The BMAL1 C terminus regulates the circadian transcription feedback loop

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The circadian clock is driven by cell-autonomous transcription/translation feedback loops. The BMAL1 transcription factor is an indispensable component of the positive arm of this molecular oscillator in mammals. Here, we present a molecular genetic screening assay for mutant circadian clock proteins that is based on real-time circadian rhythm monitoring in cultured fibroblasts. By using this assay, we identified a domain in the extreme C terminus of BMAL1 that plays an essential role in the rhythmic control of E-box-mediated circadian transcription. Remarkably, the last 43 aa of BMAL1 are required for transcriptional activation, as well as for association with the circadian transcriptional repressor CRYPTOCHROME 1 (CRY1), depending on the coexistence of CLOCK protein. C-terminally truncated BMAL1 mutant proteins still associate with mPER2 (another protein of the negative feedback loop), suggesting that an additional repression mechanism may converge on the N terminus. Taken together, these results suggest that the C-terminal region of BMAL1 is involved in determining the balance between circadian transcriptional activation and suppression.

circadian clock | real-time monitor

The mammalian circadian clock is a highly dynamic system that generates periodic fluctuations in the mRNA expression levels of hundreds of genes to confer near 24-h rhythmicity to behavior, physiology, and metabolic processes, thereby allowing mammals to anticipate the momentum of the day (1). The master clock resides in the suprachiasmatic nuclei (SCN) of the brain and, in turn, synchronizes circadian clocks in peripheral tissues (2). Even fibroblasts in culture contain an active circadian clock that has the same genetic makeup of the central clock in the SCN (3–6). To keep pace with the day–night cycle, the SCN clock, but not peripheral clocks, are entrained by light.

Circadian rhythms are generated by a molecular oscillator that consists of intertwined positive and negative transcription/translation feedback loops involving a set of clock genes (7) and clock-controlled output genes that link the oscillator to clock-controlled processes (8). BMAL1 (MOP3) and CLOCK are basic helix–loop–helix PAS transcription factors that heterodimerize and (by means of binding to E-box promoter elements) transactivate the *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) genes and an orphan nuclear receptor *Rev-Erbα* core oscillator gene. Subsequently, PER and CRY proteins act as negative elements by inhibiting the activity of the CLOCK/BMAL1 heterodimer, whereas REV-ERBα negatively regulates *Bmal1* gene expression (1, 9). The above feedback mechanism is supported by biochemical, molecular, and genetic evidence; however, formal proof of its requirement in the maintenance of circadian clock oscillations has not been shown thus far.

Genetic ablation of *mBmal1* results in complete disruption of the mammalian circadian clock at the behavioral and molecular levels (10). However, except for the PAS elements, which are required for association with CLOCK (11), relatively little is known about protein domains that regulate BMAL1 function. A recent study revealed that constitutive high expression of BMAL1 protein in *Rev-Erbα*-disrupted mice still allows robust circadian molecular and behavioral rhythms (12). Moreover, it has been shown that sumoylation of BMAL1 influences posttranscriptional features of BMAL1 and that this process is CLOCK-dependent (13). Taken together, these results suggest that posttranslational modification of BMAL1 is an important prerequisite for its performance in the circadian oscillator.

To understand the mechanism of BMAL1 function in the clock, we generated several BMAL1 mutants and analyzed their effect on circadian clock performance in mammalian cells. We show that the C terminus of BMAL1 is involved in both positive and negative regulation of transcriptional activity, which in turn is mandatory for molecular clock oscillation in the living cell.

Results and Discussion

To dissect the role of BMAL1 in circadian clock performance, we decided to perform a random mutagenesis-based structure/function analysis of this protein in mammalian cells undergoing multiple cycles of clock oscillation. To monitor circadian oscillations in real-time mode, we used *mPer2* or *mBmal1* promoter-driven luciferase reporter vectors (*mPer2*:*Luc* and *mBmal1*:*Luc*, respectively). After transient expression of *mPer2*:*Luc* (or *mBmal1*:*Luc*) in Rat-1 cells for 48 h, followed by synchronization of individual cellular clocks with 100 nM dexamethasone (14, 15), we could detect clear rhythmic expression of the reporter gene for five cycles with a periodicity of ≈21 h (Fig. 1*A*). First, we validated this system for use as a BMAL1 mutant screening tool by analyzing the effect of constitutive overexpression of Flag-tagged WT mBMAL1 (WT-BMAL1) on circadian oscillations. After dexamethasone synchronization, WT-BMAL1-overexpressing Rat-1 fibroblasts are able to produce a robust circadian rhythm with a period and amplitude comparable to cells transfected with an empty vector (Fig. 1*B*). Thus, constant high levels of BMAL1 are well tolerated by the molecular oscillator, which contrasts with the severe impact of constitutive overexpression of circadian clock components of the negative limb of the oscillator (i.e., mCRY1 and mPER2) (16, 17). In line with the observation that *Rev-Erbα*$^{-/-}$ mice (constitutively expressing *Bma1*) maintain behavioral and molecular rhythms (12), this finding indicates that mammalian core oscillator function does not require cyclic transcription of *Bmal1*. Consistent with the above observations, circadian bioluminescence rhythms were also not attenuated after cotransfection of WT-*Bmal1* and WT-*Clock* expression vectors (Fig. 5, which is published as supporting information on the PNAS web site).

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Abbreviations: CFP, cyan fluorescent protein; HA, hemagglutinin; YFP, yellow fluorescent protein.

