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Up-regulation of *per* mRNA Expression by Parathyroid Hormone through a Protein Kinase A-CREB-dependent Mechanism in Chondrocytes*

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In bone, clock genes are involved in the circadian oscillation of bone formation and extracellular matrix expression. However, to date little attention has been paid to circadian rhythm in association with expression of clock genes during chondrogenesis in cartilage. In this study, we investigated the functional expression of different clock genes by chondrocytes in the course of cartilage development. The mRNA expression of types I, II, and X collagens exhibited a 24-h rhythm with a peak at zeitgeber time 6, in addition to a 24-h rhythmicity of all the clock genes examined in mouse femurs *in vivo*. Marked expression of different clock genes was seen in both osteoblastic MC3T3-E1 and chondrogenic ATDC5 cells *in vitro*, whereas parathyroid hormone (PTH) transiently increased *period 1* (*per1*) mRNA expression at 1 h in both cell lines. Similar increases were seen in the mRNA levels for both *per1* and *per2* in prehypertrophic chondrocytes in metatarsal organotypic cultures within 2 h of exposure to PTH. PTH significantly activated the mouse *per1* (*mper1*) and *mper2* promoters but not the *mper3* promoter in a manner sensitive to both a protein kinase A inhibitor and deletion of the cAMP-responsive element sequence (CRE) in ATDC5 cells. In HEK293 cells, introduction of brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (*bmal1*)/*clock* enhanced mouse type II collagen first intron reporter activity without affecting promoter activity, with reduction effected by either *per1* or *per2*. These results suggest that PTH directly stimulates *mper* expression through a protein kinase A-CRE-binding protein signaling pathway for subsequent regulation of *bmal1*/*clock*-dependent extracellular matrix expression in cartilage.

Recent studies have revealed that the endogenous circadian rhythmicity generated at the cellular level by circadian core oscillators resides not only in the hypothalamic suprachiasmatic nucleus of the anterior hypothalamus (1, 2), which is recognized as the mammalian central clock, but also in various peripheral tissues including heart (3), adipose tissue (4), pancreas (5), and liver (2). The suprachiasmatic nucleus is not essential for driving peripheral oscillations but rather acts as a synchronizer of peripheral oscillators, whereas the physiological rhythmicity may be under the direct control by their own local clock genes in peripheral tissues (7). In mice, the rhythmic transcription of two orthologs of the *Drosophila Period* (*per*) gene appears to be essential for circadian rhythms. Expression of mouse *per* (*mper*) genes is known to be positively regulated by other clock proteins belonging to the basic helix-loop-helix period/aryl hydrocarbon receptor nuclear translocator/single minded class, which are Clock and brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (Bmal1),[2] respectively. In addition, mPer proteins constitute multimeric complexes with products of the *cryptochrome* (*cry*) genes, *mcry1* and *mcry2*, which in turn negatively regulate the gene transcription mediated by Clock/Bmal1 (8–11).

Bone is formed through a well organized and highly regulated process (12). During embryogenesis mesenchymal precursor cells differentiate into skeletal elements by forming a cartilaginous model framework, which then induces the bone formation process known as endochondral ossification in the vertebral column and long bone (13). This endochondral ossification is responsible for the formation of the cartilaginous rudiment that is a tightly regulated region in both the differentiation and maturation of chondrocytes. Within this cartilaginous rudiment, for example, chondrocytes successively differentiate through a well organized mechanistic procedure with migration from resting cells at the rudiment edge to proliferating, hypertrophic and calcifying cells around the central region, which finally leads to mineralization of the cartilage matrix in the area of the hypertrophic chondrocytes. Shortly after the mineralization process takes place, however, most hypertrophic chondrocytes undergo sustained apoptosis. Upon death of chondrocytes after mineralization, circulating precursors of osteoblasts, osteoclasts, and capillaries begin to invade the cartilage matrix to produce new bones, leading to the growth of endochondral bones (12, 13).

Previous reports have demonstrated that expression patterns of two major extracellular matrices in bone, type I collagen and

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[2] The abbreviations used are: Bmal1, brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1; ChIP, chromatin immunoprecipitation; CRE, cAMP-responsive element; CREB, cAMP-responsive element-binding protein; Cry, cryptochrome; DIG, digoxigenin; M-CSF, macrophage colony stimulating factor; Per, Period; PKA, protein kinase A; PKC, protein kinase C; PPR, parathyroid hormone/parathyroid hormone-related peptide receptor; PTH, parathyroid hormone; RANKL, receptor activator nuclear factor-κB ligand; Runx2, runt-related transcription factor 2; ZT, zeitgeber time; FBS, fetal bovine serum; HEK, human embryonic kidney; RT, reverse transcriptase.

osteocalcin, display circadian rhythms (14, 15). Molecular clock components have been shown to mediate leptin-regulated bone formation (16), whereas to date no evidence is available for a role of the expression of circadian clock genes in the proliferation and/or differentiation of chondrocytes in the literature. Because parathyroid hormone (PTH) is well known to regulate a variety of cellular functions through PTH/PTH related protein (PTHrP) receptors (PPRs) in both osteoblasts and chondrocytes, it is conceivable that circadian clock genes may be functionally expressed to play a role in the mechanisms associated with PTH-induced modulation of cellular functions in chondrocytes as well as osteoblasts. In the present study, an attempt has been made to demonstrate the expression and functionality of different circadian clock genes in chondrocytes to elucidate circadian oscillation of cartilage development during endochondral ossification.

EXPERIMENTAL PROCEDURES

Animals—The protocol employed here meets the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques. Male ddY mice obtained at 4 weeks of age were maintained for 2 weeks under controlled temperature and humidity with a 12-h light (08:00–20:00 h)/12-h dark (20:00–08:00 h) cycle with access to standard laboratory food and water *ad libitum*. Animals were then decapitated to obtain total RNA from femurs at the following zeitgeber times (ZT): 0, 6, 12, and 18, where ZT 0 is defined as the time of lights on and ZT 12 lights off, respectively. Four animals were usually used for each time point on different days.

Cell Cultures—Osteoblastic MC3T3-E1 and chondrogenic ATDC5 cells were purchased from RIKEN Cell Bank. MC3T3-E1 cells were cultured in α-minimal essential medium (Invitrogen) containing 10% FBS. For induction of differentiation, culture media were replaced with medium containing 50 μg/ml ascorbic acid and 5 mM β-glycerophosphate. ATDC5 cells were plated at a density of 1×10^4 cells/cm^2 in a 1:1 mixture of Dulbecco's modified Eagle's and Ham's F-12 medium (Invitrogen) containing 5% FBS. For induction of differentiation, culture media were replaced with medium containing 10 μg/ml transferrin, 3×10^{-8} M sodium selenite, and 10 μg/ml bovine insulin (Sigma).

Primary Osteoclasts—Bone marrow was prepared from tibias and femurs of 4-week-old Std-ddY male mice and cultured for 24 h with macrophage colony-stimulating factor (M-CSF) (R&D Systems) at 10 ng/ml in α-minimal essential medium containing 10% FBS. After culturing for 24 h, nonadherent cells in supernatants were collected, followed by lamination on Ficoll gradient, and subsequent centrifugation at $500 \times g$ for 15 min. The monocyte/macrophage progenitor fraction was collected and suspended in minimal essential medium (Invitrogen) containing 10% FBS, M-CSF at 20 ng/ml, and receptor activator of nuclear factor-κB ligand (RANKL) (R&D Systems) at 20 ng/ml. Cells were plated at a density of 1×10^5 cells/cm^2, followed by culturing at 37 °C under 5% CO_2 for 5 days.

Embryonic Metatarsal Rudiment Organ Culture—Metatarsal organ culture was conducted as described previously (17). The three central metatarsal rudiments were isolated from ddY mouse embryos at 15.5 days post-gestation. Each of three metatarsals was placed in a well of a 24-well plate containing 1 ml of organ culture medium: minimal essential medium supplemented with 0.05 mg/ml ascorbic acid, 1 mM β-glycerophosphate, and 0.25% FBS. These explants were grown at 37 °C in a humidified 5% CO_2 incubator for 5 days.

In Situ Hybridization Analysis—*In situ* hybridization was carried out as described previously (17). In brief, mounted sections were fixed with 4% paraformaldehyde, followed by HCl, proteinase K, and triethanolamine/acetic anhydride treatment. After prehybridization, sections were covered with digoxigenin (DIG)-labeled cRNA probes at 65 °C for 16 h. The slides were treated with RNase A, blocked with 1.5% blocking buffer, incubated with anti-DIG-AP-Fab fragments (Roche) at 4 °C for 16 h, and treated with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyphosphate for different periods. Moreover, neonatal mice were subcutaneously injected with 400 μg/kg PTH, followed by dissection of tibiae 1 h after administration and subsequent detection of Per1 mRNA expression using a DIG-labeled cRNA probe.

