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Et al.

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Circadian rhythmicity in mammals is under the control of a molecular pacemaker constituted of clock gene products organized in transcriptional autoregulatory loops. Phase resetting of the clock in response to light involves dynamic changes in the expression of several clock genes. The molecular pathways used by light to influence pacemaker-driven oscillation of clock genes remain poorly understood. We explored the functional integration of both light- and clock-responsive transcriptional regulation at the promoter level of the *Period* (*Per*) genes. Three *Per* genes exist in the mouse. Whereas *mPer1* and *mPer2* are light-inducible in clock neurons of the hypothalamic suprachiasmatic nucleus, *mPer3* is not. We have studied the promoter structure of the three *mPer* genes and compared their regulation. All three *mPer* promoters contain E-boxes and respond to the CLOCK/brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like protein 1 (BMAL1) heterodimer. On the other hand, only *mPer1* and *mPer2* promoters contain bona fide cAMP-responsive elements (CREs) that bind CRE-binding protein (CREB) from suprachiasmatic nucleus protein extracts. The *mPer1* promoter is responsive to synergistic activation of the cAMP and mitogen-activated protein kinase pathways, a physiological response that requires integrity of the CRE. In contrast, activation of *mPer* promoters by CLOCK/BMAL1 occurs regardless of an intact CRE. Altogether, these results constitute strong evidence that CREB acts as a pivotal endpoint of signaling pathways for the regulation of *mPer* genes. Our results reveal that signaling-dependent activation of *mPer* genes is distinct from the CLOCK/BMAL1-driven transcription required within the clock feedback loop.

Circadian rhythmicity is a conserved physiological feature of almost all organisms (1–3). Light is the most prominent stimulus that has contributed in shaping circadian physiology during evolution (4, 5). Through several photoreception systems, light is capable of synchronizing circadian oscillations to the environment (4, 6). In mammals the core pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus, whose neurons receive photic input signals from the retina by way of the retinohypothalamic tract (7).

Although several nonphotic stimuli have also been shown to reset the mammalian circadian system (8–13), light is the major entraining signal and it delays the pacemaker if administered at early night and advances it at late night (6). The effect is intimately connected to the clock mechanism because light has no effect when applied during the subjective day. The process of synchronization involves the transcriptional activation of several genes. In mice, brief exposure to light during the subjective night causes rapid induction of immediate-early genes, such as c-*fos* (14), and of clock genes, such as the homologs of the *Drosophila period* gene (15–17). Three *period* genes exist in the mouse, and although *mPer1* is induced by a light pulse within 15–30 min and *mPer2* within 2 h (15–17), the *mPer3* gene is not light-responsive (18, 19). Arousal (11) and serotonin receptor activation (20) induce acute down-regulation of *mPer1* and *mPer2* expression in the SCN, identifying them as common targets for both photic and nonphotic cues.

Whereas *mPer1* and *mPer2* seem to play a crucial role in the molecular organization of the pacemaker (21–25), *mPer3* seem to operate on clock output pathways (26). *Per* genes are known to be positively regulated by other clock proteins belonging to the basic helix-loop-helix–period/aryl hydrocarbon receptor nuclear translocator/single-minded (PAS) class. These are CLOCK and brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like protein 1 (BMAL1) which, associated as heterodimers, bind to E-box enhancer elements (27–29). In addition, mPER proteins constitute multimeric complexes with the products of the *Cryptochrome* genes, *mCRY1* and *mCRY2*, which in turn inhibit transcription mediated by CLOCK/BMAL1 (30, 31). The *mPer* genes exhibit circadian cycling expression in the SCN (15, 19) and in several peripheral tissues, e.g., liver and skeletal muscle (19, 32), and in cultured cell lines stimulated with a number of stimuli (33–37).

Several lines of evidence indicate that the *mPer1* gene plays a central role in conveying the light-entraining information to the central clock. *mPer1* is the only clock gene that has been convincingly shown to be induced very rapidly after light stimulation (15–17, 38). In addition, light-induced resetting of locomotor activity and glutamate-induced resetting of firing rhythms can be blocked by *mPer1* antisense oligonucleotides (39). Finally, some reports on *mPer1*-deficient mice confirm this view (40).