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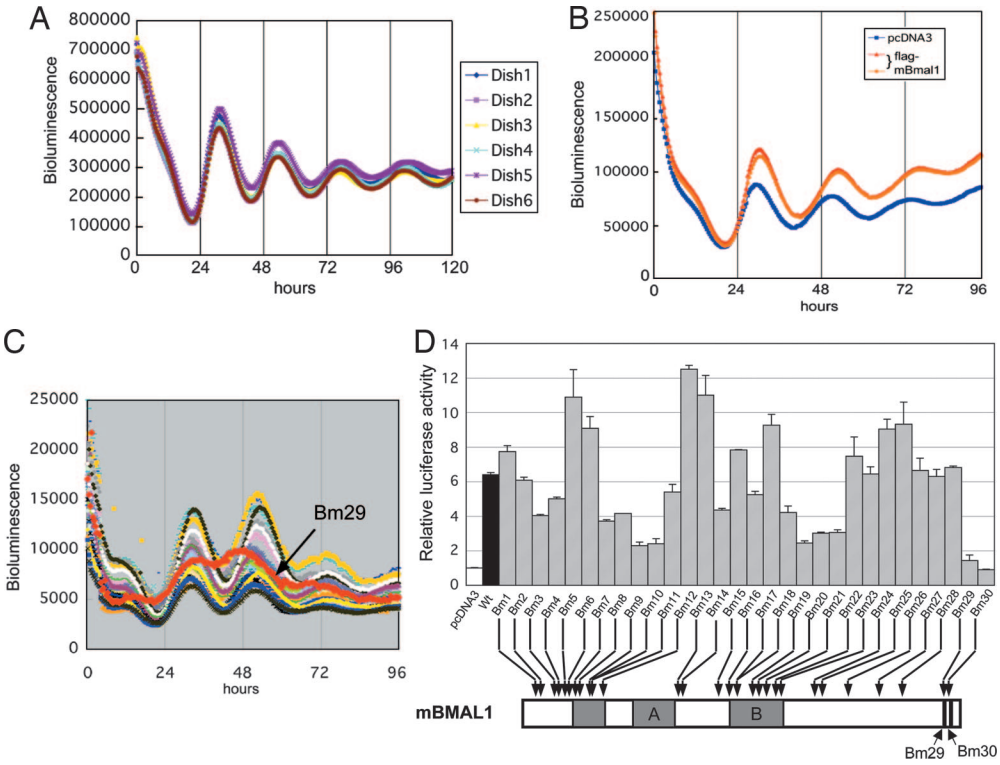

Fig. 1. Screening for functional protein domains in BMAL1. (*A*) Bioluminescence oscillations of dexamethasone-synchronized Rat-1 cells expressing the *mPer2*:*Luc* reporter demonstrate the reproducibility of the system. (*B*) Effect of overexpression of WT *Flag-mBmal1* on *mPer2*:*Luc* rhythms (red lines). As a control, we used cells transfected with the empty pcDNA3 vector (blue line). (*C*) Functional screen for mutant BMAL1 proteins (containing random transposon-based in-frame insertions of 19 aa) that affect circadian core oscillations. Different colors indicate different proteins. Note the severely impaired *mPer2*:*Luc* rhythm obtained with Flag-BMAL1-Bm29, which is indicated in red and marked by an arrow. (*D*) Functional screen for mutant BMAL1 proteins (containing random transposon-based in-frame insertions of 19 aa) that affect transcription activation. *Upper* shows transcription activity of WT Flag-mBmal1 (black bar) and Flag-mBmal1-Bm1 to Flag-mBmal1-Bm30 (gray bars). Baseline luciferase activity is indicated by the open bar. Each bar indicates the mean of three independent samples, and error bars indicate the SEM. *Lower* shows the positions of the 19-aa insertions.

Next, we performed a molecular genetic screen to identify protein domains that are critical for BMAL1 function. To this end, we generated an expression library of random mutant (Flag-tagged) BMAL1 proteins by using a commercially available Tn5 transposon-based insertion system that introduces 19 aa in-frame (18). In this way, we obtained 30 in-frame protein mutants, named Flag-BMAL1-Bm1 to Flag-BMAL1-Bm30, that were sequenced to determine the position of the 19-aa insertion. Then, mutant BMAL1 proteins were overexpressed in Rat-1 cells and cotransfected with *mPer2*:*Luc* (or *mBmal1*:*Luc*) clock reporter genes, followed by continuous and quantifiable monitoring of circadian clock parameters (e.g., period length and amplitude). We identified two mutant BMAL1 proteins (Flag-BMAL1-Bm29 and -Bm30, carrying insertions after Ala-599 and Leu-606 of mBMAL1, respectively; Fig. 1*D*) that impaired circadian rhythmicity in a dominant negative manner, as evident from the severe dampening of *mPer2*:*Luc*-driven bioluminescence rhythms (Fig. 1*C*).