Cloning and Constructs—The *mper1* (−1803 to +40), *mper2* (−1670 to +53), and *mper3* (−1594 to +128) promoters and the mutated promoters in terms of the cAMP-responsive element (CRE) within the *mper1* and *mper2* promoters were generous gifts from Dr. Sassone-Corsi (Institut de Génétique et de Biologie Moléculaire et Cellulaire, France). The 5′-flanking region (−3383 to +41) and first intron regions ((+959 to +1343) and (+959 to +2954)) of the mouse type II collagen were isolated from mouse genomic DNA by PCR using the following primers: 5′-GTTTCTTCCACTCTCAGGATGC-3′ and 5′-GCAGGAGGAGGCAGGAGACCCGG-3′ for the 5′-flanking region, and 5′-GTGTGTAGCCGTGGATGGATATGGG-3′, 5′-CCGCCTCCTTCTAAAGCATCTCTGG-3′ (to +1343) and 5′-ACTGCCTAGGACAACCCGAGAAA-3′ (to +2954) for the first intron region. The PCR-amplified DNA products were cloned into the pGL3 basic vector (Promega). Mouse *clock*, *per1*, *per2*, and *cry1* and hamster *bmal1* expression plasmids were kindly donated by Dr. Reppert (University of Massachusetts Medical School, Massachusetts, MA).

Luciferase Assay—Reporter vectors were co-transfected with a TK-*Renilla* luciferase construct into MC3T3-E1, ATDC5, or HEK293 cells using Lipofectamine and Plus reagent (Invitrogen). Two days after transfection, cells were lysed, and luciferase activity was determined using specific substrates in a luminometer according to the manufacturer's protocol (Promega). Transfection efficiency was normalized by determining the activity of *Renilla* luciferase.

RT-PCR—RT-PCR was conducted as described previously (18). In brief, cDNA was synthesized with the oligo(dT) primer and reverse transcriptase (Invitrogen) from extracted total RNA. PCR amplification was performed using specific primers (Table 1), and PCR products were subcloned into a TA cloning vector (Promega) for determination of DNA sequences. Although the results obtained by RT-PCR are by definition not quantitative, apparent semi-quantitative RT-PCR analysis was

TABLE 1
Primers used for RT-PCR

Genes	Accession No.	Upstream (5′-3′)	Downstream (5′-3′)	Estimated base pair
Col I	NM_007742	GCAATCGGGATCAGTACGAA	CTTTCACGCCTTTGAAGCCA	485
Col II	NM_031163	GGAAAGTCTGGGGAAAGAGG	CAGTCCCTGGGTTACCAGAA	457
Col X	NM_009925	GCCAGGTCTCAATGGTCCTA	AAAAGCAGACACGGGCATAC	482
Osteocalcin	S67455	AAGCAGGAGGGCAATAAGGT	AGCTGCTGTGACATCCATAC	498
Runx2	NM_009820	CCGCACGACAACCGCACCAT	CGCTCCGGCCCACAAATCTC	289
Osterix	AF184902	GGCAGTCACTAAGATCCCA	CCCAGACTCCATGGCTTTAC	563
ALP	NM_007432	GCCCTCTCCAAGACATATA	CCATGATCACGTCGATATCC	373
TRAP	BC019160	ACACAGTGATGCTGTGTGGCAACTC	CCAGAGGCTTCCACATATATGATGG	465
Per1	AB002108	CCATGGACATGTCTACT	AGAGGACCAGGGGACAT	982
Per2	AF036893	CTACCTGGTCAAGGTGCAAGAG	GGTTTGAATCTTGCCACTGG	698
Per3	AF050182	TCCTGATGGTAAGACATTCCAG	GCGTGAACAATCACACTCACTT	500
Brnal1	AB015203	TCAAGAATGCAAGGGAGGCC	AACAGGTAGAGGCGAAGTGCCC	599
Clock	NM_007715	CTATGCTTCCTGGTAACGCG	GCCTATTATTGGTGGTGCCC	718
Cry1	NM_007771	CGTCTGTTTGTGATTCGGGG	ATTCACGCCACAGGAGTTGC	669
Cry2	NM_009963	AGAAGGTGAAGAGGAACAGCAC	TAGATGTATCGAGAGGGGAAGC	515
Dec1	NM_011498	CGTCTGTTTGTGATTCGGGG	AACCACTACCGAGACATGCC	700
Dec2	NM_024469	ACAGGACAGAAACCTCCAAATCG	TCTTTCAGCTGAGCAATGCATTC	432
Dbp	NM_016974	TCTTTCAGCTGAGCAATGCATTC	ACGTTCTTCGGGCACCTAGC	680

performed at cycles below 30 with relatively high linearity using primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase for comparative reference. PCR products were quantified by using a densitograph, followed by calculation of the ratios of expression of mRNA for each gene over that of glyceraldehyde-3-phosphate dehydrogenase.

Electrophoretic Mobility Shift Assay—Gel retardation electrophoresis was conducted as described previously (18). In brief, nuclear extracts were prepared by the method of Schreiber *et al.* (19) with minor modifications. Cells were collected in 10 mM HEPES-NaOH buffer (pH 7.9) containing 10 mM KCl, phosphatase inhibitors, and protease inhibitors. Following the addition of 10% Nonidet P-40 to make a final concentration of 0.6%, cells were centrifuged at 20,000 × *g* for 5 min. Pellets were suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 400 mM KCl and inhibitors for phosphatases and proteases. Suspensions were kept in an ice bath for 30 min, followed by centrifugation at 20,000 × *g* for 5 min. The supernatants thus obtained were collected as nuclear extracts. Assays were carried out using double-stranded oligonucleotide containing consensus core sequence for the E-box from the Dec1 promoter (5′-CTAGGTCCAACACGTGAGACTCTCGA-3′), type II collagen promoter (5′-ATCCATGGTTGCACGTGGCCCGTGCA-3′), and type II collagen first intron (5′-AATTTTCTTCCACGTGTTTGGGCACG-3′), as a probe labeled by [α-^{32}P]deoxy-ATP for detection of DNA binding activity. After determining protein contents, an aliquot of nuclear extracts was incubated at a fixed amount of 3 μg of protein and analyzed by electrophoresis on a 6% polyacrylamide gel. Gels were fixed and dried, followed by exposure to x-ray films for different periods to obtain autoradiograms appropriate for subsequent quantification by a densitograph.

Western Blotting—Western blotting was conducted as described previously (18). In brief, nuclear extracts prepared from cultured osteoblasts were treated with SDS, and lysates were subjected to electrophoresis on a polyacrylamide gel containing SDS, followed by transference to a polyvinylidene fluoride membrane. The membranes were then subjected to blocking with skimmed milk, followed by reaction with the anti-phospho-CRE-binding protein (CREB) antibody or the anti-CREB antibody (New England Biolabs). Finally, membranes were reacted with the anti-rabbit immunoglobulin G antibody conjugated with peroxidase and detected with the aid of ECL detection reagents for subsequent exposure to x-ray films.

Northern Blotting—Northern blotting was conducted as described previously (18). In brief, extracted RNA was resolved on 1% formaldehyde/agarose gel, and transferred onto positively charged nylon transfer membranes. After fixation of RNA to the blot by UV cross-linking, blotted membranes were prehybridized at 68 °C for 1 h, and subsequently hybridized with a denatured DIG-labeled RNA probe of mPer1 at 68 °C for 16 h. Membranes were then washed and incubated with anti-DIG-AP-Fab, followed by incubation with CDP-star and subsequent exposure to x-ray films for appropriate periods to detect chemiluminescence.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP experiments were performed essentially according to the protocol provided with the ChIP assay kit (Upstate Biotechnology) using ATDC5 cells. ATDC5 cells were treated with formaldehyde for cross-linking, followed by sonication in lysis buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin). Immunoprecipitation was performed with the anti-FLAG antibody (Sigma), followed by extraction of DNA with phenol/chloroform. PCR was performed using sequences found in the 5′-flanking region (−3215 to −2821) and first intron region (+1042 to +1365) of the mouse type II collagen gene, in addition to the *mper* promoter (−663 to −383), as primers.

Data Analysis—Results are all expressed as the mean ± S.E. and statistical significance was determined by two-tailed and unpaired Students' *t* test or one-way analysis of variance with Bonferroni/Dunnett post-hoc test.

