCRY1 on the mMetAP2 transcription enhanced by the mCLOCK:mBMAL1 heterodimer was investigated. Both proteins suppressed mMetAP2 promoter activity in a dose-dependent manner. Cotransfection of mPER2 partially suppressed the induction of mMetAP2 by the mCLOCK:mBMAL1 heterodimer, whereas cotransfection of mCRY1 abolished the induction.

Activation of the Deleted mMetAP2 Promoter Region by Clock Genes. In addition to the mMetAP2 promoter vector, the Bp 1212-LUC (−1272 to −60) 5′ flanking region of the mMetAP2 gene, we made shorter constructs, such as Bp 782-LUC (−842 to −60), which included five E-boxes, and Bp 422-LUC (−482 to −60), which included two E-boxes, in the 5′ flanking region (Fig. 3). To investigate which E-box is important for the transcription of mMetAP2 mRNA, a luciferase assay was performed using each promoter vector. These reporter vectors retained 2.3- to 3.9-fold greater levels of activity compared with each control. White boxes, the location of the E-box (CANNTG) in the promoter region. Each value is the mean with SE of three experiments.

Contrasting Rhythms in Transcription Factor Binding and Chromatin Remodeling for the mMetAP2 Gene. We examined the binding of mCLOCK and mBMAL1 to the mMetAP2 E-box in tumor mass nuclear extracts from Sarcoma180-bearing mice using chromatin immunoprecipitation with antibodies against clock gene proteins. Fig. 5A shows a schematic representation of the mCLOCK:mBMAL1 heterodimer-binding, acetyl histone (AcH3), and RNA polymerase.

oratories, Inc., San Francisco, CA) and mGAPDH (Santa Cruz Biotechnology). The immunocomplexes were additionally reacted with peroxidase-conjugated secondary antibodies and made visible with 4-chloro naphthol as a peroxidase substrate. Immunoblot analysis with mMetAP2 or GAPDH polyclonal antibody revealed a single band of M, 67,000 or 38,000.

Statistical Analysis. ANOVA and Bonferroni’s test were used for multiple comparisons. A 5% level of probability was considered to be significant.
II-binding regions. A significant 24-hour rhythm was demonstrated for the binding of mCLOCK and mBMAL1 with higher levels of binding from the mid-light to early dark phase and low levels from the mid-dark to early light phase (Fig. 5C; \( P < 0.01 \)). Then, we examined the amount of Ach3 in the mMetAP2 promoter region to assay for rhythmic changes in chromatin structure using the same method. There was a significant 24-hour rhythm with higher levels from the mid-light to early dark phase and lower levels from the mid-dark to early light phase (Fig. 5C; \( P < 0.01 \)). We also examined the binding of polymerase II to the mMetAP2 promoter to assay for dynamic changes in coactivator assembly necessary for transcriptional activity. There was also a significant 24-hour rhythm in the binding of polymerase II with higher levels of binding from the mid-light to early dark phase and lower levels from the mid-dark to early light phase (Fig. 5C; \( P < 0.01 \)).

24-Hour Rhythm of mMetAP2 mRNA and mMetAP2 Protein Expression in Tumor-Bearing Mice. There was a significant 24-hour rhythm of mMetAP2 mRNA expression with higher levels from the mid-light to early dark phase and lower levels from the late-dark to early light phase in all of the tumor (Sarcoma180, Lewis lung carcinoma, and B16 melanoma)-bearing mice. (Fig. 6A; \( P < 0.01 \), respectively). This result indicates that the 24-hour rhythm of mMetAP2 mRNA expression corresponded to that of mMetAP2 transcription in Sarcoma180 masses (Fig. 5C and Fig. 6A). We also determined the 24-hour rhythm of mMetAP2 protein production in implanted tumor masses. In the three different types of tumor masses, the abundance of mMetAP2 protein varied with higher levels from the late-dark to early light phase and lower levels from the late-light to early dark phase (Fig. 6B). This result indicates that the peak of mMetAP2 protein occurs \( \sim 12 \) hours after the peak of mMetAP2 mRNA. On the other hand, the 24-hour rhythm of mMetAP2 protein expression corresponded to that of mMetAP activity.

DISCUSSION

Although the purpose of the present study was to investigate the mechanism underlying the 24-hour rhythm of mMetAP2 activity, the arrangement of the \( 5' \) flanking region of the mMetAP2 gene has not been clarified as yet in the mouse. Therefore, cloning of the \( 5' \) flanking region of mMetAP2 was performed in the mouse. Comparison of the arrangement of the \( 5' \) flanking region of the gene with the known rat sequence revealed \( \sim 99\% \) homology between the mouse and rat (23). The arrangement and position of eight E-boxes in the \( 5' \) flanking region of mMetAP2 were also conserved between the mouse and rat. Recently, we demonstrated the mechanism underlying the 24-hour rhythm of vascular endothelial growth factor expression in hypoxic tumor cells (24). The negative limbs of the feedback loop, PER and CRY protein, inhibit the hypoxia-induced activation of vascular endothelial growth factor transcription. On the other hand, mMetAP2 mRNA expression may not be enhanced by hypoxia, because there was no hypoxia-responsive element in the \( 5' \) flanking region of the mMetAP2 gene.