Although resetting of the circadian system seems to involve changes in gene expression, little is known about the signal transduction pathways that initiate this transcriptional response. Signaling pathways for light-dependent clock resetting include glutamate release from retinohypothalamic tract terminations within the SCN, *N*-methyl-D-aspartate receptor activation, and Ca^{2+} influx (41, 42). Calcium influx may be linked to cAMP-responsive element (CRE)-mediated transcription activated by the extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase (MAPK) pathway (43). Indeed, a light pulse during the night activates the MAPK signaling cascade (44) and consequently induces CRE-binding protein (CREB) phosphorylation (45) in SCN neurons.

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Abbreviations: SCN, suprachiasmatic nucleus; ARNT, aryl hydrocarbon receptor nuclear translocator; BMAL1, brain and muscle ARNT-like protein 1; ZT, Zeitgeber time; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; CRE, cAMP-responsive element; CREB, CRE-binding protein; EGF, epidermal growth factor; TPA, phorbol 12-tetradecanoate 13-acetate.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF491941 and AF491942).

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We have studied the regulation of *mPer* promoters by signaling stimuli. Our analysis has identified significant differences and similarities among the three promoters. We demonstrate that CREB acts as a major effector of converging signaling pathways to the *mPer1* promoter and that this regulation is independent of CLOCK/BMAL1 action.

Materials and Methods

Plasmids. The *mPer1*, *mPer2*, and *mPer3* promoter regions were isolated and cloned in pGL3-Basic Vector (Promega). The *mPer1* region spans from −1803 to +40 (+1 is the putative transcription start site), and the sequence is identical with that in GenBank accession no. AB030818 (46). The mPer2 and mPer3 regions are from −1670 to +53 and from −1594 to +128, respectively, and were deposited in GenBank (accession nos. AF491941 and AF491942). Mutation in the CREs within *mPer1* and *mPer2* promoters was generated by deletion of the central 4 nt (TGACGTCA → TGCA). Oligonucleotides corresponding to CRE-*mPer1* (5′-tccgcttTGACGTCAcctccct-3′), CRE-*mPer2* (5′-ccaccatTGACGTCAatgtaag-3′), or their mutated forms (5′-tccgctcTCACAAAAcctccct-3′ and 5′-ccaccgcTGACAAAatgtaag-3′) were inserted in the pGL3-Promoter Vector (Promega). Mouse *Clock* ORF was amplified by PCR from a cDNA kindly provided by J. Takahashi (Northwestern University, Chicago), cloned in the pSG5 vector (Stratagene) with a FLAG epitope sequences at the 5′ end. Mouse *Bmal1* was amplified by reverse transcription–PCR from mouse brain RNA. This cDNA was cloned in pCS2+MTK, a derivative of pCS2 with five Myc-tag sequences at the 5′ end of the cDNA.

Cell Culture and Transient Transfections. Human choriocarcinoma JEG3 cells were cultured in monolayers with Dulbecco's modified Eagle's medium (GIBCO/BRL) supplemented with 10% FCS. Cells were transfected by the calcium phosphate coprecipitation technique with 1 μg of reporter construct. Medium was replaced with 0.5% FCS medium 12–16 h before cells were treated with various agents: forskolin (10 μM), epidermal growth factor (EGF; 50 ng/ml), 4-bromo-calcium ionophore A23187 (1 μM), phorbol 12-tetradecanoate 13-acetate (TPA) (100 ng/ml), and/or 20% FCS for 6 h before harvest. In specific experiments inhibitors PD 98059 (30 μM; Calbiochem) and SB 203580 (10 μM; Calbiochem) were added to cells 1 h before treatments. Luciferase activity was measured as described (47). All experiments were performed in triplicate.

Nuclear Extract Preparation and DNA-Binding Assays. Adult male Wistar rats were kept on a 12-h light:12-h dark cycle for 3 weeks before dissection. SCNs and underlying optic chiasms were isolated under the dissecting microscope. Blocks $\approx 1 \times 1 \times 1$ mm were rapidly frozen in liquid nitrogen and stored at −80°C. SCN (48) and Rat-1 fibroblasts (49) nuclear extracts were prepared as described. Western analyses and immunostaining were as described (50) with anti-phospho-CREB or anti-CREB antibodies (New England Biolabs). For the gel retardation assays, CRE oligonucleotide probes or their mutated forms (same sequences as above) were used as described (51). In competition or in antibody-supershifting assays, unlabeled oligonucleotides or anti-CREB, anti-ATF1, anti-ATF2 antibodies (Santa Cruz Biotechnology) were added to the extracts 30 min before the labeled oligonucleotides. Recombinant CREB was produced as described (52).