RESULTS

Rhythmic Expression of Clock Genes and Marker Genes in Bone and Cartilage—To investigate whether mRNA expression for bone and cartilage marker genes exhibit daily rhythms in addition to circadian clock genes, total RNA was obtained from adult ddY mouse femurs every 6 h throughout a single 24-h period, followed by determination of the mRNA levels by semi-quantitative RT-

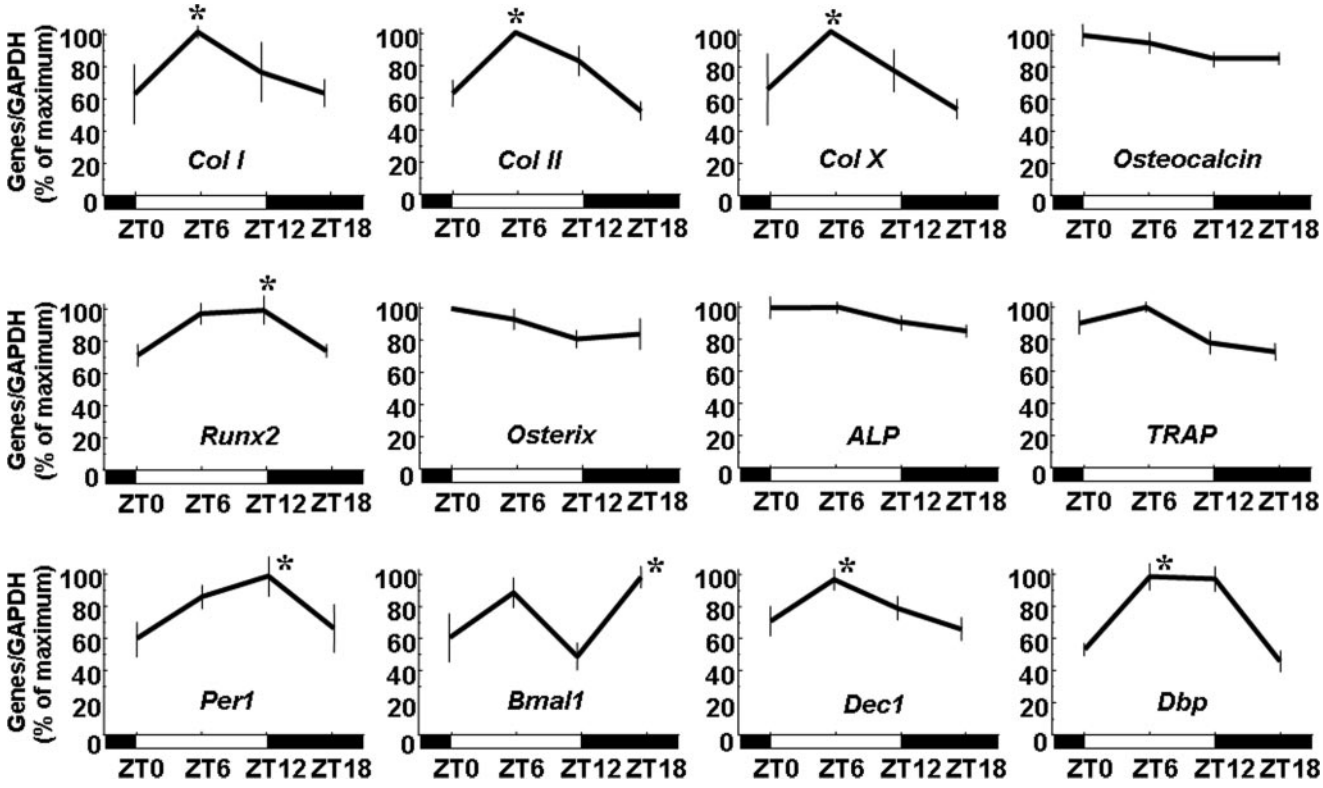

FIGURE 1. **Rhythmic expression of different genes in mouse femurs *in vivo*.** Total RNA was obtained from ddY mouse femurs every 6 h throughout a single 24-h period, followed by determination of mRNA levels by semi-quantitative RT-PCR using primers specific for each differentiation marker and circadian clock gene. Values are the mean ± S.E. in separate experiments using 4 different animals per each time point. *, $p < 0.05$, significantly higher than the lowest control value for each gene. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

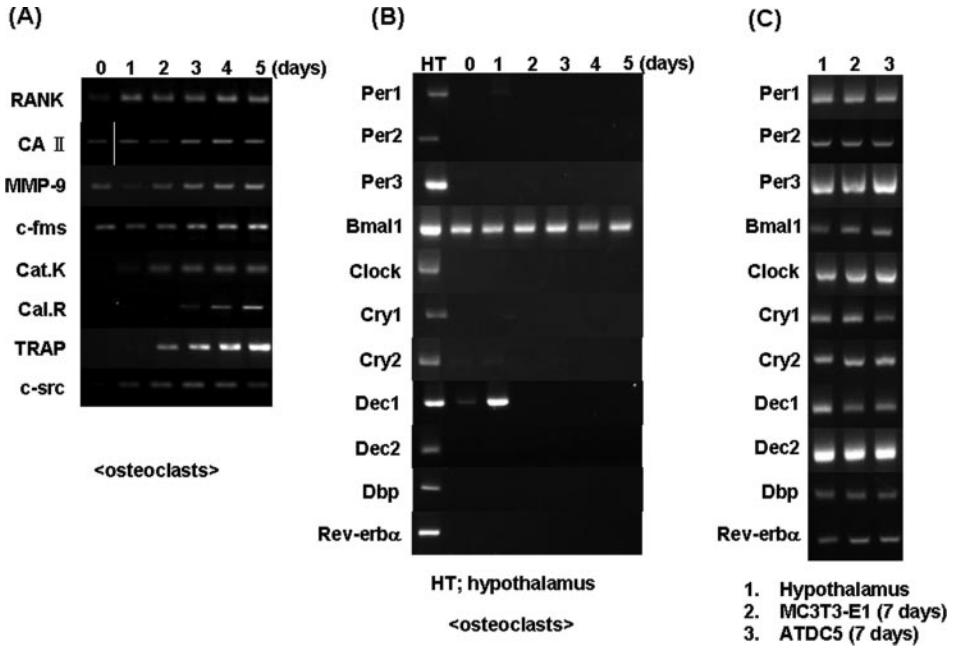

FIGURE 2. **Expression of circadian clock genes in bone cells.** The monocyte/macrophage progenitor cells isolated from mouse bone marrows were cultured for 5 days in the presence of 20 ng/ml M-CSF and 50 ng/ml RANKL, followed by determination of expression of osteoclastic differentiation markers (*A*) and clock genes (*B*) by RT-PCR. *C*, MC3T3-E1 cells were cultured for 7 days in the presence of 50 μg/ml ascorbic acid and 5 mM β-glycerophosphate, and ATDC5 cells were cultured in the presence of 10 μg/ml transferrin, 3×10^{-8} M sodium selenite, and 10 μg/ml bovine insulin for 7 days, followed by determination of expression of circadian clock genes. Typical pictures are shown in the figure with similar results in three separate determinations. *TRAP*, tartrate-resistant acid phosphatase.

PCR. As shown in Fig. 1, all the clock genes (*per1*, *bmal1*, *dec1*, and *dbp*) examined in this study exhibited 24 h rhythmicity in mouse femurs *in vivo*. Maximum expression was observed at ZT 12 for Per1, at ZT 16 for *dec1* and *dbp*, and at ZT 18 for *bmal1*, respectively. The expression of mRNA for type I collagen, type II collagen, and type X collagen similarly exhibited a 24-h rhythm with a peak at ZT 6 and a gradual decrease thereafter up to ZT 18. By contrast, no rhythmic expression was observed in mRNA for the tartrate-resistant acid phosphatase, alkaline phosphatase, or osteocalcin genes in mouse femurs. These genes appear either to be constitutively expressed or to oscillate with amplitudes below statistical significance. Interestingly, mRNA expression of runt-related transcription factor 2 (Runx2) showed a 24-h rhythm with a peak at ZT 12, but no such pattern in osterix mRNA expression.

