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A novel autofeedback loop of *Dec1* transcription involved in circadian rhythm regulation

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Abstract

An autofeedback loop associated with transcription of clock gene(s), *Per(s)*, is generally accepted as the molecular machinery of circadian rhythm generation, in which CLOCK/BMAL act as positive regulators and PER/CRY as negative ones. We show here an autofeedback loop of *Dec1* encoding a basic helix–loop–helix transcription factor: CLOCK/BMAL increased the promoter activity of *Dec1*, and DEC1 and DEC2 as well as PERs and CRYs suppressed the induced expression. Three CACGTG E-boxes are responsible for both the activation and the suppression of *Dec1* transcription. Forced expression of *Clock*/*Bmal* increased endogenous *Dec1* mRNA level, and overexpression of *Dec1* resulted in suppression of *Dec2*, *Per2*, and *Dbp* expression. The level of *Dec1* expression in the heart of *Clock*/*Clock* mutant mice was continuously low throughout the day. These findings suggest that *Dec1* is positively regulated by CLOCK/BMAL and is involved in circadian rhythm regulation by suppressing CLOCK/BMAL-induced gene expression. The autofeedback loop of *Dec1* may be interlocked with the core feedback loop of *Per* in some situations.

Keywords: DEC1; Circadian rhythm; Clock; bHLH transcription factor

Circadian rhythms in behavior and physiology have an adaptive significance in living organisms from prokaryotes to humans [1]. These rhythms are regulated by a circadian clock(s) which has a period slightly deviated from an exact 24 h and which responds to environmental time cues such as a daily light–dark cycle. In mammals, clock genes *Clock*, *Bmal1*, *Per(s)*, and *Cry(s)* and their protein products comprise a molecular feedback loop, in which a CLOCK/BMAL1 heterodimer binds to a CACGTG E-box and activates transcription of *Per(s)* and *Cry(s)* [2,3]; protein products of *Per(s)* and *Cry(s)* in turn suppress the transactivation by CLOCK/BMAL1 [4,5]. It is assumed that one cycle of this autofeedback loop generates a circadian cycle. However, this simple feedback loop model is still insufficient to explain the stability and precision of circadian rhythms in living cells, especially in the mammalian clock.

Recently, we found that *Dec1* and *Dec2* encoding basic helix–loop–helix (bHLH) transcription factors were expressed in the suprachiasmatic nucleus (SCN) in a circadian fashion, with peaks in the subjective day, and that *Dec1* expression was enhanced by a light pulse in a phase-dependent manner similar to *Per1* [6]. Furthermore, DEC1 and DEC2 suppressed *Per* transactivation by CLOCK/BMAL1 through competition for E-boxes and/or protein–protein interaction with BMAL1. However, it is unknown whether DEC1 expression is regulated by CLOCK, BMAL, PER, CRY, and DEC1/2. In the present study, we demonstrated the existence of a novel autofeedback loop associated with *Dec1*

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transcription, with CLOCK/BMAL as positive elements and DECs as negative ones.

Materials and methods

Cell cultures. NIH3T3 cells were supplied by the cell bank of the Institute of Physical and Chemical Research (Tsukuba, Japan). Human bone marrow-derived mesenchymal cells were obtained from BioWhittaker. These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Human umbilical vein endothelial cells were obtained from Clonetics and cultured in M199 medium supplemented with 20% fetal bovine serum, 60 μg/ml endothelial cell growth supplement (Collaborative Biomedical), and 50 mg/ml heparin as described previously [7].

Plasmid constructions. A series of deletion constructs of the human *Dec1* promoter connected to the luciferase reporter gene were prepared as described previously [8]. The oligonucleotides of three CACGTG E-boxes with 6-bp of flanking sequence on each side were linked together and subcloned into pGL3-TK [9] (a generous gift from T. Sueyoshi) to make pDEC1-E-ABC-TK. The oligonucleotides of three tandem sequences containing E-box A, E-box B, or E-box C in the *Dec1* promoter were also subcloned into pGL3-TK (pDEC1-E-Ax3-TK, pDEC1-E-Bx3-TK, or pDEC1-E-Cx3-TK). *Cry1* expression and *Per1* expression vectors were generously supplied by M. Ikeda and by H. Tei [10], respectively. Construction of pGL3-TK containing three E-boxes in the mouse *Per1* promoter (pE54-TK) and expression vectors of *Clock*, *Bmal1*, *Per2*, *Cry2*, and *Bmal2* (*Clif*) was described previously [6,11]. Mouse *Dec1* cDNA was amplified by RT-PCR using a pair of primers: 5′-CCACCATGGAACGGATCCCCAGC-3′ and 5′-AGGAAAGCAAAGCAGCAGGA-3′. The PCR product was subcloned into the pGEM T-Easy vector (Promega) and subjected to sequencing for identification. Human and mouse *Dec1* and *Dec2* cDNAs were subcloned into pcDNA3.1/Zeo (Invitrogen).