In parallel, we tested the capacity of these 30 mutant BMAL1 proteins to drive E-box-mediated transcription in the NIH 3T3 cell-based transcription activation assay. Interestingly, the second screen uncovered more mutant proteins with a clear molecular phenotype than the real-time assay. For example, BMAL1 mutant proteins with insertions in the basic helix–loop–helix (Flag-BMAL1-Bm7/8/9/10) and PAS-B domains (Flag-BMAL1-Bm19/20/21) exhibited a reduced transcriptional activation capacity (possibly due to a lack of association with CLOCK), yet they did not disturb the performance of the molecular oscillator in the real-time assay (Fig. 1*D*). In addition, we isolated three BMAL1 mutant proteins that showed 2-fold greater activity in the presence of coexpressed CLOCK (as compared with WT BMAL1), suggesting that those are gain-of-function BMAL1 mutants. In this case, insertions were located upstream and downstream of the basic helix–loop–helix region (-Bm5 and -Bm12, respectively) and downstream of the PAS-A domain (-Bm13). Importantly, however, we identified Flag-BMAL1-Bm29 and -Bm30 (shown in the previous experiment to have a dominant negative effect on the circadian core oscillator) as the two mutant BMAL1 proteins that failed to drive E-box-mediated transcription. Thus, the C terminus of BMAL1 must contain a domain (or domains) required for transcription activation, the disruption of which causes a dominant negative effect on core oscillator function.

To obtain more insight into the role of the C-terminal region of BMAL1 in controlling the generation of circadian rhythmicity, we generated by *in vitro* mutagenesis three BMAL1 "deletion" constructs in which amino acid Glu-447, Leu-554, or Ile-584 of mBMAL1 was substituted by a stop codon (Fig. 2*A*). Western blot analysis of cell lysates from COS7 cells transiently expressing Flag-BMAL1(E447X), Flag-BMAL1(L554X), or Flag-BMAL1(I584X) revealed proteins of expected molecular weight at WT amounts (Fig. 6*A*, which is published as supporting information on the PNAS web site). In contrast to WT Flag-BMAL1, all three mutant BMAL1 proteins remained inactive in the E-box-mediated transcription assay (Fig. 2*B*). These results support a previous study in which the C-terminal region of BMAL1 was shown to be required for its transcriptional activation function (19). Importantly, similar to Flag-BMAL1-Bm29 (Fig. 7, which is published as supporting information on the PNAS web site), they inhibited WT Flag-BMAL1/CLOCK-driven transcription activation in a dose-dependent manner, pointing to a dominant negative effect of deletion of the C terminus on the transcription activation property of BMAL1 (Fig. 2*C*).

We next investigated whether these deletion mutant proteins also affected real-time core oscillations in Rat-1 cells. Overexpression of Flag-BMAL1(L554X) dramatically blunted *mPer2*:*Luc*- and *Bmal1*:*Luc*-mediated luminescence rhythms in a dose-dependent (dominant negative) manner, but, importantly, whereas *mPer2*:*Luc* expression levels dropped to basal values (Fig. 2*D*), *Bmal1*:*Luc* luminescence levels were markedly elevated (Fig. 2*E*). These results fit well with the recently reported mechanism of transcriptional regulation of *Bmal1* expression (12), which proposes that the CLOCK/BMAL1 heterodimer up-regulates transcription of the *Rev-Erbα* gene, causing REV-ERBα-mediated suppression of *Bmal1* transcription through REV-ERBα response element enhancer elements in the *Bmal1* promoter. Similar results were