Expression of mRNA for Clock Genes in Bone Cells—To examine whether circadian clock genes are

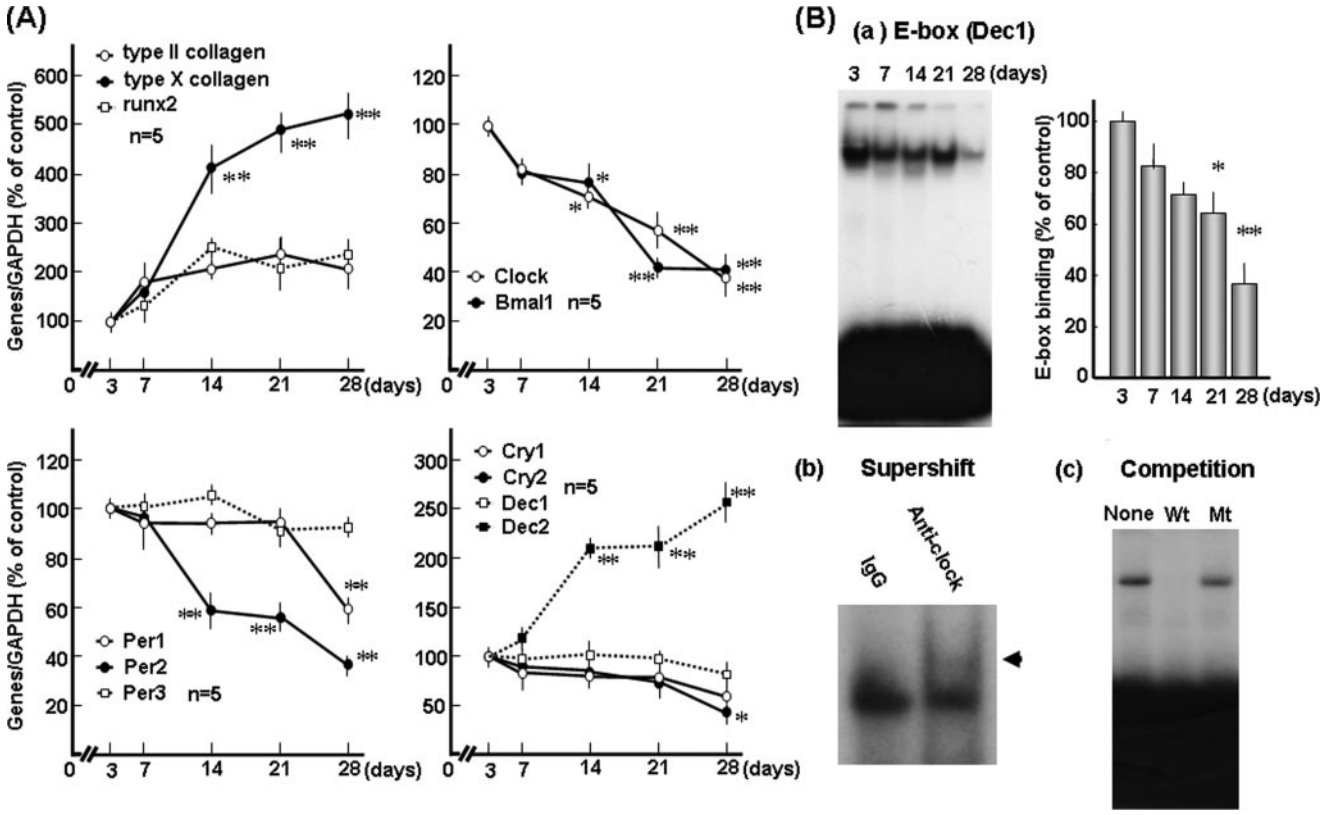

FIGURE 3. **Expression of circadian clock genes in chondrogenic ATDC5 cells.** *A*, ATDC5 cells were cultured for 3 to 28 days, followed by isolation of mRNA and subsequent RT-PCR using primers specific for each gene. Values are the mean ± S.E. of percentages over the corresponding values obtained at 3 days in five independent determinations. *B*: *a*, ATDC5 cells were cultured for 3 to 28 days, followed by preparation of nuclear extracts and subsequent gel retardation electrophoresis using E-box probe. A typical autoradiogram is shown in the *left panel*, whereas values are the mean ± S.E. of percentages over the corresponding values obtained at 3 days in four independent experiments in the *right panel*. *b* and *c*, supershift analysis was carried out using the anti-clock antibody, whereas competition analysis was conducted using unlabeled wild type (*Wt*) and mutant (*Mt*) oligonucleotides for the E-box sequence. Typical pictures are shown in the figure with similar results in three separate determinations. *, $p < 0.05$; **, $p < 0.01$, significantly different from each control value obtained in cells cultured for 3 days. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

indeed expressed in bone cells, including osteoclasts, osteoblasts, and chondrocytes, RT-PCR analysis was conducted using specific primers for each clock gene. For the purpose of preparation of primary osteoclasts devoid of osteoblasts, monocyte/macrophage progenitor cells were cultured for 5 days in the presence of 20 ng/ml M-CSF and 50 ng/ml RANKL, followed by determination of expression profiles of each clock gene. RT-PCR analysis revealed that expression of mRNA was drastically increased for all the osteoclastic marker genes examined in primary cultured osteoclasts differentiated from hematopoietic progenitors in the presence of both M-CSF and RANKL (Fig. 2*A*). Under these conditions, *bmal1* mRNA was constitutively expressed at each of the differentiation stages, with transient expression of *dec1* mRNA 1 day after differentiation induced by RANKL and M-CSF. However, no marked expression was seen for the other molecular clock genes, including *clock*, *per1–3*, *cry1–2*, *dbp*, and *rev-erbα*, in cultured osteoclasts throughout the entire range of differentiation stages in primary osteoclasts (Fig. 2*B*). Moreover, osteoblastic MC3T3-E1 and chondrogenic ATDC5 cells were cultured for 7 days in individual differentiation media, followed by non-quantitative determination of expression of circadian clock genes. Expression of mRNA was seen for all clock genes examined in both MC3T3-E1 and ATDC5 cells cultured for 7 days, in addition to adult mouse hypothalamus used as a positive control (Fig. 2*C*).

Expression Profile of mRNA for Clock Genes in ATDC5 Cells—We next examined the expression profiles of circadian clock genes in ATDC5 cells. ATDC5 cells are chondrogenic cells and gradually display a chondrocytic phenotype in the presence of insulin in proportion to the length of the culture period up to 28 days. Expression of mRNA was markedly increased for type X collagen during culture periods of from 7 to 14 days with a gradual increase thereafter up to 28 days, whereas a relatively steady level of mRNA expression was seen for both type II collagen and Runx2 for a period of up to 28 days (Fig. 3*A*). Under these conditions, expression of mRNA was drastically decreased for *per2*, *bmal1*, and *clock* from 7 to 14 days with a gradual decline thereafter up to 28 days, whereas mRNA levels were significantly decreased for *per1* and *cry2* at 28 days. By contrast, expression of *dec2* mRNA was markedly increased from 7 to 14 days with a sustained increase up to 28 days. No significant alternation was seen in mRNA for *per3*, *cry1*, or *dec1* on any day examined. We next examined DNA binding to the E-box sequence in ATDC5 cells cultured for different periods up to 28 days. Consistent with the mRNA expression for both *clock* and *bmal1*, DNA binding activity of the E-box was decreased in proportion to the duration of culture from 3 to 28