Transient transfection and luciferase assay. NIH3T3 cells were seeded at 2×10^4 cells per 16-mm well 24 h before transfection. Luciferase reporter plasmid (2 ng per well) and mouse *Clock* expression and *Bmal1* expression vectors (each 50 ng per well) were co-transfected with or without one of the following vectors (10 ng per well): human *Dec1*, mouse *Dec1*, human *Dec2*, mouse *Dec2*, mouse *Per1*, rat *Per2*, human *Cry1*, or human *Cry2*, using Trans IT polyamine (Pan Vera). For the internal standard, 0.2 ng of phRL-TK (Promega) or pRL-SV40 (Promega) was co-transfected. The total concentration of DNA was adjusted to 112.2 ng per well with an empty vector (pcDNA3.1/Zeo). After incubation for 3 h, the medium was replaced with a fresh one. The cells were incubated for 48 h and subjected to luciferase reporter assay using Dual-Luciferase Reporter Assay System (Promega). Luciferase activities were normalized by the internal control activities. The values represent means ± SD for four wells.

RNA isolation, Northern blot analysis, and real-time quantitative RT-PCR. Human umbilical vein endothelial cells were infected with adenovirus AdCMV.GFP, AdCMV.CLOCK, or AdCMV.CLIF/BMAL2, as described previously [11]. Total RNA was prepared from the cells with the RNeasy kit (Qiagen) 48 h after adenovirus infection and subjected to Northern blot analysis of *Dec1*. Human *Dec1* cDNA used as a hybridization probe was amplified by RT-PCR using a pair of primers: 5′-TTACAACTTTGGGTCACTTGG-3′ and 5′-TCCATAGCCACTG TCTGTGTC-3′. The radioactivity of the hybridized area was quantified using Bio-imaging Analyzer System (Fuji Photo Film).

Human bone marrow-derived mesenchymal cells were infected with adenovirus carrying human *Dec1* at a multiplicity of infection (m.o.i.) of 100 as described previously [12]. Total RNA was extracted from the cells 6 h after adenovirus infection using Trizol (Invitrogen) and subjected to real-time quantitative RT-PCR using ABI Prism 7900 sequence detection system. Two-month-old male wild type and *Clock*/*Clock* mutant C57/BL6 mice were maintained under 12 h:12 h light–dark (LD) cycle for at least 2 weeks before the day of the experiment. The mice kept under LD or constant dark (DD) conditions were killed by decapitation at different times of day. The experimental procedures on animal care and treatment were performed with permission, and following the rules and guidelines of Hiroshima University and Hokkaido University. Total RNA was extracted from mouse heart and subjected to real-time quantitative RT-PCR. The pairs of nucleotides for amplification of cDNAs were 5′-GCATCAGAAGATAATTG CTTTACAGAA-3′ and 5′-TCTCAAACCGGGAGAGGTATTG-3′ for *Dec2*, 5′-GGTACTTGGAGAGCTGCAATGAG-3′ and 5′-CTTA TCACTGGACCTTAGCGCTG-3′ for *Per2*, and 5′-CATCGCTTCT CAGAAGAGGAACTT-3′ and 5′-TCCGGCACCTGGATTTTTC-3′ for *Dbp*. The sequences of TaqMan fluorogenic probes were 5′-FAM-C GTTCCACTCGGGATTTCAAACATGC-TAMRA-3′ for *Dec2*, 5′-F AM-TGCCACCCTGAAGAGGAAATGCGA-TAMRA-3′ for *Per2*, and 5′-FAM-CCCCAGCCAATCATGAAGAAGGCA-TAMRA-3′ for *Dbp*. The kit for the TaqMan probe and primers for GAPDH was obtained from PE Applied Biosystems. The TaqMan probe and primers for mouse and human *Dec1* and mouse GAPDH were as described previously [8,13]. The values were normalized by the amount of GAPDH expression and represent means ± SD.

Electrophoretic mobility shift assay. Human DEC1, human DEC2, and luciferase protein as a control product were synthesized using TNT Coupled Reticulocyte Lysate Systems (Promega). The double-stranded oligonucleotides of DEC1 E-box C (5′-ctagGTCCAACACGTG AGACTCtcga-3′) were end-labelled using [^{32}P]dCTP (Du Pont-New England Nuclear) and DNA polymerase I Klenow fragment (TAKARA). Synthesized human DEC1 or DEC2 protein was incubated with approximately 30,000 cpm of ^{32}P-labelled probe for 10 min at room temperature in 10 μl of 10 mM Tris/HCl (pH 8.0), 0.5 mM dithiothreitol, 10% glycerol, 1 μg of poly(dI–dC), 50 mM NaCl, and 5 mM $MgCl_2$, and then the mixtures were subjected to 5% polyacrylamide gel electrophoresis in 12 mM Tris–HCl/125 mM glycine/1 mM EDTA electrophoresis buffer. In competition experiments, a 50- or 200-fold molar excess of unlabelled E-box A (5′-ctagAATAAACACGTG TCAAAT-3′), E-box B (5′-ctagCCCGGCCACGTGAAGCGTtcga-3′), E-box C, or mutated E-box C (E-box Cm; 5′-ctag GTCCAAtcgctcAGACTCtcga-3′) was added. Rabbit antibodies to human DEC1 and DEC2 were produced by immunizing synthetic peptide fragments Cys-Leu-Gln-Gly-Gly-Thr-Ser-Arg-Lys-Pro-Ser-Asp-Pro-Ala-Pro and Cys-Lys-Pro-Lys-Arg-Ser-Met-Lys-Arg-Asp-Asp-Thr-Lys-Asp, respectively. The obtained antibodies were purified by affinity column chromatography. Supershift analysis was performed 10 min after addition of 0.3 μg of the polyclonal antibodies for human DEC1 or DEC2.