Results

Similarities and Differences Between the *mPer* Promoters. We have analyzed the sequence of the 5′ flanking regulatory regions of the three mouse *Per* genes. Three CACGTG E-boxes and one canonical CRE (TGACGTCA) are within 1,803 bp of the *mPer1* gene promoter, centered at 1,728 bp upstream of the transcription start site (Fig. 1). Within the 1,670-bp promoter region of *mPer2* and 1,594 bp of *mPer3* genes, seven and four E-boxes (CANNTG) were found, respectively (Fig. 1). None of these E-boxes corresponds to the CACGTGA sequence, the strict consensus binding site for CLOCK/BMAL1 heterodimers (28). A canonical CRE is also present within *mPer2* promoter, but not in the *mPer3* promoter (Fig. 1). The CRE has a common location within both *mPer1* and *mPer2* promoters, being consistently upstream from the E-boxes. This reciprocal location of CREs and E-boxes is characteristic also in promoters of other genes, such as renin and transforming growth factor β2 (53, 54).

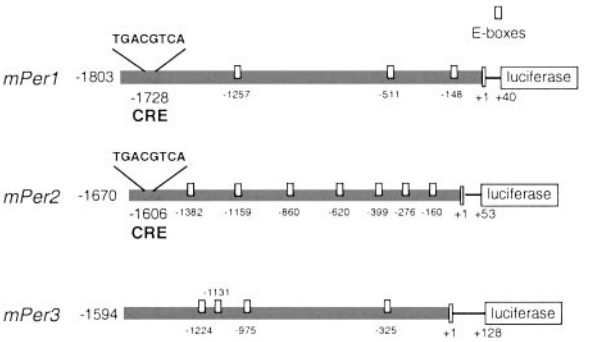

Fig. 1. Schematic representation of *mPer1*, *mPer2*, and *mPer3* promoters. The upstream sequence of *mPer1*, *mPer2*, and *mPer3* genes was fused to a luciferase reporter. On the left is indicated the size of the genomic fragment upstream of the transcription start site (+1) that is included in the construction. The numbers below the box representing the promoter sequence are the positions of the CRE (TGACGTCA) and E-boxes (small white boxes).

***mPer1* and *mPer2* CREs Bind CREB in SCN Nuclear Extract.** The CREs present in the promoters of the *mPer1* and *mPer2* genes efficiently bind recombinant CREB protein, whereas their mutated forms do not (Fig. 2*A*). A nuclear extract from Rat-1 fibroblasts was also used to assess binding to the *mPer1* CRE (Fig. 2*B*). Specificity of binding was confirmed by competition with unlabeled CRE oligonucleotides. The canonical somatostatin CRE (55) and the *mPer1* and *mPer2* CREs successfully competed for binding, whereas a mutated *mPer1* CRE was not an effective competitor. Analogous results were obtained with the *mPer2* CRE (not shown). We also performed binding assays using nuclear extracts from rat SCN collected at Zeitgeber time (ZT) 5 or ZT17, or 1 h after a 30-min light pulse at ZT17 (Fig. 2*C*). SCN extracts display a robust CRE-binding activity. Complex formation for both *mPer1* and *mPer2* CREs is constant independent of circadian phase or photic stimulation. Binding specificity was assessed by competition with the somatostatin CRE. To identify the nature of the natural *mPer* CRE-binding activity supershift assays were performed with the SCN extracts. Whereas preincubation with anti-CREB antibodies drastically decreased complex formation on both *mPer1* or *mPer2* CREs, neither anti-ATF1 nor anti-ATF2 antibodies had any effect (Fig. 2*D*). These results show that CREB naturally binds the *mPer* promoter sequences in the SCN.