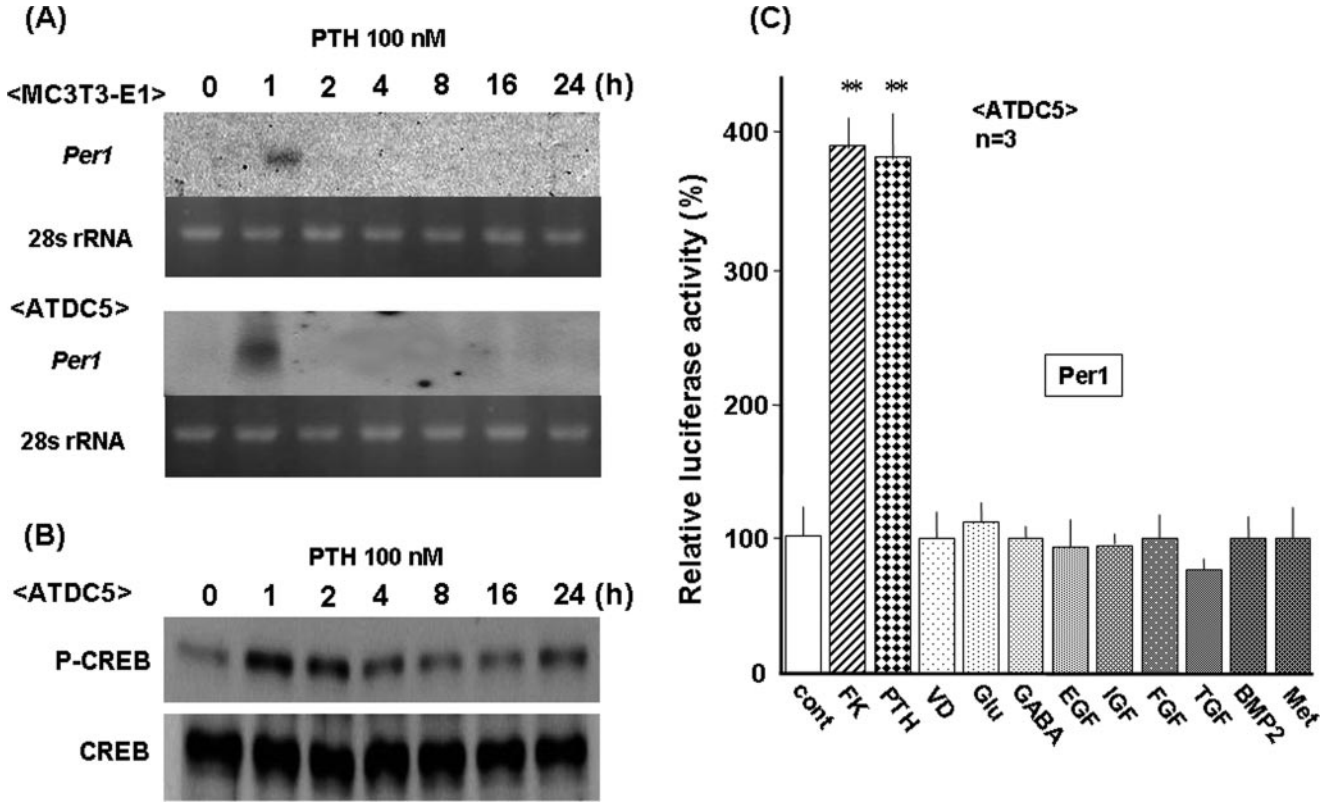

FIGURE 4. **Effect of PTH on expression of mPer1 mRNA in MC3T3-E1 and ATDC5 cells.** *A*, MC3T3-E1 and ATDC5 cells cultured for 7 days were exposed to 100 nM PTH for a period up to 24 h, followed by isolation of total RNA at the indicated time points and subsequent Northern blot analysis using a probe for mPer1. *B*, ATDC5 cells cultured for 7 days were treated with 100 nM PTH for different periods up to 24 h, followed by preparation of cell lysates at the indicated time points and subsequent immunoblotting analysis using antibodies against CREB and CREB phosphorylated at Ser-133. Typical pictures are shown in this figure, whereas similar results were invariably obtained in at least four independent determinations. *C*, ATDC5 cells were transfected with a construct of mPer1 promoter regions linked to the luciferase reporter gene, followed by incubation with 10 μM forskolin (*FK*), 10 nM PTH, vitamin D_3 (*VD*), and melatonin (*Met*), 1 mM Glu and GABA, 10 ng/ml epidermal growth factor (*EGF*), insulin-like growth factor (*IGF*), fibroblast growth factor (*FGF*), and transforming growth factor (*TGF*), and 100 ng/ml BMP-2 for 48 h and subsequent determination of luciferase activity. Values are the mean ± S.E. obtained in three independent experiments. **, $p < 0.01$, significantly different from the control (cont) value obtained in cells treated with vehicle alone.

days (Fig. 3*B*, *a*). To verify the nuclear proteins involved in E-box binding, supershift analysis was carried out in nuclear extracts prepared from ATDC5 cells cultured for 14 days using the anti-clock antibody. The anti-clock antibody was effective in inducing an upward shift of the probe-protein complex on gels (Fig. 3*B*, *b*). Moreover, E-box binding was completely inhibited by excess unlabeled double-stranded oligonucleotide containing the E-box sequence, whereas oligonucleotide with mutations of the E-box core sequence did not markedly affect E-box binding even at a molar concentration ratio of 30-fold (Fig. 3*B*, *c*).

Effect of PTH on Per mRNA Expression in MC3T3-E1 and ATDC5 Cells—To determine whether PTH induces *per1* in osteoblasts and chondrocytes, osteoblastic MC3T3-E1 and chondrogenic ATDC5 cells were cultured for 7 days, followed by the addition of 100 nM PTH and subsequent collection of total RNA at different time points after the PTH addition for determination of the mRNA levels by Northern blotting. mPer1 mRNA levels were markedly increased 1 h after the addition of PTH-(1–34), with a recovery to the baseline within 2 h in both MC3T3-E1 and ATDC5 cells (Fig. 4*A*), whereas no marked expression was seen at any time point after the addition of PTH-(3–34) (data not shown). The addition of PTH-(1–34) stimulated phosphorylation of CREB at Ser-133 in ATDC5 cells with maximal phosphorylation at 1 h and a return to the basal level within 4 h, whereas no marked alteration was seen for the expression of CREB at any time point after PTH addition up to 24 h (Fig. 4*B*). We next determined the effects of different drugs known to affect chondrocytic functions on *per* expression, in addition to PTH. ATDC5 cells were transfected with a construct of *mper* promoter regions linked to the luciferase reporter gene, followed by treatment with different drugs for 48 h. Of the drugs tested, the adenylyl cyclase activator forskolin and PTH induced a significant elevation of *mper1* promoter activity, with no significant alternations by other reagents. These included vitamin D_3, glutamate, GABA, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, transforming growth factor, BMP-2, and melatonin (Fig. 4*C*). In addition, only forskolin significantly elevated *mper2* promoter activity in addition to PTH, whereas *mper3* promoter activity was not markedly affected by any of the regents examined (data not shown).

Effect of PTH on mPer Promoter Activity—ATDC5 cells were transfected with either of the *mper* promoter constructs and treated with PTH at 10 nM for 48 h. Both *mper1* and *mper2* promoter activities were significantly increased after exposure to PTH, with no response for the *mper3* promoter (Fig. 5*A*). To examine the possible signaling pathway responsible for *mper1* and