Results and discussion

A novel autofeedback loop associated with Dec1 transcription

As reported previously [6], *Dec1* in the SCN is expressed in a circadian fashion. Since there are three CACGTG E-boxes in the promoter region of human *Dec1* [14] and at least two in similar regions of the mouse *Dec1* promoter (T. Kawamoto, unpublished observation), we examined whether the promoter activity of human *Dec1* is inducible by CLOCK/BMAL1. The luciferase activity of the *Dec1* promoter containing E-boxes A, B, and C (pDEC1-3620) was increased 3-fold by *Clock Bmal1* co-transfection. Furthermore, the induced activity was suppressed by DEC1 or DEC2 (Fig. 1A). The activity of the *Dec1* promoter containing

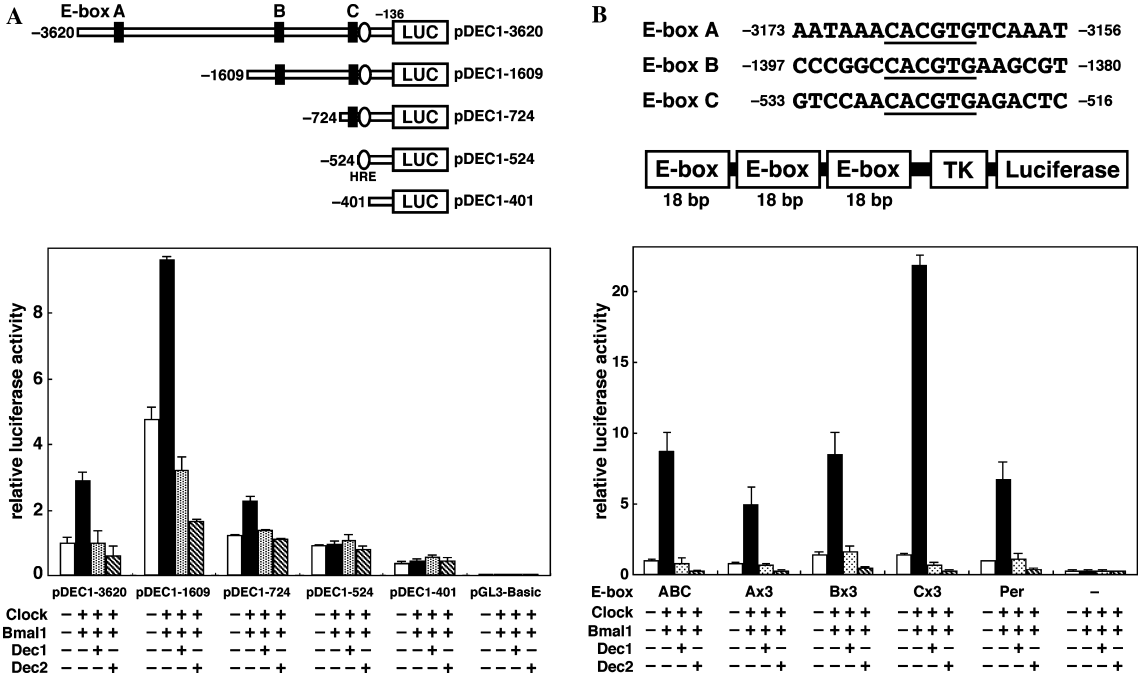

Fig. 1. Effect of co-expression of *Clock Bmal1*, *Dec1*, and *Dec2* on the promoter activity of human *Dec1*. (A) The diagram shows the structure of luciferase reporter constructs containing various lengths of 5′-flanking regions of *Dec1*. Three CACGTG E-boxes (A–C) and a hypoxia response element (HRE) are shown. The numbers indicate relative positions from the translation initiation codon. The reporter constructs were co-transfected with the *Clock* expression and *Bmal1* expression vectors together with human *Dec1* or *Dec2* expression vector into NIH3T3 cells. The total amount of transfected DNA was adjusted to the same value with an empty vector (pcDNA3.1/Zeo). After the cells were incubated for 48 h luciferase activities were determined. The values represent relative levels of means ± SD for four wells. (B) Activity of three CACGTG E-boxes in the *Dec1* promoter. Three E-boxes (A–C) with 6 bp of flanking sequences in the *Dec1* promoter were subcloned into pGL3-TK [9]. The reporter construct of *Dec1* E-boxes ABC, Ax3, Bx3, or Cx3 (pDEC1-E-ABC-TK, pDEC1-E-Ax3-TK, pDEC1-E-Bx3-TK, or pDEC1-E-Cx3-TK), or three E-boxes in the mouse *Per1* promoter (*Per*) connected to the *TK* promoter (pE54-TK), were co-transfected with the *Clock* expression and *Bmal1* expression vectors together with the *Dec1* or *Dec2* expression vector.