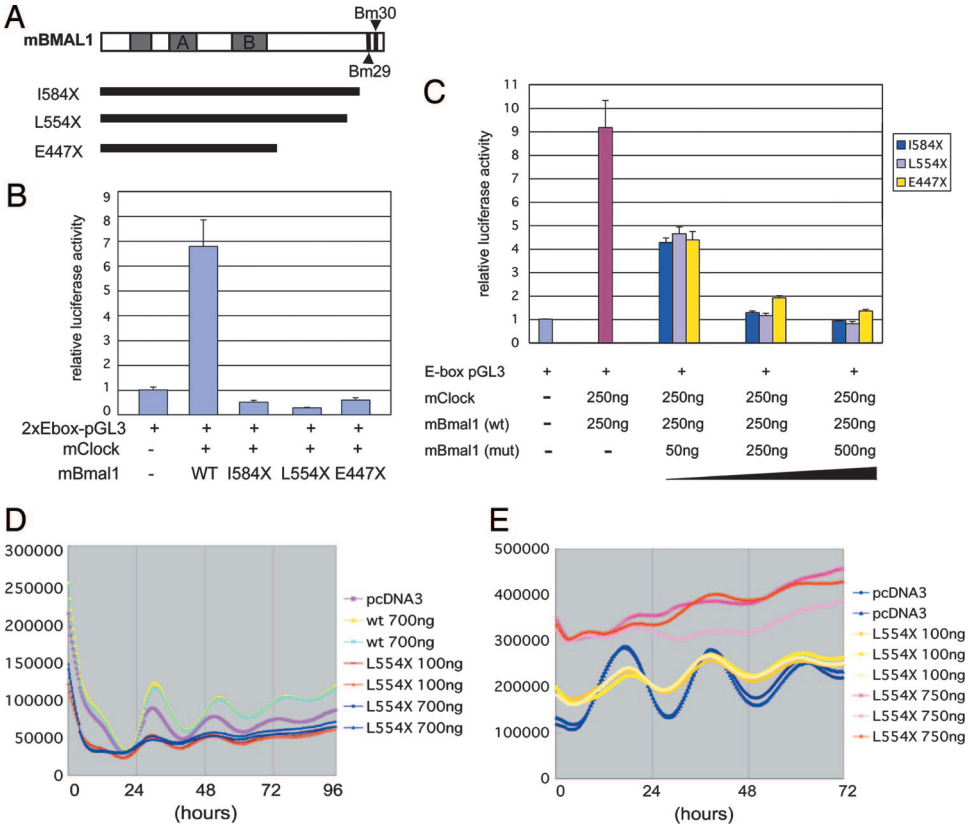

Fig. 2. Dominant negative effect of C-terminally truncated BMAL1. (*A*) Schematic representation of the BMAL1 protein (including the positions of 19-aa insertions in Flag-BMAL1-Bm29 and -Bm30) and three mutant proteins lacking the C-terminal region as a result of the introduction of stop codons. (*B*) NIH 3T3 cell-based promoter assay for CLOCK/BMAL1 transcriptional activity using the 2xE-box-pGL3 reporter construct, *Flag-Clock*, and WT and mutant *Flag-Bmal1* constructs (as indicated). Relative luciferase activities are calculated as a severalfold induction from the basal expression level. Each bar indicates the mean of three independent samples, and error bars indicate the SEM. (*C*) Same as *B* except that WT *Flag-Bmal1* is coexpressed with *Flag-Bmal1(E447X)*, *Flag-Bmal1(L554X)*, or *Flag-Bmal1(I584X)* constructs (amounts as indicated). (*D*) Effect of overexpression of WT *Flag-mBmal1* and *Flag-mBmal1(L554X)* on *mPer2*:*Luc* rhythms in dexamethasone-synchronized Rat-1 cells. Each line represents one dish, and all dishes presented were simultaneously analyzed by using the same photomultiplier. (*E*) Effect of overexpression of *Flag-mBmal1(L554X)* on *mBmal1*:*Luc* rhythms in dexamethasone-synchronized Rat-1 cells.

obtained when Flag-BMAL1(L554X) was replaced by Flag-BMAL1(E447X) or Flag-BMAL1(I584X) (data not shown). Taken together, these data indicate that the C-terminal 43 aa of BMAL1 protein contain a domain that is essential for mammalian circadian oscillator performance and that, consistent with recent reports that show interaction of p300/CREB-binding protein coactivators by means of the C-terminal region of BMAL1 (19–21), this domain must be involved in transcription activation.

Next, we investigated whether the impaired transcription activation properties of the C-terminally truncated BMAL1 proteins originated from an inability to physically interact with CLOCK or from improper subcellular localization of the heterodimer. In a coimmunoprecipitation experiment, Flag-tagged mutant BMAL1 proteins pulled down cyan fluorescent protein (CFP)-tagged CLOCK equally as well as WT Flag-tagged BMAL1 (Fig. 3*A*), indicating that the C terminus is not involved in CLOCK/BMAL1 heterodimerization. Furthermore, transient COS7 cell expression studies revealed nuclear localization of Flag-BMAL1(E447X), Flag-BMAL1(L554X), and Flag-BMAL1(I584X) (Fig. 3*B Upper*), suggesting that the C terminus neither contains nuclear localization sequences nor facilitates nuclear localization of BMAL1 by means of cotransport with other proteins. Transiently expressed CLOCK displays a nucleocytoplasmic distribution and becomes completely nuclear after coexpression with BMAL1 (11). In line with their retained ability to bind CLOCK, the truncated BMAL1 proteins caused complete nuclear accumulation of CLOCK (Fig. 3*B Lower*). Taken together, these results indicate that nuclear accumulation of BMAL1, as well as its interaction with CLOCK, does not involve the BMAL1 C terminus and that, as a consequence, defective CLOCK binding and improper nuclear localization are not responsible for the dominant negative behavior of the truncated BMAL1 proteins in the assays reported above.

Other proteins known to interact *in vivo* with CLOCK and BMAL1 are mPER2 and mCRY1 proteins. These interactions, as well as mPER and mCRY association, are believed to be important for proper performance of the circadian feedback loops (22). Therefore, we investigated the physical interactions of mutant BMAL1 proteins with mPER2 and mCRY1. Coimmunoprecipitation experiments revealed that mPER2 protein physically associates with Flag-BMAL1(E447X) and Flag-BMAL1(L554X) and, accordingly, does not require the C-terminal region of BMAL1 (Fig. 3*C*). Instead, the PER2-binding site of BMAL1 must reside in the N-terminal half of BMAL1, including the PAS domain. A different situation is encountered for mCRY1, where anti-hemagglutinin (HA) antibodies hardly pull down WT Flag-BMAL1 or Flag-CLOCK from lysates containing either coexpressed HA-mCRY1 and Flag-BMAL1 or HA-mCRY1 and Flag-CLOCK. However, coexpression of HA-mCRY1 with WT Flag-BMAL1 and Flag-CLOCK resulted in distinct coprecipitation of the latter two proteins with HA-mCRY1 (Fig. 3*D*). Importantly, when WT Flag-BMAL1 was replaced by Flag-BMAL1(L554X), coimmunoprecipitation of (truncated) BMAL1 and CLOCK with HA-mCRY1 was dramatically reduced (Fig. 3*E*). Similarly, insertion mutants Bm29 and Bm30 hardly interacted with HA-mCRY1 in the presence of CLOCK protein (Fig. 8, which is published as supporting information on the PNAS web site). In line with a recently published study by Sato *et al.* (23), these results strongly indicate that the C-terminal region of BMAL1 is critical for the binding of mCRY1 to the CLOCK/BMAL1 complex.