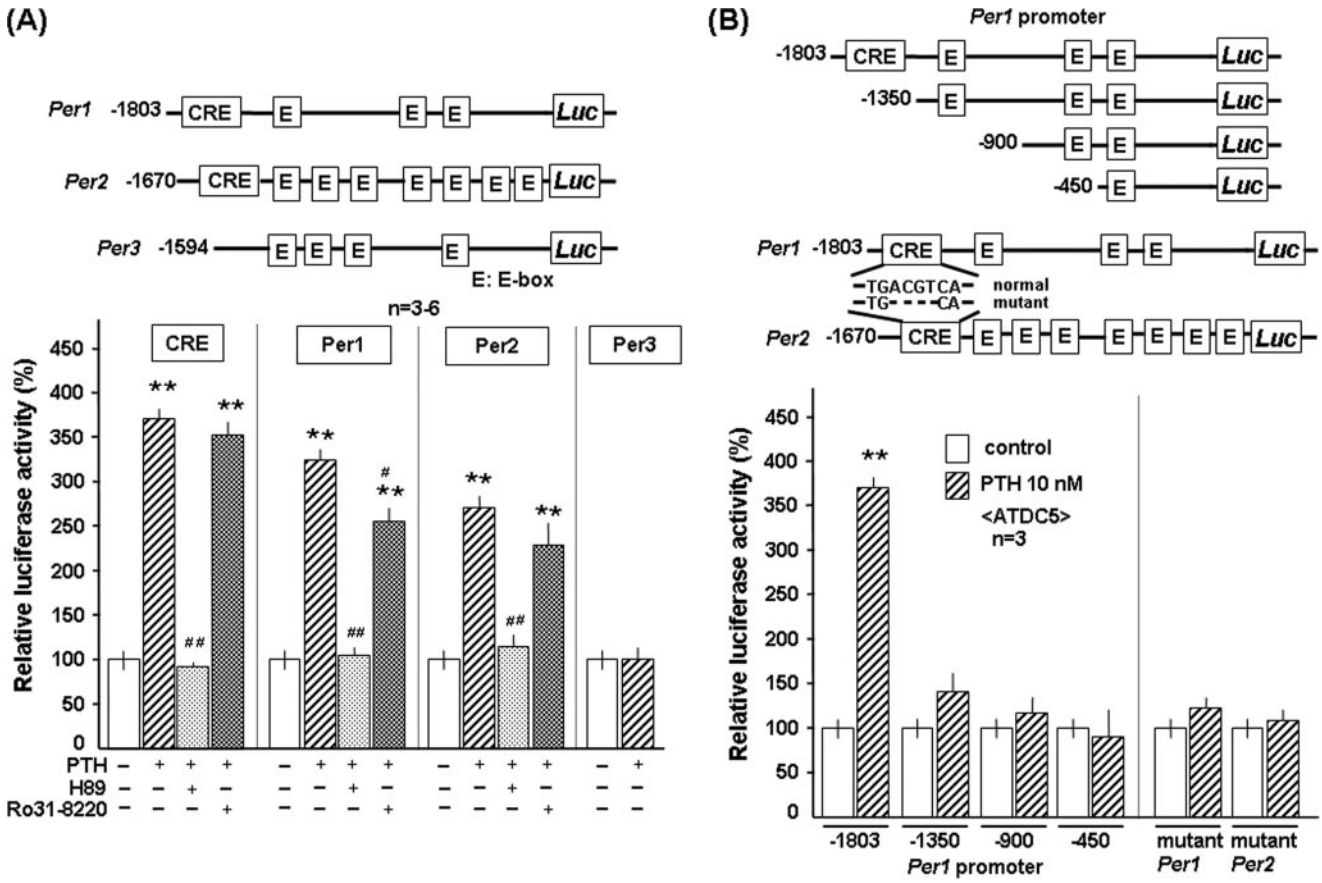

FIGURE 5. **Effect of PTH on mPer promoter activities in ATDC5 cells.** *A*, putative CRE exists within the promoter region of the *mper1* and *mper2* genes but not in the *mper3* gene. ATDC5 cells were transfected with a construct of the *mper* promoter region or a construct containing three tandems of CRE, followed by incubation with PTH at 10 nM in either the presence or absence of the PKA inhibitor H89 or the PKC inhibitor Ro31-8220 for 48 h and subsequent determination of luciferase activity. *B*, ATDC5 cells were transfected with the 5′ deletion constructs of the *mper* promoter region or mutant constructs of the four internal nucleotides of CRE within the context of the whole *mper* promoter, followed by incubation with PTH at 10 nM for 48 h and subsequent determination of luciferase activity. Values are the mean ± S.E. obtained in 3–6 independent experiments. **, $p < 0.01$, significantly different from each control value obtained in cells treated with vehicle alone. ##, $p < 0.01$; #, $p < 0.05$, significantly different from the value obtained in cells cultured in the presence of PTH alone.

mper2 transactivation by PTH, cells were transfected with the *mper* promoter, followed by treatment with the protein kinase A (PKA) inhibitor H89 and the protein kinase C (PKC) inhibitor Ro31-8220, respectively, 1 h before PTH stimulation. Inhibition of PKA by H89 almost completely blocked transactivation of both the *mper1* and *mper2* promoters by PTH, whereas the PKC inhibitor slightly but significantly inhibited the promoter activity of *mper1* without significantly affecting that of *mper2*. Moreover, PTH significantly increased the activity of pCRE-luc containing three tandems of CRE. The inhibitor of PKA, but not of PKC, completely inhibited PTH-dependent CRE reporter activity. No marked alternation was found in any promoter activities, including *mper1*, *mper2*, and CRE, in the presence of either H89 or Ro31-8220 alone. To determine the element responsive to PTH within the *mper* promoter region and to identify the *cis*- and *trans*-acting elements responsible for transcriptional activation, *mper1* and *mper2* promoter 5′ deletion constructs were generated from the full-length mPer1 promoter (−1803 to +40) with one putative CRE and three E-box sequences. PTH significantly stimulated the reporter activity of full-length *mper1* promoter containing CRE, without altering that of the other mutated *mper1* promoters not containing CRE (Fig. 5*B*). These included a −1350 mutant with three E-boxes, a −900 mutant with two E-boxes, and a −450 mutant with one E-box. To further assess the importance of CRE in the responsiveness of the *mper1* promoter to PTH, mutant constructs were made at the four nucleotides within CRE in the context of whole *mper1* and *mper2* promoters. This CRE mutation completely abolished the responsiveness of both the *mper1* and *mper2* promoters to PTH. In addition, MC3T3-E1 cells transfected with either of *mper* promoter constructs were similarly treated with PTH at 10 nM or forskolin at 10 μM. *mPer1* promoter activities were significantly increased by PTH and forskolin, whereas marked induction was observed by only forskolin for the *mper2* promoter. In contrast, no response was seen for the *mper3* promoter, as shown in ATDC5 cells. Moreover, the significant induction of *mper1* promoter activity by PTH and forskolin was completely abolished when CRE mutant constructs of the mper1 promoter were used (data not shown).

Effect of PTH on Per mRNA Expression in Metatarsals and Tibiae—To determine whether PTH induces *mper ex vivo* as seen in ATDC5 cells *in vitro*, mouse metatarsals were isolated before vascularization, followed by organotypic culture for 5 days and subsequent treatment with 100 nM PTH for *in situ* hybridization analysis. Small cells were labeled by a cRNA probe for type I collagen known to be specifically expressed by osteoblasts in the chondrocyte layer (data not shown). In cul-

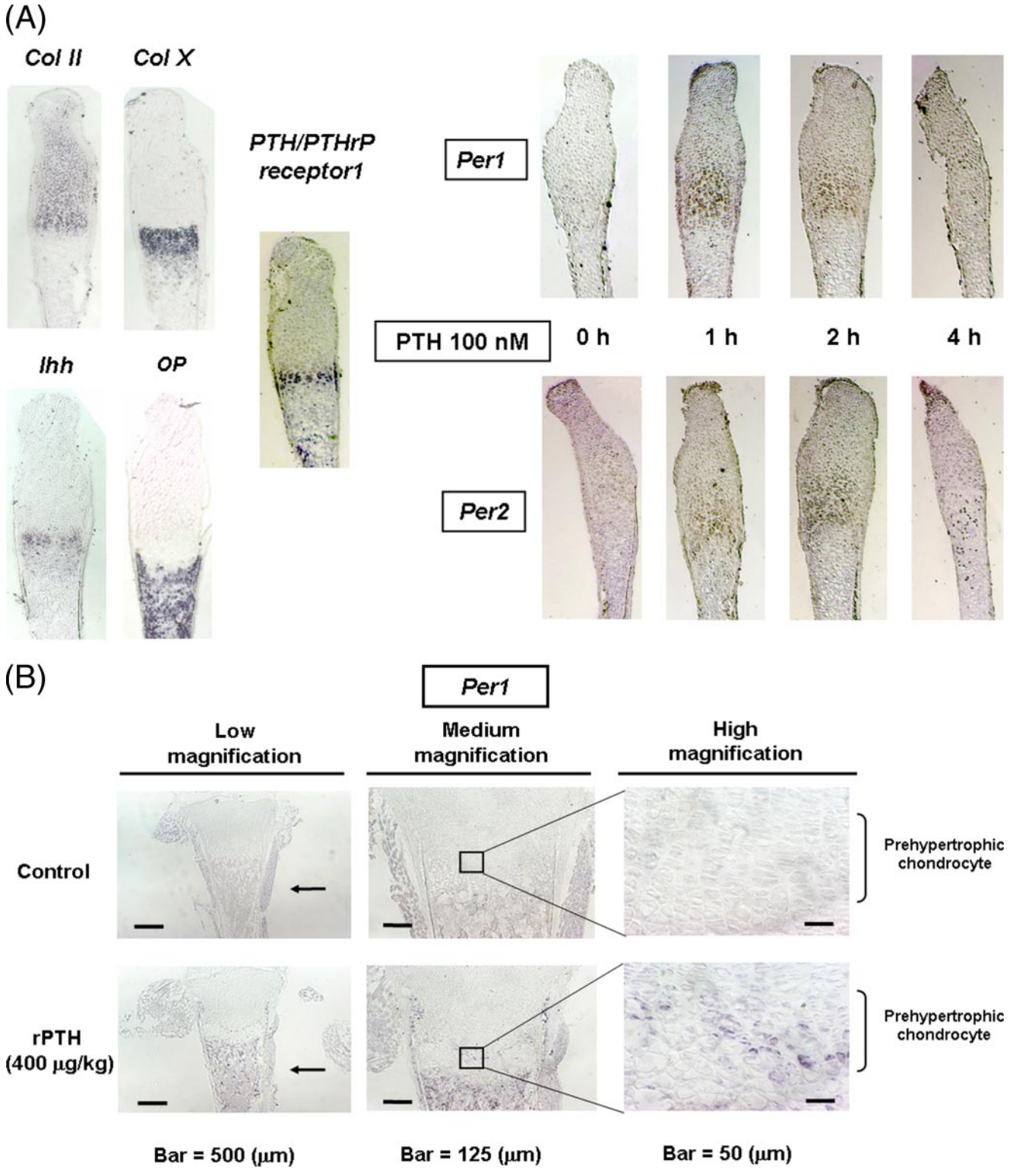

FIGURE 6. **Effect of PTH on expression of mPer1 and mPer2 mRNA in chondrocytes.** *A*, metatarsals were isolated from fetal mice before vascularization, followed by organotypic culture for 5 days and subsequent exposure to 100 nM PTH for different periods up to 4 h. Frozen sections were dissected after fixation with formalin in a cryostat, followed by *in situ* hybridization using cRNA probes for mPer1 and mPer2, in addition to different chondrocytic markers. *B*, neonatal mice were subcutaneously injected with 400 μg/kg PTH, followed by dissection of tibiae 1 h after administration and subsequent *in situ* hybridization using cRNA probes for mPer1. *Black arrows* indicate osteoblasts in cancellous bone. Typical micrographic pictures are shown in this figure, whereas similar results were invariably obtained in at least five independent determinations. *Ihh*, Indian hedgehog; *OP*, osteopontin.