E-boxes B and C (pDEC1-1609), or E-box C (pDEC1-724), was increased 2-fold by CLOCK/BMAL1 and reduced by DEC1 or DEC2. In contrast, the luciferase activities of the constructs that did not contain any E-boxes (pDEC1-524 and pDEC1-401) were not increased by CLOCK/BMAL1, nor were they suppressed by DEC1 or DEC2.

To examine the involvement of the CACGTG E-boxes in induction of *Dec1* transcription, we subcloned E-boxes A, B, and C with their flanking sequences upstream of the thymidine kinase (*TK*) promoter connected to the luciferase reporter gene (pDEC1-E-ABC-TK). We also made a construct containing three copies of E-box A, E-box B, or E-box C upstream of the *TK* promoter (pDEC1-E-Ax3-TK, pDEC1-E-Bx3-TK, or pDEC1-E-Cx3-TK). The luciferase activity of pDEC1-E-ABC-TK was increased 9-fold by CLOCK/BMAL1 and the induced activity was reduced by DEC1 or DEC2 (Fig. 1B). Among the E-box Ax3, Bx3, and Cx3, E-box Cx3 showed the highest activity. The activities of these constructs were equal to or stronger than those of three E-boxes in the mouse *Per1* promoter (pE54-TK) [6]. These results indicate that E-boxes A, B, and C in the *Dec1* promoter are responsible for the induction by CLOCK/BMAL1, and that the feedback of the protein product of *Dec1* inhibits the induced *Dec1* transcription.

Direct binding of DEC1 and DEC2 to E-boxes A, B, and C in the Dec1 promoter

In electrophoretic mobility shift assay, a shifted band was observed with human DEC1 or DEC2 protein when a ^{32}P-labelled DNA containing E-box C with its surrounding sequences was used as a probe (Fig. 2A, lanes 2 and 12). Unlabelled E-boxes A, B, and C oligonucleotides competed with the radiolabelled E-box C probe dose-dependently (Fig. 2A, lanes 3–8 and 13–18). However, competition by unlabelled E-box B was less effective than that of unlabelled E-box A or C. Unlabelled oligonucleotides with mutations in E-box C (E-box Cm) were ineffective even at a 200-fold molar excess (Fig. 2A, lanes 10 and 20). Furthermore, anti-DEC1 antibodies, but not anti-DEC2 antibodies, supershifted mobility of the bands of E-box C and DEC1 complex (Fig. 2B, lanes 3 and 4). In contrast, anti-DEC2 antibodies, but not anti-DEC1 antibodies, supershifted the bands of E-box C and DEC2 complex (Fig. 2B, lanes 6 and 7). These results demonstrated that DEC1 and