A light stimulus that phase-shifts circadian rhythms has been shown to induce phosphorylation of CREB at Ser-133, a critical event in transcriptional activation (45, 56). To link this notion to the activation of *mPer* genes, we analyzed CREB phosphorylation in our SCN nuclear extracts. We used antibodies raised against CREB phosphorylated at Ser-133 (45). CREB is phosphorylated at ZT5, but not at ZT17, and is induced by a light pulse at ZT17 (Fig. 2*E*). These results implicate CREB as a signaling-responsive switch for *mPer1* transcriptional response in the SCN.

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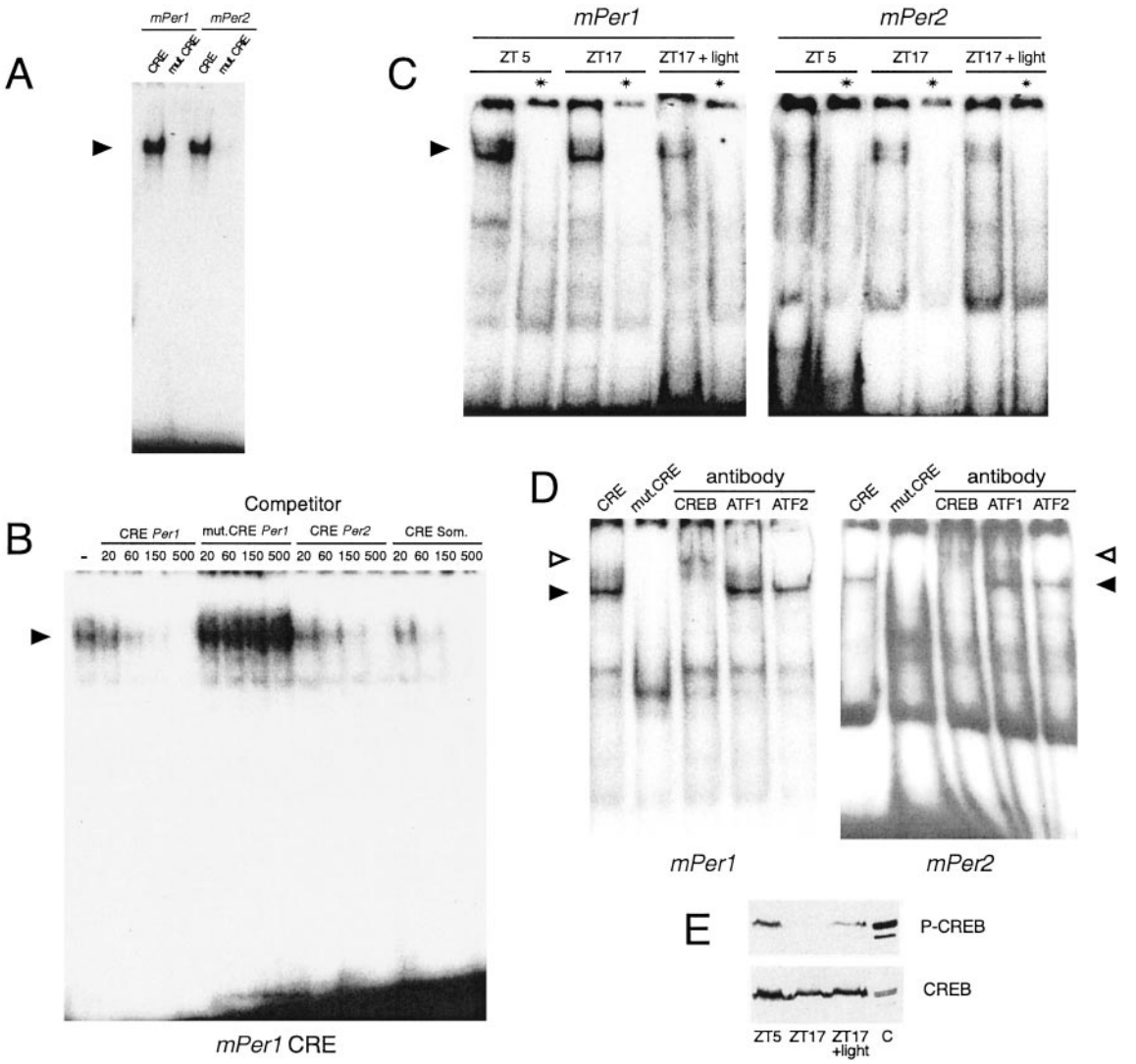