tured metatarsals, type II collagen mRNA was preferentially expressed by proliferating to prehypertrophic chondrocytes, whereas Indian hedgehog mRNA was selectively expressed by prehypertrophic chondrocytes with highly selective expression of type X collagen mRNA by hypertrophic chondrocytes, in addition to osteopontin mRNA expression by hypertrophic

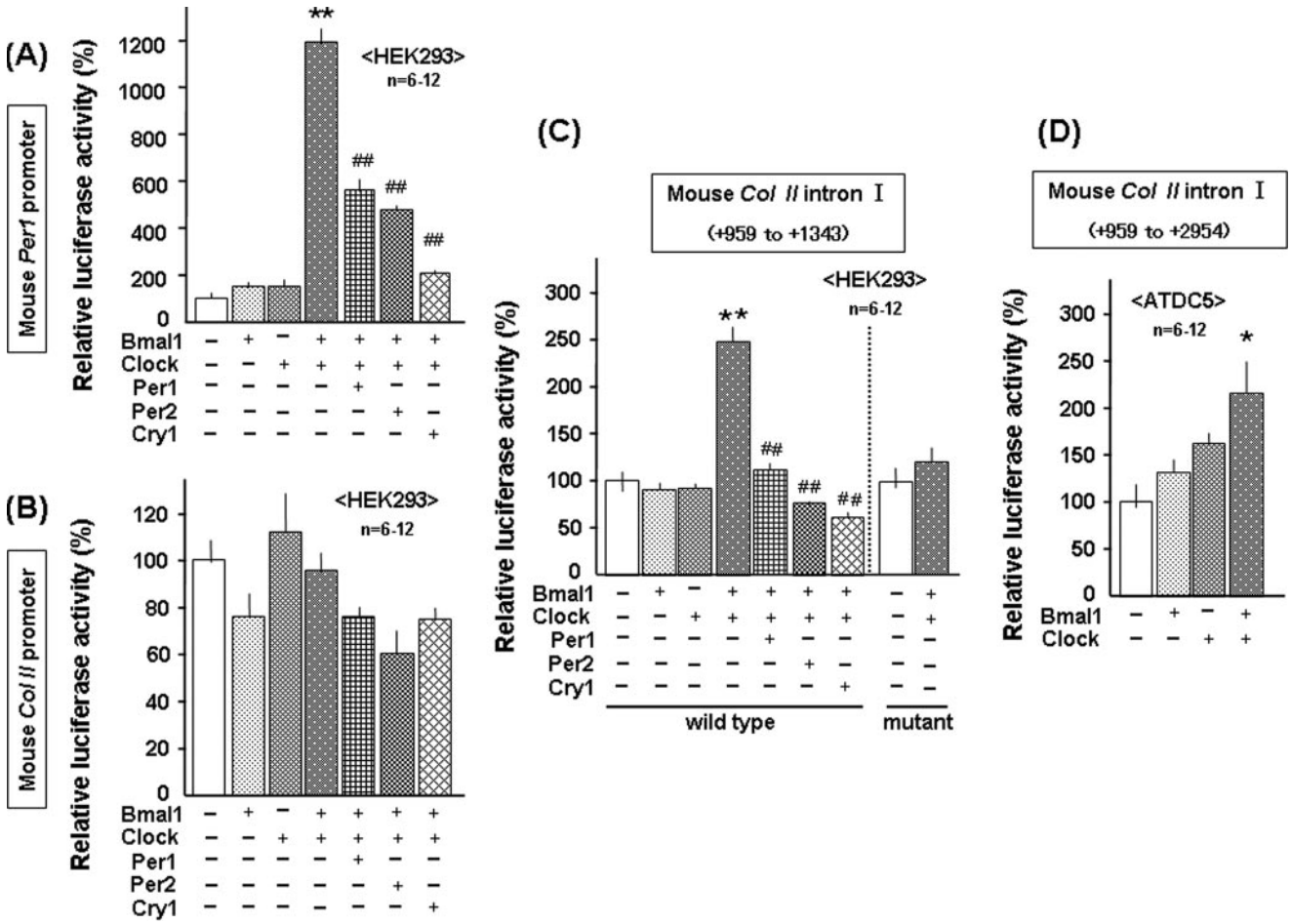

FIGURE 7. **Effect of circadian clock genes on mouse type II collagen first intron promoter activity.** HEK293 cells were transfected with a construct of *mper1* promoter region (*A*), mouse type II collagen promoter region (*B*), or mouse type II collagen first intron region (+959 to +1343) (*C*) in either the presence or absence of Clock, Bmal1, Per1, Per2, and Cry1 expression vectors, followed by incubation for 48 h and subsequent determination of luciferase activity. *D*, ATDC5 cells were transfected with a construct of the mouse type II collagen first intron region (+959 to +2954) in either the presence or absence of Clock and Bmal1 expression vectors, followed by incubation for 48 h and subsequent determination of luciferase activity. Values are the mean ± S.E. obtained in 6–12 independent experiments. **, $p < 0.01$, significantly different from each control value obtained in cells transfected with the empty vector alone. ##, $p < 0.01$, significantly different from the value obtained in the presence of Bmal1/Clock expression vectors.

chondrocytes (Fig. 6*A*). In metatarsals cultured for 5 days, PPR was predominantly expressed by prehypertrophic chondrocytes. Under the experimental conditions used here, mRNA levels peaked for both the *per1* and *per2* selectively expressed by prehypertrophic chondrocytes at 1–2 h with a return to basal levels within 4 h after the addition of PTH-(1–34). However, PTH-(3–34) failed to induce *mper* expression in cultured metatarsals at any time point after stimulation (data not shown). In sections treated with sense RNA probes, no marked expression was detected for any of the genes examined (data not shown).

To further demonstrate the induction of *mper* by PTH *in vivo* as seen in cultured ATDC5 cells *in vitro* and metatarsals *ex vivo*, neonatal mice were subcutaneously injected with 400 μg/kg PTH, followed by dissection of tibiae and subsequent *in situ* hybridization analysis. *In situ* hybridization analysis revealed that up-regulation of *mper1* mRNA expression was mainly detected in prehypertrophic chondrocytes, as seen in cultured metatarsals, 1 h after PTH administration (Fig. 6*B*). Interestingly, *mper1* mRNA expression was also increased by the systemic administration of PTH in osteoblasts attached to cancellous bone in the tibiae.

Possible Involvement of mPER in Type II Collagen Transcription—To examine the possible role of mPer in the expression of chondrocytic marker genes, promoter regions were subjected to a Motif data base search. The mouse type II collagen promoter contains a putative E-box recognized by the Bmal1/Clock heterodimer, whereas the first intron region of mouse type II collagen also has a putative E-box. In HEK293 cells transfected with a particular construct made up of different promoter regions, *mper1* promoter activity was significantly enhanced by Bmal1/Clock expression, whereas no marked increase was found in cells transfected with Bmal1 or Clock alone (Fig. 7*A*). In contrast, mPer1, mPer2, and mCry1 significantly impaired the Bmal1/Clock-dependent enhancement of *mper*1 promoter activity. In particular, mCry1 almost completely inhibited the increase in *mper1* promoter activity by Bmal1/Clock. Under these conditions, the mouse type II collagen promoter was neither activated by Bmal1/Clock nor inhibited by the clock genes tested (Fig. 7*B*). However, wild-type mouse type II collagen first intron reporter activity was significantly enhanced by Bmal1/Clock expression in a manner sensitive to inhibition by the introduction of mPer1, mPer2, or mCry1, as was also seen with *mper1* promoter activity. In con-

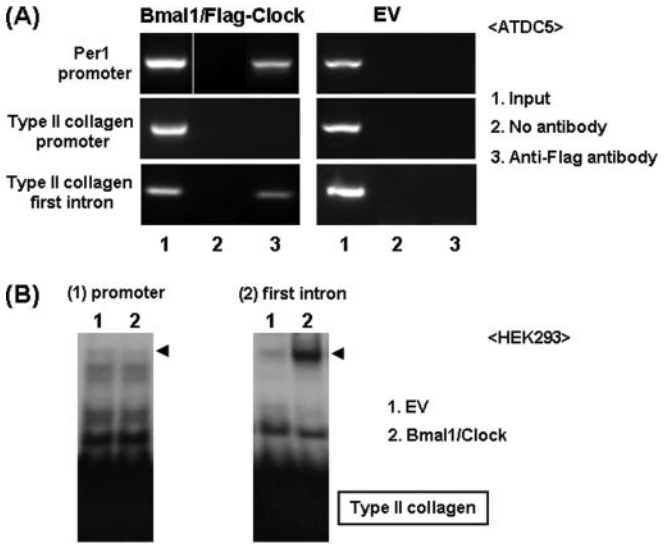

FIGURE 8. **DNA binding of circadian clock genes to mouse type II collagen first intron.** *A*, ATDC5 cells were transiently transfected with Bmal1/Clock or empty vector (*EV*), followed by incubation for 48 h and subsequent ChIP assay using the anti-FLAG antibody. Typical pictures are shown in this figure, whereas similar results were invariably obtained in at least three independent determinations. *B*, HEK293 cells were transiently transfected with Bmal1/Clock, followed by incubation for 48 h and subsequent electrophoretic mobility shift assay using the E-box probe on mouse type II collagen promoter and the first intron region. Typical autoradiograms are shown in the figure, whereas similar results were invariably obtained in at least three independent determinations.

trast, Bmal1/Clock did not significantly induce the mouse type II collagen first intron reporter activity when the reporter with the mutated E-box core sequence was used in HEK293 cells (Fig. 7*C*).