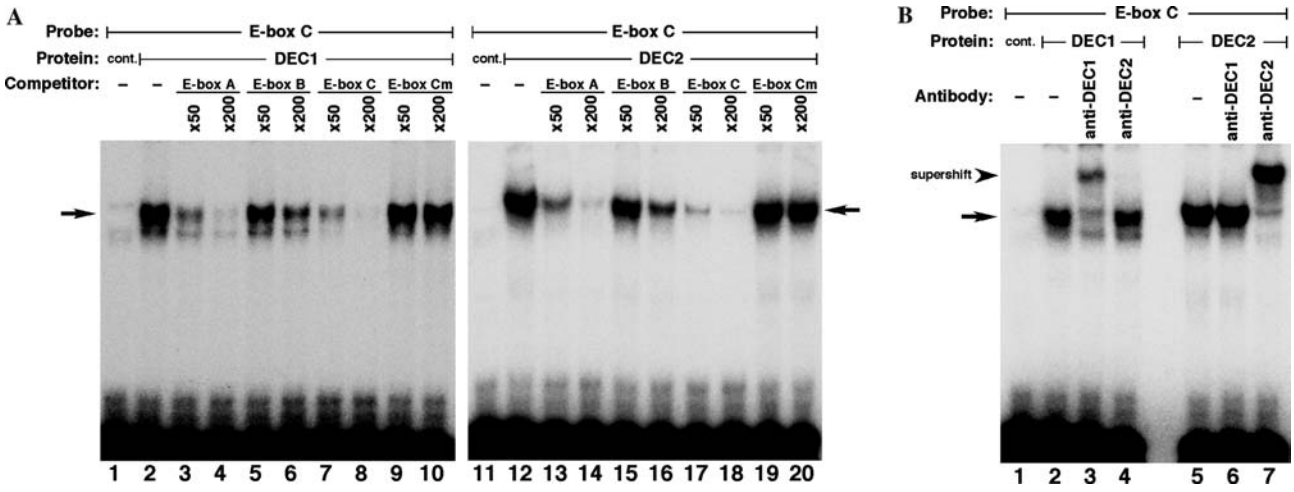

Fig. 2. Analysis of DEC1 and DEC2 binding to the CACGTG E-boxes in the *Dec1* promoter. (A) The ^{32}P-labelled DEC1 E-box C probe was incubated with human DEC1 (lanes 2–10) or DEC2 (lanes 12–20) protein synthesized by in vitro transcription/translation system, or a synthesized luciferase protein as a control product (cont.) (lanes 1 and 11). The competition experiments were performed using a 50- or 200-fold molar excess of unlabelled oligonucleotides of E-box A (lanes 3, 4, 13, and 14), E-box B (lanes 5, 6, 15, and 16), E-box C (lanes 7, 8, 17, and 18), or mutated E-box C (E-box Cm) (lanes 9, 10, 19, and 20). Arrows indicate the binding of radiolabelled E-box C to DEC1 or DEC2. (B) Supershift analysis of DEC1 or DEC2 was performed using anti-DEC1 or anti-DEC2 antibodies (lanes 3, 4, 6, and 7). The supershifted bands are indicated by an arrowhead.

DEC2 specifically bind to E-boxes A, B, and C in the *Dec1* gene and suppress its induced promoter activity. Deletion analysis of DEC1 protein showed that the N-terminal region of DEC1 containing the bHLH domain is sufficient for the suppression of CLOCK/BMAL1-induced promoter and for the binding to the CACGTG E-box (T. Kawamoto, unpublished observation).

There are two pathways for DEC1 to recognize the target genes: One is binding to the CACGTG E-boxes in the promoter regions of the target genes [6,15,16], the other is protein–protein interaction with positive transcription factors such as USF [17] and BMAL1 [6]. Although DECs can interact with BMAL1, the high binding affinity of DECs to the CACGTG E-box suggests that the competition with the CLOCK/BMAL1 heterodimer for the E-box is a major mechanism for suppression by DECs.

Enhancement of Dec1 expression by a CLOCK/BMAL2 heterodimer

Recently, one of our group (K.M.) identified a novel bHLH/PAS domain transcription factor, BMAL2 (CLIF), that shares high homology with BMAL1 [11]. BMAL2 forms a heterodimer with CLOCK to up-regulate clock-controlled gene expression through CACGTG E-boxes. To examine the activity of BMAL2 on the *Dec1* promoter, the reporter construct pDEC1-3620 or pDEC1-ABC-TK was co-transfected with a *Bmal2* expression vector together with a *Clock* expression vector. The luciferase activities of both reporter genes were increased by CLOCK/BMAL2 (Fig. 3A), and the induced activities were suppressed by DEC1 or DEC2. Enhancement by CLOCK/BMAL2 was comparable to that by CLOCK/BMAL1.

Since BMAL2 is suggested to play an important role in the peripheral circadian clock in umbilical vein endothelial cells [11], we examined the effect of CLOCK/BMAL2 overexpression in the cells. Infection of adenovirus expressing *Clock*-induced *Dec1* mRNA dose-dependently (2- to 3-fold), and overexpression of *Bmal2* increased the expression of *Dec1* mRNA 2-fold (Fig. 3B). Coinfection of adenoviruses expressing *Clock* and *Bmal2* further increased the *Dec1* mRNA level (3- to 4-fold), but infection of *Gfp*-expressing adenovirus did not increase *Dec1* mRNA. These results suggest that *Dec1* expression is actually regulated by CLOCK/BMAL in living cells.

Suppression of Clock-related genes by Dec1 overexpression

Forced expression of human *Dec1* in rabbit mesenchymal cells decreased the mRNA level of endogenous *Dec1* [13]: It is not known whether the overexpression of *Dec1* decreases the mRNA levels of rabbit *Dec2* and other clock genes, since their nucleotide sequences for rabbit cDNAs have not yet been determined. We then examined the effect of *Dec1* overexpression on other clock-related genes using human mesenchymal cells: Infection of adenovirus carrying human *Dec1* increased the mRNA level of human *Dec1* by 8-fold in human mesenchymal cells (Fig. 3C). The increased mRNA seems to be derived from the transfected adenovirus, and endogenous *Dec1* expression must be suppressed as in the case of the experiments with rabbit mesenchymal