Fig. 2. Specific binding of CREB to wild-type CRE of *mPer1* and *mPer2* promoters. (*A*) Gel mobility-shift assay using wild-type or mutated *mPer1* or *mPer2* CRE oligonucleotides, plus bacterially expressed CREB protein. The specific complex is indicated by an arrowhead. (*B*) Competition gel mobility-shift assay using labeled *mPer1* CRE oligonucleotide together with nuclear extract from Rat-1 fibroblasts. Competition is made by preincubating the labeled DNA with increasing amounts (20–500 ng) of unlabeled wild-type *mPer1, mPer2*, or somatostatin CRE or mutated *mPer1* oligonucleotides. The specific complex is indicated by an arrowhead. (*C*) Gel mobility-shift assay using *mPer1* or *mPer2* CRE oligonucleotides along with nuclear extracts from the SCN of rats killed during the day (ZT5), and during the night (ZT17) either in the darkness or 1 h after the beginning of a 30-min light pulse. (*D*) Supershift assay on SCN nuclear extracts with *mPer1* or *mPer2* CRE after preincubation of SCN nuclear extract with anti-CREB, anti-ATF1, and anti-ATF2 antibodies. The specific complex is indicated by a closed arrowhead and the supershifted by an open arrowhead. The first lane is the control without antibody and the second lane is a complex with mutated oligonucleotide. (*E*) Immunoblotting of SCN protein extracts from rats killed as in *C*, with an anti-phosphorylated CREB (P-CREB) antibody. ''C'' is a control containing P-CREB (EGF-stimulated fibroblasts).

Distinct Effects of CLOCK/BMAL1 and Signaling Pathways on *mPer* Promoter Activation. Cotransfection of JEG-3 cells with *mPer1, mPer2*, and *mPer3* promoter reporters along with CLOCK and BMAL1 expression vectors resulted in transcriptional stimulation of all three *mPer* promoters (Fig. 3*A*), as reported for *mPer1* (27). CLOCK/BMAL1-dependent activation of *mPer1* is significantly higher than *mPer2* and *mPer3*.

To explore the ability of *mPer* promoters to respond to stimulation of different signaling pathways, we treated transfected cells with adenylate cyclase activator forskolin, EGF, 4-bromo-calcium ionophore A23187, TPA, and 20% FCS. Only forskolin caused significant elevation of *mPer1* promoter activity (Fig. 3*B*). However, remarkable up-regulation was observed when cells were concurrently treated with forskolin and EGF or TPA. These results indicate that maximal induction of *mPer1* promoter activity requires a cooperative activation of both cAMP and MAPK pathways. No response was observed for either *mPer2* or *mPer3* after treatments (Fig. 3*B*), which is noteworthy considering the similarity in the organization of *mPer1* and *mPer2* promoters (Fig. 1) and the capacity of the *mPer2* CRE to bind CREB (Fig. 2).

Essential Role of the *mPer1* CRE in the Response to Signaling Inducers. To assess the role played by the CRE in the remarkable responsiveness of the *mPer1* promoter, we generated a deletion of the four internal nucleotides of the CRE within the context of the whole *mPer1* promoter (Fig. 4*A*). This mutation of the CRE abolishes CREB binding (not shown) and fully blocks responsiveness of the *mPer1* promoter (Fig. 4*A*). This result underscores the importance of the CRE in signaling