To further verify the possible involvement of clock genes in type II collagen transcription in chondrocytic cells, mouse type II collagen first intron reporter (+959 to +2954) containing both the chondrocyte-specific *cis*-acting elements and Bmal1/Clock recognition sites was used in ATDC5 cells. In ATDC5 cells, mouse type II collagen first intron reporter (+959 to +2954) activity was significantly enhanced by Bmal1/Clock expression, whereas no marked increase was found in cells transfected with Bmal1 or Clock alone (Fig. 7*D*).

To examine the possible association of Bmal1/Clock with the intron E-box of type II collagen *in vivo*, we next performed ChIP assays using ATDC5 cells expressing FLAG-tagged Bmal1/Clock. As shown in Fig. 8*A*, the association of Clock was detected in ATDC5 cells transfected with Bmal1/Clock within the type II collagen first intron, in addition to the *mper1* promoter, but not the type II collagen promoter. In contrast, no association of Clock was found in ATDC5 cells transfected with empty vectors alone within the type II collagen first intron in addition to the *mper* promoter.

The binding of Bmal1/Clcok to the E-box found in the promoter and first intron region of mouse type II collagen was evaluated by electrophoretic mobility shift assay in HEK293 cells transfected with Bmal1/Clock. DNA binding was detected in nuclear extracts of HEK293 cells transiently expressing Bmal1/Clock in the E-box located in the first intron, but not in the promoter, of mouse type II collagen (Fig. 8*B*).

DISCUSSION

The essential importance of the present findings is that PTH stimulates expression of both *mper1* and *mper2* mRNA through transactivation mediated by a PKA-CREB signaling pathway in both cultured chondrocytes and metatarsals, with inhibition by mPer of Bmal1/Clock-dependent mouse type II collagen first intron reporter activity but not type II collagen promoter reporter activity. Circadian rhythm was found for the expression of particular osteoblastic and chondrocytic marker genes in adult mouse femurs, and in addition, a subcutaneous injection of PTH markedly elevated *mper1* mRNA expression predominantly in prehypertrophic chondrocytes in the tibia of neonatal mice *in vivo*. To our knowledge, this is the first direct demonstration of the expression of molecular clock genes by chondrocytes in terms of direct modulation of cellular functions. Although several previous studies have already demonstrated the functional expression of molecular clock genes in peripheral tissues, including bone, no direct evidence for a role of these clock genes in the cellular functionality of chondrocytes has been previously reported.

The prevailing view is that PPR is a member of the B superfamily of G protein-coupled receptors with seven transmembrane domains in association with at least three different isoforms of the trimeric G protein. These include G_s protein for the activation of adenylyl cyclase/PKA, G_q/G_{11} protein for the activation of phospholipase C/PKC, and G_i protein for the inhibition of adenylyl cyclase (20, 21). Previous studies have demonstrated that PPR may differentially regulate the expression of various genes on the basis of intracellular signaling pathways in target cells. The PKC signaling pathway appears to regulate the expression of type X collagen, Bcl-2, and vitamin D_3 receptors, for example, whereas the cAMP/PKA signaling pathway plays a pivotal role in controlling the mineralization and synthesis of glycosaminoglycans, in addition to inducing alkaline phosphatase and Indian hedgehog, in primary chondrocytes and chondrogenic cells such as ATDC5 cells (22–24). However, it is widely accepted that the roles of PKA *versus* PKC signaling activated by PTH/PTH-related protein vary according to experimental conditions, such as cell densities and differentiation stages, in terms of regulating proliferation and differentiation of chondrocytes (25, 26). Although PKC signaling is associated with the enhancement of cell proliferation and DNA synthesis in addition to calcium signaling (27), cAMP-PKA signaling appears to be the dominant pathway for the actions of PTH on osteoblasts (28). Indeed, in osteoblasts PTH transiently activates various immediate early genes, including c-*fos*, c-*jun*, c-*myc*, interleukin-6, and leukemia inhibitory factor (29–31), through the cAMP-PKA signaling pathway.

The present study clearly demonstrates the involvement of cAMP-PKA signaling in the regulation by PTH of *mper* gene expression. For instance, pretreatment with the PKA inhibitor, H89, almost completely prevented the PTH-dependent enhancement of both the *mper1* and *mper2* reporter activities, with the PKC inhibitor, Ro-31-8220, being rather ineffective. Forskolin strongly potentiated the *mper* reporter activity to an extent similar to PTH, whereas the PTH analog PTH-(3–34), which retains the ability to signal through PKC and calcium

pathways without PKA signaling activity (32), had no effect on *mper1* mRNA expression or *mper1* promoter activity (PTH-(1–34)). Moreover, the cAMP-PKA signaling pathway has been shown to stimulate the phosphorylation of CREB on serine 133, which is required for the recruitment of the CREB binding protein/p300 co-activator for gene transcription (33). Taken together, it appears that PTH activates *mper1* gene expression through a mechanism related to cAMP-PKA-CREB signaling in both osteoblasts and chondrocytes.

A data base analysis revealed a putative E-box, which would be able to bind a Bmal1/Clock heterodimer, exists within 3 kb of the 5′-flanking region and the first intron of the type II collagen gene. The present findings from reporter and ChIP assays, however, suggest that the Bmal1/Clock heterodimer binds to the E-box located in the first intron but not that in the 3 kb of the 5′-flanking region. In addition to the mouse type II collagen gene described above, the E-box element is present within the first intron region but not in the 5′-flanking region of the type X collagen gene. Moreover, the type I collagen gene, an osteoblastic marker, contains an E-box element in the 5′-flanking region. Several independent lines of evidence indicate that effective transactivation would be mediated by the E-box element located in the intron regions but not that in the 5′-flanking regions with regard to Bmal1/Clock target genes. E-box elements located in the first and second introns of Dbp are critical for circadian transactivation by the Clock protein (34), in fact, the Bmal1/Clock heterodimer transactivates the peroxisome proliferator-activated receptor α gene through an E-box-rich region located in the second intron (6). As the Per protein would be translocated into the nucleus to inhibit the activity of the Bmal1/Clock heterodimer (8), it may be speculated that the mPer transiently up-regulated by PTH negatively regulates the transactivation of chondrocytic marker genes by Bmal1/Clock through the E-box located in the intron region in chondrocytes.

In this study, we conducted reporter activity assays using HEK293 cells, which are a human embryonic kidney cell line with high transfection efficiency. The mouse type II collagen first intron reporter construct used in HEK293 cells does not contain chondrocyte specific *cis*-acting elements reported in the literature. However, the chondrocyte-specific type II collagen first intron construct containing the Bmal1/Clock recognition sites was up-regulated by overexpression of Bmal1/Clock in ATDC5 cells. It is therefore conceivable that Bmal1/Clock binds the E-box located in the mouse type II collagen first intron for subsequent positive regulation of transcription, and mPer proteins in turn negatively regulate the transcription mediated by Bmal1/Clock even in chondrogenic cells, as described previously (8–11). The exact molecular mechanism as well as the functional significance of differential expression profiles of clock genes during *in vitro* maturation of chondrogenic ATDC5 cells, however, remains to be elucidated in future studies.

It appears circadian clock genes are functionally expressed by chondrocytes to regulate extracellular matrix in cartilage. Molecular clock signaling is thus a potential target for the development of a drug useful for the treatment and therapy of a variety of bone and cartilage diseases relevant to abnormal development and maturation of chondrocytes as well as osteoblasts in human beings.

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