These findings prompted us to speculate that the C-terminal region of BMAL1 is the interface for activation as well as for (mCRY-mediated) suppression of E-box gene transcription. To test the above concept, we generated two additional deletion BMAL1 mutants named Flag-BMAL1(E608X) and Flag-BMAL1(F619X), which lack 19 and 8 aa of the C terminus, respectively (Figs. 4*A* and 6*B*). Interestingly, in a transcription activation assay using 2xE-box-driven luciferase reporter, transcriptional activity was gradually recovered by these mutant proteins as compared with Flag-BMAL1(L554X) (Fig. 4*B*). Particularly, Flag-BMAL1(F619X)/CLOCK heterodimers exhibited transactivation activity up to

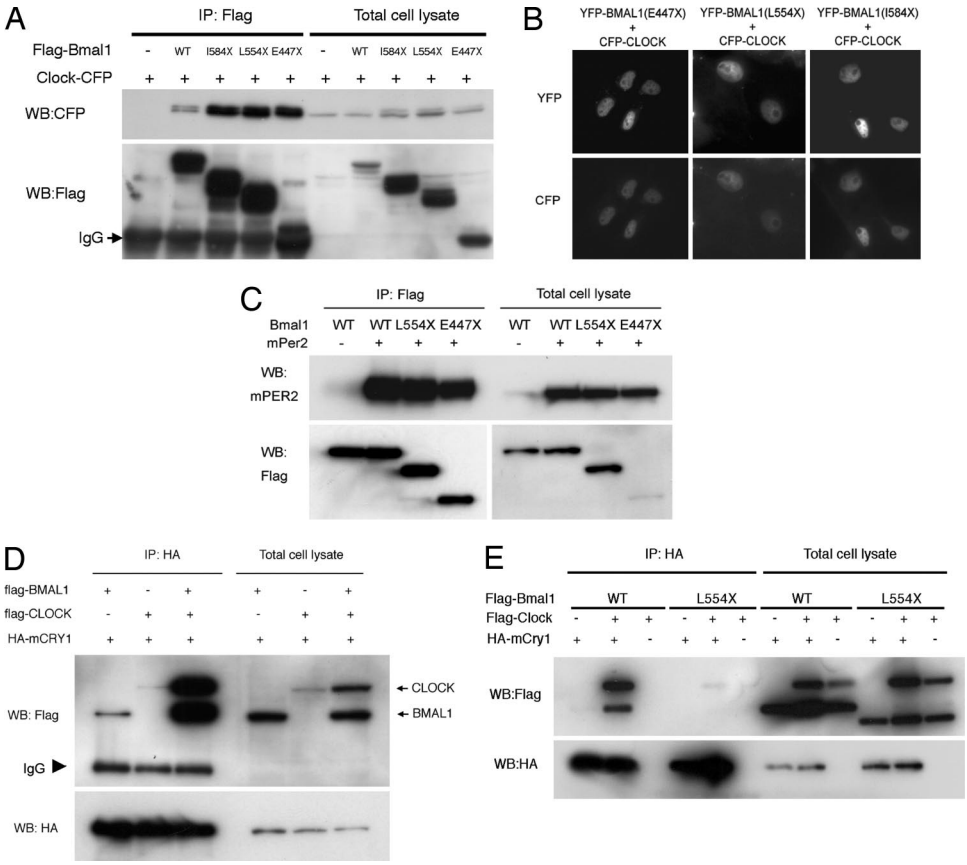

Fig. 3. Role of the C terminus of BMAL1 in the binding of other clock proteins. (*A*) Immunoprecipitation (IP) studies showing physical interaction of Flag-mCLOCK with WT mBMAL1 or mutant Flag-BMAL1(E447X), Flag-BMAL1(L554X), and Flag-BMAL1(I584X). Proteins were expressed in COS7 cells, precipitated from cell lysates with anti-Flag M2 monoclonal antibody conjugated to agarose beads, and analyzed on Western blots by using anti-CFP and anti-Flag antibodies. The Western blot of the total lysate served as a control. (*B*) Immunofluorescence images of COS7 cells expressing CFP-clock and YFP-BMAL1(E447X), YFP-BMAL1(L554X), or YFP-BMAL1(I584X). *Upper* shows YFP-BMAL1 mutant proteins, and *Lower* shows CFP-CLOCK. (*C*) Immunoprecipitation studies showing physical interaction of mPER2 with WT Flag-BMAL1 protein or Flag-BMAL1(E447X) and Flag-BMAL1(L554X). Procedures were as described in *A* except that anti-mPER2 antibodies were used to detect mPER2. (*D*) Immunoprecipitation study showing physical interaction of the Flag-BMAL1/Flag-CLOCK complex with HA-mCRY1. Procedures were as described in *A* except that anti-HA antibodies were used to precipitate and detect mCRY1. (*E*) Immunoprecipitation study showing impaired physical interaction between HA-mCRY1 and Flag-BMAL1(L554X)/Flag-CLOCK. Procedures were as described *D*.