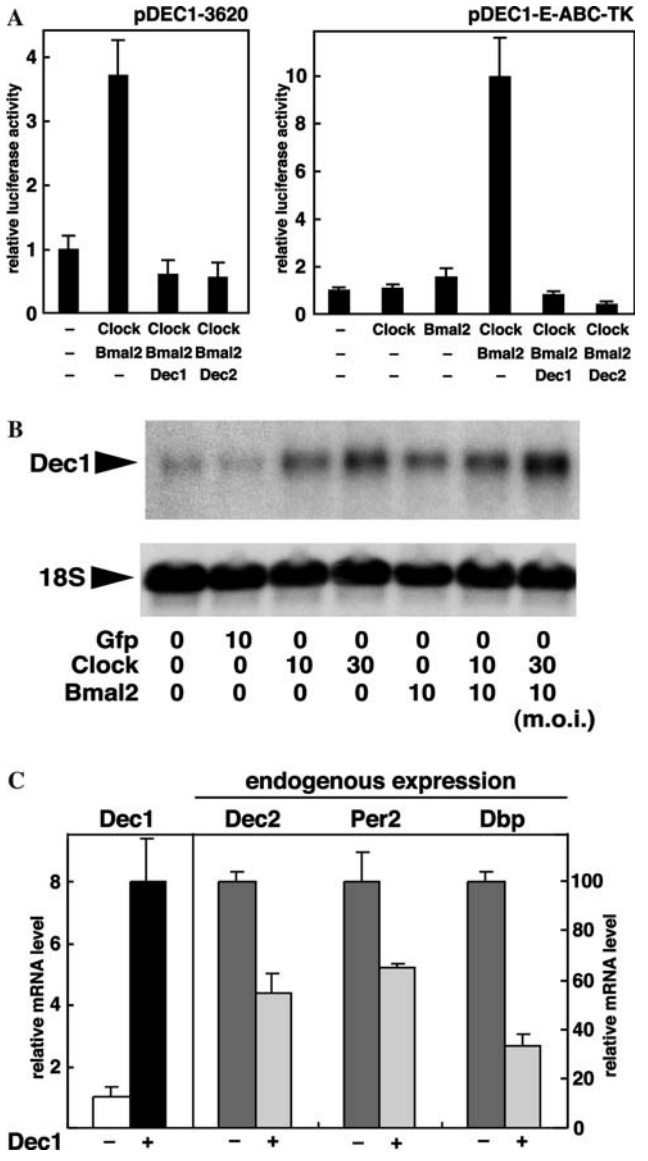

Fig. 3. Induction of *Dec1* transcription by CLOCK and BMAL2 heterodimer and suppression of clock-related genes by DEC1. (A) The reporter construct pDEC1-3620 or pDEC1-E-ABC-TK was co-transfected with some of the following expression vectors into NIH3T3 cells as indicated: *Clock*, *Bmal2*, *Dec1*, and *Dec2*. The cells were incubated for 48 h and luciferase activities were determined. (B) Human umbilical vein endothelial cells were infected with adenovirus expressing *Gfp*, *Clock*, and/or *Bmal2* at the indicated m.o.i. Total RNA was isolated 48 h after infection and subjected to Northern blot analysis using ^{32}P-labelled human *Dec1* cDNA as a probe. The blot was also hybridized with 18S rRNA to normalize for loading. Similar results were obtained in repeated studies. (C) Human bone marrow-derived mesenchymal cells were infected with adenovirus expressing human *Dec1* at m.o.i. 100 (+) or mock-infected (−). Total RNA was extracted from the cells 6 h after infection and subjected to real-time quantitative RT-PCR of *Dec1*, *Dec2*, *Per2*, and *Dbp* mRNA.

cells [13]. In these cells, the mRNA level of human *Dec2* was decreased by 45% (Fig. 3C): Li et al. [18] also showed that forced expression of *Dec1* in 293T cells suppressed *Dec2* expression. In addition, the over-expression of *Dec1* in the mesenchymal cells decreased the mRNA levels of human *Per2* and *Dbp* by 35% and 67%, respectively. These results indicate that DEC1 may suppress the expression of CLOCK/BMAL-inducible genes involved in the circadian core loop and/or in the output pathway.

Decreased expression of Dec1 in Clock/Clock mutant mice

To further confirm that a CLOCK/BMAL heterodimer actually regulates *Dec1* transcription in vivo, we compared the mRNA level of *Dec1* in *Clock*/*Clock* mutant mice with that in wild type mice. Circadian rhythms ($P < 0.05$) of *Dec1* expression in the heart of the wild type mice were observed under both LD and DD conditions (Fig. 4A). However, the expression of *Dec1* in the tissue of the *Clock*/*Clock* mutant mice was continuously low throughout the day and the level was less than the circadian trough in the wild type mice, indicating the critical role of CLOCK for *Dec1* expression. Similar results were obtained in other tissues including the SCN, which will be published elsewhere (M. P. Butler et al., manuscript in preparation; M. Noshiro et al., manuscript in preparation).

The autofeedback loop of Dec1 transcription interlocking with the core feedback loop associated with PERs and CRYs

To examine whether CLOCK/BMAL1-induced *Dec1* transcription is suppressed by PERs and CRYs, we co-transfected pDEC1-E-ABC-TK with a *Per* or *Cry* expression vector together with the *Clock* expression and *Bmal1* expression vectors, and compared the abilities of PERs and CRYs with those of DEC1 and DEC2. The CLOCK/BMAL1-induced promoter activity was suppressed by PER1, PER2, CRY1, and CRY2 (Fig. 4B). Suppression of CLOCK/BMAL1-induced transcription by DEC1 was stronger than that by PER1 but similar to PER2-induced suppression. Suppression by DEC2 was more robust than that by PERs or CRYs. Thus, the negative elements of the circadian core loop, PERs and CRYs, were also the negative elements for the feedback loop of *Dec1* transcription. Taking DECs as negative regulators of *Per* transcription into consideration [6] (Fig. 3C), these findings suggest that at least two autofeedback loops, DEC loop and PER loop, are involved in circadian rhythm regulation, and that the two loops are interlocked to each other (Fig. 5).