In the present study, a significant 24-hour rhythm was demonstrated for mMetAP2 mRNA expression in implanted tumor masses. The clock genes control downstream events by regulating the expression of clock-controlled output genes. The CLOCK:BMAL1 heterodimer acts through an E-box enhancer element of the output genes to activate transcription, and its activation is inhibited by PER or CRY protein (14, 15). The expression of clock genes in implanted cells is subordinated to the dominance exerted by the central clock of the host animal (25). The rhythmic patterns of clock gene expression in tumor masses are similar to those reported in healthy tissues, such as liver and skeletal muscle (15, 26–28). In addition, the 24-hour rhythm of mCRY1 protein expression was antiphase to that of mMetAP2 mRNA expression in the present study (9, 24). Therefore, the transcription of mMetAP2 mRNA may be regulated by clock gene proteins in tumor masses. Monitoring clock gene protein levels is not enough to estimate whether mMetAP2 mRNA is increased by the mCLOCK:BMAL1 heterodimer in vivo, e.g., mPer and mCry mRNA expression are enhanced by clock gene proteins of the
The chromatin immunoprecipitation assay can detect a time-dependent change in the amount of clock gene protein combining with the promoter domain, which includes the E-box, although a conventional gel shift assay cannot (29). Therefore, a chromatin immunoprecipitation assay was performed to investigate whether mCLOCK and mBMAL1 directly combine with the E-box in tumor-bearing mice and the binding of the mCLOCK:mBMAL1 heterodimer mediating the rhythmic drive of mMetAP2 transcription is regulated by time-dependent changes in chromatin structure. Consequently, mCLOCK and mBMAL1 directly combined with the E-box in the mMetAP2 promoter region. The 24-hour rhythm of the binding of mCLOCK: mBMAL1 showed the same waveform as that of mMetAP2 mRNA expression. Although mCLOCK shows a rhythm of small amplitude, mBMAL1 shows a rhythm of large amplitude (21, 27, 30). Therefore, the rhythm in the binding of mCLOCK:mBMAL1 to the mMetAP2 promoter may be caused by the large change in the mBMAL1 protein level in addition to the small change in the mCLOCK protein level. Similar findings are demonstrated for the transcription of Per1 or Cry1 by mCLOCK:mBMAL1 (26, 29). The mCLOCK:mBMAL1 heterodimer exerts its transcriptional activity by forming a transcriptional coactivator complex with p300 histone acetyltransferase (29). mCRY1 protein disrupts the transcriptional coactivator complex, p300, thereby reducing histone acetyltransferase activity and altering chromatin structure to decrease mCLOCK:mBMAL1 transcriptional activation. In the case of mMetAP2, the rhythmic changes in the amount of AcH3 and polymerase II binding were similar to those in the mMetAP2 mRNA expression. These results suggest that transcription of mMetAP2 mRNA is controlled by clock gene proteins. In addition, the transcriptional regulation is accompanied by expression of AcH3 and the binding of polymerase II.

We reported previously that mMetAP activity in Sarcoma180-bearing mice showed a significant 24-hour rhythm (9). A higher level of activity was observed from the late-dark to early light phase and a lower level from the late-light to early dark phase in implanted Sarcoma180 masses. Because a specific assay method for only mMetAP2 activity has not been established, it was impossible to separately measure mMetAP2 and the subtype mMetAP1. Consequently, the activity of mMetAPs in the previous study included both mMetAP2 and mMetAP1 activity. Therefore, we measured the protein levels of mMetAP2 in three different types of tumor masses using a specific antibody for mMetAP2. The protein levels of mMetAP2 also showed a 24-hour rhythm associated with the rhythmicity of mMetAP activity in Sarcoma180-bearing mice. On the other hand, the peak of mMetAP2 protein was 12 hours later than the peak of mMetAP2 mRNA in Sarcoma180. Similar findings were confirmed in different cell lines, such as Lewis lung carcinoma and B16 melanoma. Furthermore, CRY2 protein behaves similarly to mMetAP2 protein (26). mCRY2 mRNA shows a peak at ZT2, and its protein shows a peak at ZT14. The mechanism involved is unclear at present. It is suggested that the synthesis of mMetAP2 protein from its mRNA requires more time than that of other proteins.

Although MetAP2 was initially implicated in the proliferation of endothelial cells during the tumor-angiogenesis stage, it has since been identified in tumor cells and normal cells and may be implicated in the proliferation of both (4, 12, 31). There was a significant 24-hour rhythm of mMetAP2 mRNA expression in all of the tumor (Sarcoma180, Lewis lung carcinoma, and B16 melanoma)-bearing mice. In addition, a significant 24-hour rhythm has been demonstrated for mMetAP2 mRNA levels in healthy liver (32). The rhythmic patterns of clock gene expression in tumor masses are similar to those reported in healthy tissues (15, 26–28). These results suggest that the 24-hour rhythm of mMetAP2 in healthy tissue may also be controlled by the clock feedback loops.
The present study suggests that the 24-hour rhythm of mMetAP2 is controlled by the clock feedback loops. The transcription of the mMetAP2 promoter is enhanced by the clock gene proteins of the mCLOCK:mBMAL1 heterodimer, and mPER2 or mCRY1 inhibits mMetAP2 promoter activation by mCLOCK:mBMAL1 in vitro. In addition, the transcription of mMetAP2 mRNA is generated by the mCLOCK:mBMAL1 heterodimer in vivo. TNP-470, inhibiting MetAP2 activity, has significant clinical advantages as a therapeutic agent for treating tumors, and research into the design of tumor therapies using TNP-470 was reported recently (33). The present study also supports the notion that choosing the most appropriate time of day for drug administration will aid in establishing rational chemothrapeutics with TNP-470 for the treatment of tumors.

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