≈70% of WT Flag-BMAL1/CLOCK activity, which was used as a control (Fig. 4*B*). Immunoprecipitation studies showed that interaction of both Flag-BMAL1(F619X)/CLOCK and Flag-BMAL1(E608X)/CLOCK complexes with HA-mCRY1 were still extremely weak (Fig. 4*C*). Thus, Flag-BMAL1(F619X) appeared to maintain transcription activation capacity, although it lost the capability to bind and thus be inhibited by mCRY1. To test the consequence of this mutation for circadian core oscillator performance, we overexpressed Flag-BMAL1(L554X), Flag-BMAL1(E608X), or Flag-BMAL1(F619X) with either the *mPer2:Luc* or *mBmal1:Luc* reporter in Rat-1 cells. Each of the truncated BMAL1 proteins severely impaired oscillations, as was evident from the extremely low amplitudes of bioluminescence rhythms (Fig. 4*D*). Importantly, as could be predicted for a dominant negative form of BMAL1 that lacks transcription activation potential, we noticed an obvious correlation between the level of (flattened) *mPer2:Luc* expression (Fig. 4*D Upper*) and transcription activation capacity of the mutant BMAL1 protein (Fig. 4*B*). In line with the level of E-box gene expression (including the *Bmal1* transcription repressor *Rev-Erbα* and visualized by the *mPer2:Luc* reporter), an inverse correlation is seen between *Bmal1:Luc* expression and transcription activation capacity of the mutant BMAL1 protein (Fig. 4*D Lower*). Most important, however, is our observation that transactivation of E-box genes by BMAL1 apparently is not sufficient to generate a robust circadian oscillation of the molecular clock as long as the protein cannot be inhibited by mCRY1.

Recently, Sato *et al.* (23) performed a large-scale screening study with panels of mutant BMAL1 and CLOCK proteins to identify amino acids that are critical for the biological function of these proteins. Amino acid substitutions at position 611 or 612 of the BMAL1 protein were shown to result in a reduced sensitivity of the protein to CRY1-mediated transcription repression. Our complementary data are in complete agreement with the idea that the C-terminal region of BMAL1 acts as a sensor domain for CRY-dependent transcriptional repression and thus has an essential role in cyclic regulation of transcriptional activity.

Concluding Remarks

We have shown here that the C-terminal 43 aa of BMAL1 contain a domain that is required for transcription activation as well as for inhibition of this activity by the mCRY1 protein. Deletion of this domain does not abolish mPER2 binding, which suggests that transcription suppression by mCRY and mPER proteins in the negative limb of the core oscillator uses different interfaces.

Whereas constitutive high expression of BMAL1 does not perturb the circadian core oscillator (as shown in this study and in ref. 12), deletion of the mCRY1-binding domain in a transcription activation-proficient BMAL1 protein abolishes circadian oscillations. Apparently, periodic inhibition of transactivation capacity of BMAL1 by mCRY1, rather than cyclic expression of BMAL1, is mandatory for preservation of circadian oscillations. The dual role of the BMAL1 C terminus suggests the presence of a "switch" mechanism that allows the BMAL1/CLOCK heterodimer to control the balance between activation of transcription by coactivator function, such as p300/CREB-binding protein ("on" mode), and repression by mCRY1 ("off" mode) (Fig. 4*E* and ref. 23). Because the C terminus contains (putative) casein kinase Iε (amino acids 517–526) and mitogen-activated protein kinase (amino acids 525–530, 531–535, and 596–603) phosphorylation sites (24), this periodic on/off status might be achieved through cyclic phosphorylation, controlling transcription activation potential (25) and mCRY1 binding. Although constitutive high overexpression of mCRY1 and mPER2 results in severe impairment of cellular circadian clock oscillations, constitutive low to moderate levels of both proteins in Rat-1 or NIH 3T3 cells still allows the clock to oscillate with normal