A model of multiple feedback loops for circadian rhythm regulation involving DEC loop

In the regulation of clock gene expression, BMAL1 forms a heterodimer with CLOCK and acts as a

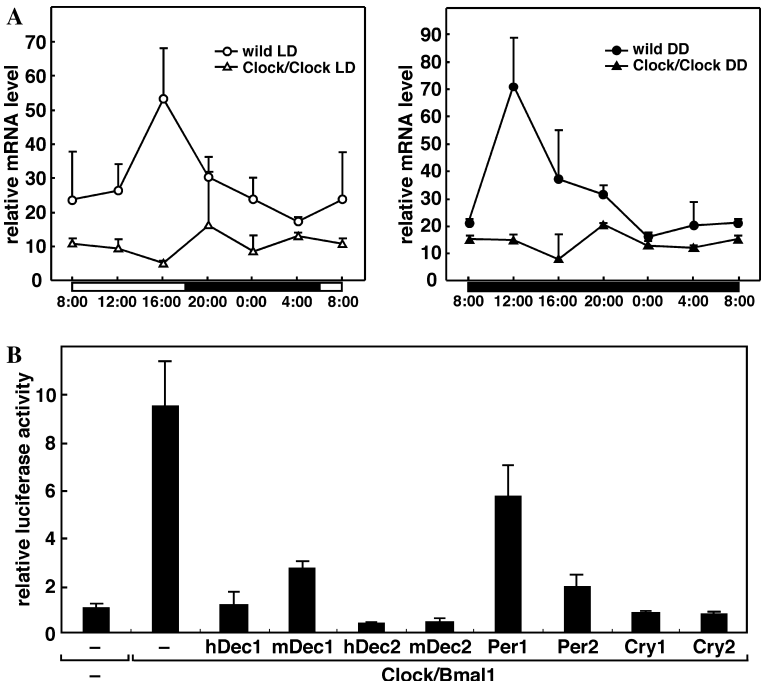

Fig. 4. CLOCK/BMAL-dependent expression of *Dec1*. (A) *Dec1* expression in the heart of *Clock*/*Clock* mutant mice. Two-month-old male wild type (circles) and *Clock*/*Clock* mutant (triangles) C57/BL6 mice were maintained under 12 h:12 h LD cycle for 2 weeks before the day of the experiment. The mice kept under LD (left) or DD (right) conditions were killed by decapitation at different times of day. Total RNA was isolated from the heart of the mice (3 mice at each time point) at indicated zeitgeber time and subjected to real-time quantitative RT-PCR of *Dec1* [13]. (B) Effects of PERs and CRYs on the CLOCK/BMAL1-induced promoter. pDEC1-E-ABC-TK was co-transfected with the *Clock* expression and *Bmal1* expression vectors together with one of the following expression vectors into NIH3T3 cells: human *Dec1* (*hDec1*), mouse *Dec1* (*mDec1*), human *Dec2* (*hDec2*), mouse *Dec2* (*mDec2*), mouse *Per1*, rat *Per2*, human *Cry1*, or human *Cry2*. The cells were incubated for 48 h, and luciferase activities were determined.

common positive factor through the CACGTG E-box, whereas PERs and CRYs act as negative ones. In the present study, we identified another autofeedback loop of *Dec* (DEC loop), in which *Dec* transcription is activated by the CLOCK/BMAL1 heterodimer and suppressed by DEC (Fig. 5). In addition to this loop, *Dec* transcription is also suppressed by PER and CRY via an interlocked loop. Similarly, *Per* and *Cry* transcriptions are activated by CLOCK/BMAL1 and suppressed by PER and CRY (by an autofeedback loop) on the one hand and by DEC (by an interlocked loop) on the other. DECs can suppress the induced promoter activity by competition with the CLOCK/BMAL1 heterodimer for the E-box binding, whereas PER and CRY inhibit the CLOCK/BMAL1 activity without binding to the E-box. The mechanisms for suppression differ between DEC and PER/CRY: DEC gives the other way to suppress the CLOCK/BMAL activity. Recently, another feedback loop for *Bmal1* in the circadian regulation has been reported [19,20]: In this loop, the expression of *Rev-Erbα* is inducible by the CLOCK/BMAL1 heterodimer, and the protein product of *Rev-Erbα* suppresses the expression of *Bmal1* through the ROR response element (Fig. 5). These factors, including two *Dec*, three *Per*, and two *Cry* homologs, may work together to constitute a network of the circadian clock system [21].