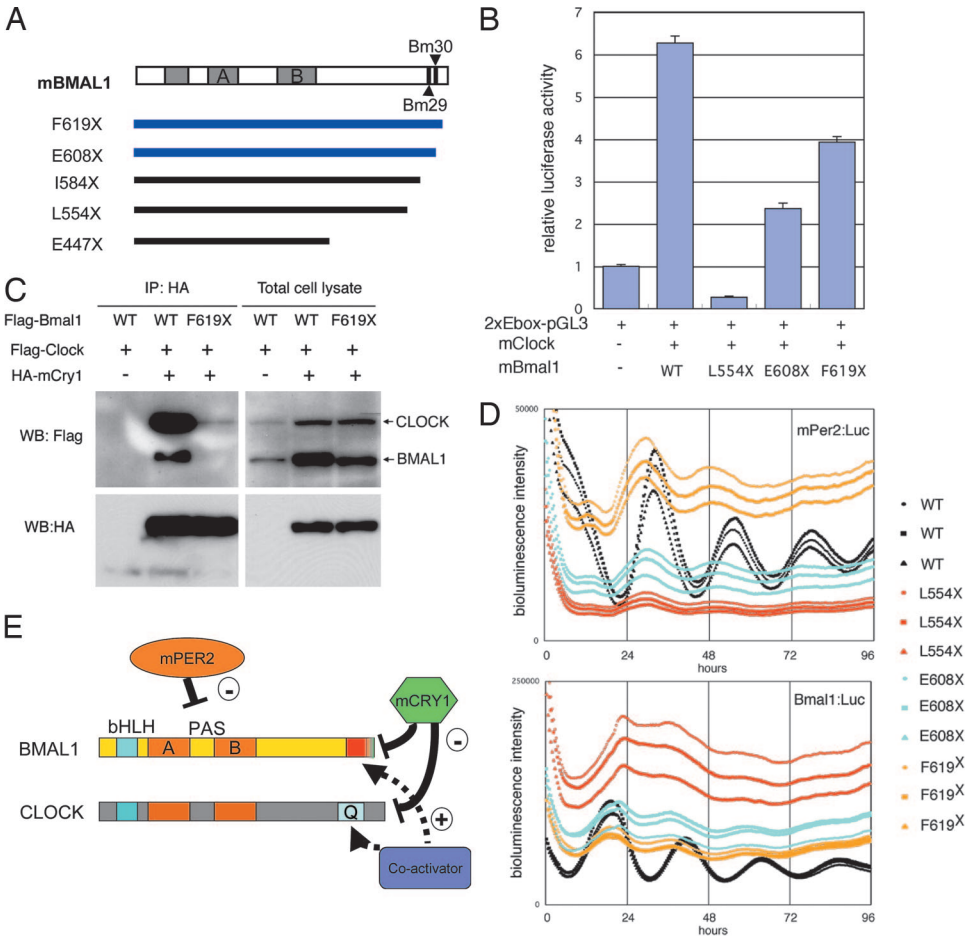

Fig. 4. Detailed analysis of the BMAL1 C terminus for circadian clock function. (*A*) Schematic representation of two additional C-terminally truncated BMAL1 mutant proteins (blue) generated by introduction of stop codons. (*B*) NIH 3T3 cell-based promoter assay for CLOCK/BMAL1 transcriptional activity using the 2xE-box-pGL3 reporter construct, *Flag-mClock*, and WT and mutant *Flag-Bmal1* constructs (as indicated). Relative luciferase activities are calculated as a severalfold induction from the basal expression level. Each bar indicates the mean of three independent samples, and error bars indicate the SEM. (*C*) Immunoprecipitation study showing lack of physical interaction between HA-mCRY1 and Flag-BMAL1(F619X)/Flag-CLOCK. Procedures were as described in the legend of Fig. 3*D*. (*D*) Effect of overexpression of WT Flag-BMAL1(WT) (black), Flag-BMAL1(L554X) (red), Flag-BMAL1(E608X) (blue), and Flag-BMAL1(F619X) (yellow) on *mPer2:Luc* (*Upper*) and *mBmal1:Luc* (*Lower*) reporter-driven bioluminescence rhythms in dexamethasone-synchronized Rat-1 cells. (*E*) Schematic model depicting protein–protein interactions for balanced regulation of transcription activation by coactivators and transcription suppression by the mPER2 and/or mCRY1 components of the negative limb of the mammalian circadian core oscillator.

period length (16, 17). Although research on the function and kinetics of cyclic posttranslational modifications of positive and negative factors is far from complete, it is tempting to speculate that, as with the cyanobacterial oscillator (26, 27), cyclic posttranslational modification, rather than cyclic gene expression, is the major requisite for mammalian core oscillator function and that transcriptional regulation of clock genes only serves to donate robustness to the system.

Materials and Methods

Plasmids and Cloning. Flag-tagged *mBmal1* and *mClock* pcDNA3.1 expression constructs were kindly provided by T. Todo (Kyoto University, Kyoto). For generation of Flag-BMAL1(E447X), (L554X), (I584X), (E608X), and (F619X) constructs, we mutagenized the *Flag-mBmal1* cDNA by using the QuikChange Mutagenesis Kit (Stratagene) and the following primer pairs: E447X forward, 5′-GTT TTA GCC AAT GTC CTG *T*AA GGC GGG GAC CC-3′; E447X reverse, 5′-GG GTC CCC GCC TTA CAG GAC ATT GGC TAA AAC-3′; L554X forward, 5′-CT TCC ACT GGA CTA T*A*A CCA GGG CAG GCT C-3′; L554X reverse, 5′-G AGC CTG CCC TGG T*T*A TAG TCC AGT GGA AG-3′; L584X forward, 5′-GGC ATC GAT ATG *TA*A GAT AAC GAC CAA GG-3′; I584X reverse, 5′-CCT TGG TCG T*TA* TCT TAC ATA TCG ATG CC-3′; E608X forward, 5′-ATC ATG AGC CTC TTG *T*AA GCA GAT GCG GGG-3′; E608X reverse, 5′-CCC CGC ATC TGC TT*A* CAA GAG GCT CAT GAT-3′; F619X forward, 5′-GGC CCC GTT GAC T*AA* AGT GAC TTG CCA TGG-3′; F619X reverse, 5′-CCA TGG CAA GTC ACT *TT*A GTC AAC GGG GCC-3′ (italicized bases indicate substituted nucleotides). Mutated Flag-mBmal1 constructs were sequenced to confirm the presence of the specific mutations and that no other unexpected mutations were introduced.

The *mPer2* promoter fragment (423 bp) was obtained from C57BL/6 mouse genomic DNA by PCR using forward primer 5′-AA GGT ACC TCT GCC GGC TGT GAG TTG CGC AG-3′ and reverse primer 5′-TT CTC GAG ACC GCT AGT CCC AGT AGC GCC G-3′, containing the KpnI and XhoI sites, respectively. Similarly, a *mBmal1* promoter fragment (530 bp) was amplified by using primers reported in ref. 6. These promoter regions were then cloned into pGL4.11 vector (Promega).

To generate CFP-*mClock* and yellow fluorescent protein (YFP)-*mBmal1* expression vectors, the ORFs of *mClock* and *mBmal1* were digested with BamHI/XhoI from the respective Flag-tagged constructs and ligated into BglII/SalI-digested pECFP-C1 and pEYFP-C1 (BD Clontech).

Cell Culture. Rat-1 fibroblast (Health Science Research Resources Bank, Osaka), NIH 3T3, and COS7 (kindly donated by T. Ishitani, Nagoya University) cells were cultured in DMEM with 10% FBS and penicillin-streptomycin.

Transposon-Based *Bmal1* Mutant Library. Random insertions of 19 aa were introduced in the BMAL1 protein by using the EZ-Tn5 In-Frame Linker Insertion Kit (Epicentre Technologies, Madison, WI). Briefly, a kanamycin resistance gene containing transposon was incubated with EZ-Tn5 transposase and Flag-mBal1 pcDNA3.1. Transposon-containing *Flag-mBmal1* colonies were selected on kanamycin-containing LB plates and harvested in LB medium. After plasmid extraction using the QIAfilter Plasmid Maxi kit (Qiagen, Valencia, CA), DNA was digested with NotI restriction

enzyme to remove the kanamycin resistance marker, self-ligated, and transformed in *Escherichia coli*. The mutant *Flag-mBmal1* constructs were sequenced by the following primers: BML1 forward, 5′-GAC CAG AGA ATG GAC ATT T-3′; BML1 reverse, 5′-CCT TGC ATT CTT GAT CCT TC-3′; BML2 forward, 5′-GTA CCA ACA TGC AAT GCA ATG-3′; BML3 forward, 5′-CTG CAT CCA AAA GAT ATT GCC-3′; BML4 forward, 5′-CTG GAC GAA GAC AAT GAG CC-3′; BML5 forward, 5′-C GCA GAA TGT CAC AGG CAA G-3′; BML6 forward, 5′-GGA GCA GGA AAA ATA GGT CG-3′; BML7 forward, 5′-CC ACT GGA CTA TTA CCA GGG-3′.

Real-Time Circadian Rhythm Monitoring. The mechanics of the bioluminescence detection system used to analyze the circadian rhythm are described in ref. 28. Rat-1 cells were cultured in 10% FBS and penicillin-streptomycin-containing medium. Cells were plated in 35-mm dishes (2×10^5 cells per dish) or 24-well plates (5×10^4 cells per well). Cells were cotransfected with the *Bmal1* or *mPer2* luciferase reporter construct (250 or 200 ng for 35-mm dishes and 24-well plates, respectively), Flag-mBmal1 mutant construct (750 or 200 ng for 35-mm dishes and 24-well plates, respectively), and FuGENE 6 transcription reagent (3 or 0.8 μl for 35-mm dishes and 24-well plates, respectively, Roche, Indianapolis, IN). After overnight culture, medium was changed to luciferine (100 nM) and Hepes (15 mM) containing DMEM without phenol-red. After additional overnight culture, cells were synchronized by treatment with 100 nM dexamethasone and set on the turntable of a home-made real-time monitoring machine (28).

Western Blot Analysis and Immunoprecipitation. Cells were washed three times with ice-cold PBS and harvested in 50 μl of SDS sample buffer (125 mM Tris·HCl, pH 6.8/2% SDS/10% glycerol/0.05% bromophenol blue/1 μM PMSF/50 μM NaF/100 μM NaVO3/40 μM DTT). As primary antibodies, we used rabbit anti-GFP polyclonal antibody (1:2,000, MBL, Nagoya, Japan), rabbit anti-mPER2 polyclonal antibody (1:1,000, Alpha Diagnostic International, San Antonio, TX), and anti-FLAG M2 mouse monoclonal antibody (1:500, Sigma). As secondary antibody, we used horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (1:2,000, Jackson ImmunoResearch). Chemiluminescence detection was performed by using ECL Western Blotting Luminol Reagent (Amersham Pharmacia).

Immunoprecipitation was performed on whole-cell lysates obtained from cells harvested in 0.2 ml of lysis buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/1% Nonidet P-40/10% glycerol/1 mM EDTA/1 mM PMSF/1 mM DTT/50 μM NaF/100 μM NaVO3/complete mini protease inhibitor). The total lysate was centrifuged at top speed in a 15,000 $\times$ *g* centrifuge for 10 min at 4°C. The supernatant was transferred to a fresh microtube, after which anti-HA (Roche) or anti-FLAG M2 (Sigma) antibodies were added and incubated for 2 h at 4°C under continuous rotation. After addition of 30 μl of protein G-agarose in lysis buffer (Roche), samples were rotated for another hour. Beads were collected, washed twice with lysis buffer, incubated with 8 μl of 3× SDS sample buffer, and boiled for 5 min before electrophoresis.

Transcription Assay. Luciferase reporter gene assays were performed in NIH 3T3 cells that were seeded on a six-well plate at a density of 2×10^5 cells per well and transfected the following day. Luciferase expression was detected by using the Dual Luciferase reporter assay system (Promega). Each transfection contained 50 ng of 2xE-box-pGL3 promoter vector, 5 ng of pRL-tk, and the indicated amount of effecter constructs per well. The total amount of DNA per well was adjusted to 1 μg by adding pcDNA3 vector as a carrier. Forty-eight hours after transfection, cells were harvested to determine luciferase activity with a luminometer.

Subcellular Localization. The WT, E447X, L554X, and I584X mBmal1 pEYFP-C1 constructs were transfected in COS7 cells (cultured on the coverslips) either alone or together with mClock pECFP-C1 construct. Cells were fixed with 4% paraformaldehyde/0.1 M phosphate buffer for 10 min at room temperature. After fixation, cells were washed three times with PBS and mounted with PermaFluor Mountant Medium (Thermo Electron, San Jose, CA). Cells were analyzed with an Axiovert 200M fluorescent microscope (Zeiss).

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