Among the genes that show circadian expression rhythms in the SCN and/or liver, only 4% are common to both organs [20,22], and *Dec1* is among these. Protein products of these common genes are considered to be components of the rhythm generating machinery or factors important for mediating the circadian signal from the machinery to the periphery. As demonstrated previously [23], PERs, CRYs, BMAL1, and CLOCK act as output molecules of the core loop as well as major components of the master clock. They can regulate output gene transcription such as the vasopressin gene in addition to *Per* transcription. Similarly, DECs may function as output molecules that can drive circadian rhythms in clock-controlled genes (Fig. 5). On the other hand, overexpression of *Dec1* (*Stra13*) enhanced chondrogenic differentiation of ATDC5 cells [13] and neuronal differentiation of P19 cells [24], but suppressed mesodermal differentiation of P19 cells, adipocyte differentiation of 3T3-L1 cells [25], and B cell activation [26]. *Dec1* (*Stra13*)-deficient mice developed age-induced autoimmune disease as a result of ineffective elimination of activated T-cells [27]. A relationship between these effects of DEC in various tissues and the role of DEC1 in the molecular clock system remains unknown.

Dec1 may also be involved in the input pathway of the molecular circadian clock. A light pulse enhances

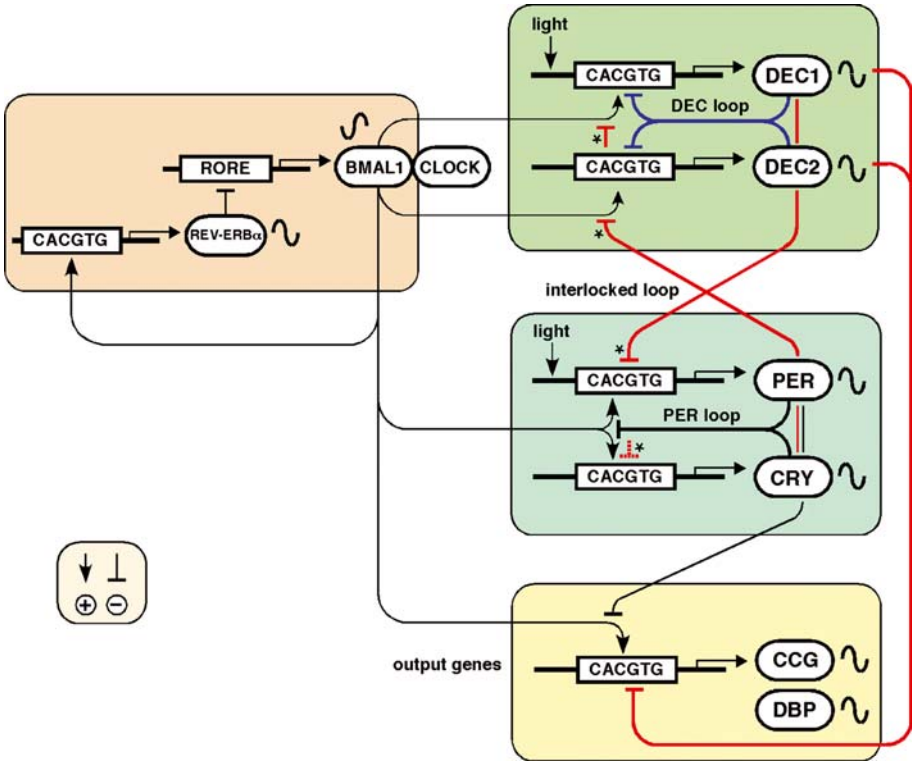

Fig. 5. A model of multiple feedback loops for circadian rhythm regulation. A heterodimer of CLOCK and BMAL1 activates expression of clock genes such as *Per*, *Cry*, *Dec1*, *Dec2*, and *Rev-Erbα*, as well as output genes such as *Dbp* and some clock-controlled genes (CCG). Induced DEC1 or DEC2 suppresses the promoter activity of *Dec* genes by autofeedback loops by binding to the CACGTG E-box (DEC loop) on the one hand and suppresses *Per* or *Cry* gene expression by interlocked loops on the other [6]. Similarly, induced PER together with CRY suppresses the expression of *Per* and *Cry* by autofeedback loops by interacting with the CLOCK/BMAL1 heterodimer (PER loop) and suppresses *Dec* promoter activity by interlocked loops. Expression of output genes such as *Dbp* is also suppressed by DECs or by PERs/CRYs [31] in a similar manner. Induced REV-ERBα suppresses the promoter activity of *Bmal1* by binding to the ROR response element (RORE) [19,20]. Light induces the expression of *Dec1*, *Per1*, and *Per2* in the SCN [6]. Induction and suppression of gene expression are indicated by + and −, respectively. Suppression by interlocked loops is shown by asterisks.

Dec1 expression, but not *Dec2*, in a phase-dependent manner [6] (Fig. 5), suggesting its important role in light entrainment of the circadian pacemaker. The light induction of *Dec1* is not a result of light-induced *Per* expression, since light-induced *Dec1* expression occurs as fast as that of *Per1*. Furthermore, the expression of *Dec1* (or *Dec2*) is inducible by hypoxia [8,28], cAMP [29], retinoic acid [24], transforming growth factor-β [15], and nerve growth factor [30] in the peripheral tissues or in the brain. DEC induced by these factors might modulate the molecular circadian system in the central as well as the peripheral tissues. These findings suggest a possible role of DEC as a coupling molecule between the circadian system and environment.

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