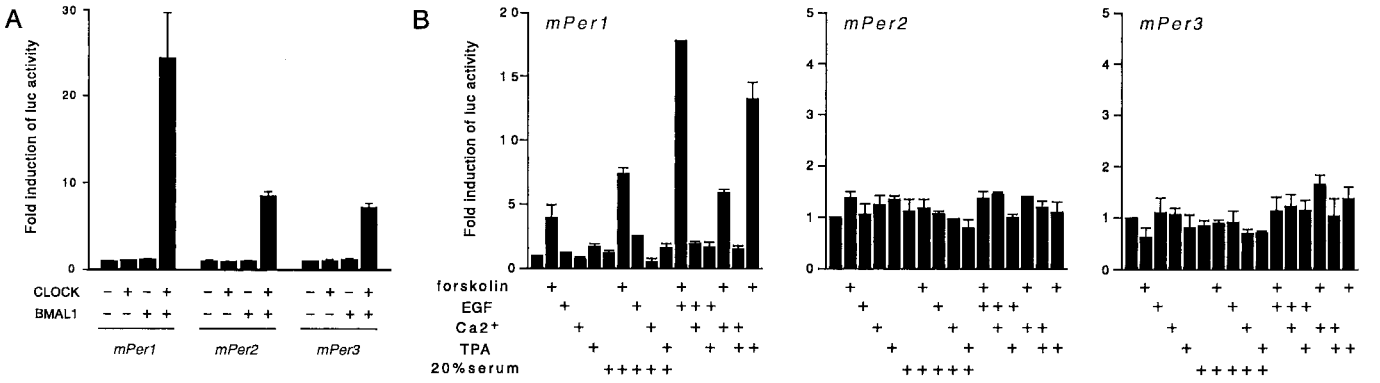

Fig. 3. Activation of *mPer1*, *mPer2*, and *mPer3* promoters by CLOCK/BMAL1 and by the induction of different signaling pathways. (*A*) JEG-3 cells were transfected with a construct of the *mPer* promoter regions linked to the luciferase reporter gene and either CLOCK or BMAL1 alone or together. Cells were washed after 12 h and processed for luciferase assay 24 h later. (*B*) For stimulation, cells transfected with either of the *mPer* promoter constructs were treated with forskolin (10 μM), EGF (50 ng/ml), 4-bromo-calcium ionophore A23187 (1 μM), TPA (100 ng/ml), 20% fetal calf serum, or combinations. Six hours later, cells were harvested for luciferase assay. Data are expressed as fold increase over the value for unstimulated cells. Each bar represents the mean and SEM of three independent samples. The figures are representative from several independent experiments with similar results.

response and demonstrates that the rest of the *mPer1* promoter, including the E-boxes, is not sufficient to elicit inducibility.

The functional importance of the CRE was further verified by using the isolated *mPer1* and *mPer2* CRE sequences inserted in heterologous promoter reporters. The *mPer1* CRE confers remarkable responsiveness on stimulation with forskolin, EGF, TPA, or serum, and synergistic activation after combined treatments, analogous to what was observed with the full *mPer1* promoter. All responses were abolished when the CRE was mutated (Fig. 4*B*). Thus, the CRE alone is capable of conveying MAPK- and cAMP-inducible activation and could thereby constitute a link between light-stimulated MAPK pathway and light induction of the *mPer1* gene. When isolated, the CRE of *mPer2* promoter can also serve as a target site of activated signaling pathways, especially cAMP (Fig. 3*B*). This observation suggests that additional sequences within the *mPer2* promoter may modulate the responsiveness of the CRE.

MAPK Cascade Inhibitors Block *mPer1* EGF and TPA Inducibility. Phosphorylation of CREB in the SCN in response to a light stimulus has been proposed to depend on the MAPK pathway (43). To examine the specificity of *mPer1* stimulation by EGF and TPA, we treated cells transfected with the full-length *mPer1* promoter with the specific MEK inhibitor PD 98059 1 h before stimulation (Fig. 5). Inhibition of MEKs blocks the synergistic activation of the *mPer1* promoter by concurrent application of EGF or TPA and forskolin. However, forskolin-dependent activation is not affected, stressing the independence from MAPKs of the cAMP-inducible activation. These results suggest the convergence of multiple signaling routes whose effects are integrated at the level of the *mPer1* promoter. In support of this interpretation, SB 203580, a specific inhibitor of the p38 MAPK cascade, also significantly inhibits EGF- and TPA-mediated activation of *mPer1*. As for the inhibitor PD 98059, cAMP inducibility of *mPer1* is not affected by SB 203580.

Activation by CLOCK/BMAL1 Is Independent of the CREs. We wished to establish whether signaling-dependent induction through the CRE would influence the clock-controlled CLOCK/BMAL1 activation. We used the full-length *mPer1* and *mPer2* promoters containing either an intact or a mutated CRE in cotransfection experiments with CLOCK and BMAL1 expression vectors. The results clearly show that CLOCK/BMAL1 activation does not require the CRE (Fig. 6). This finding indicates that specific, independent sequences within the *mPer1* promoter integrate signaling stimuli and clock-dependent regulation.

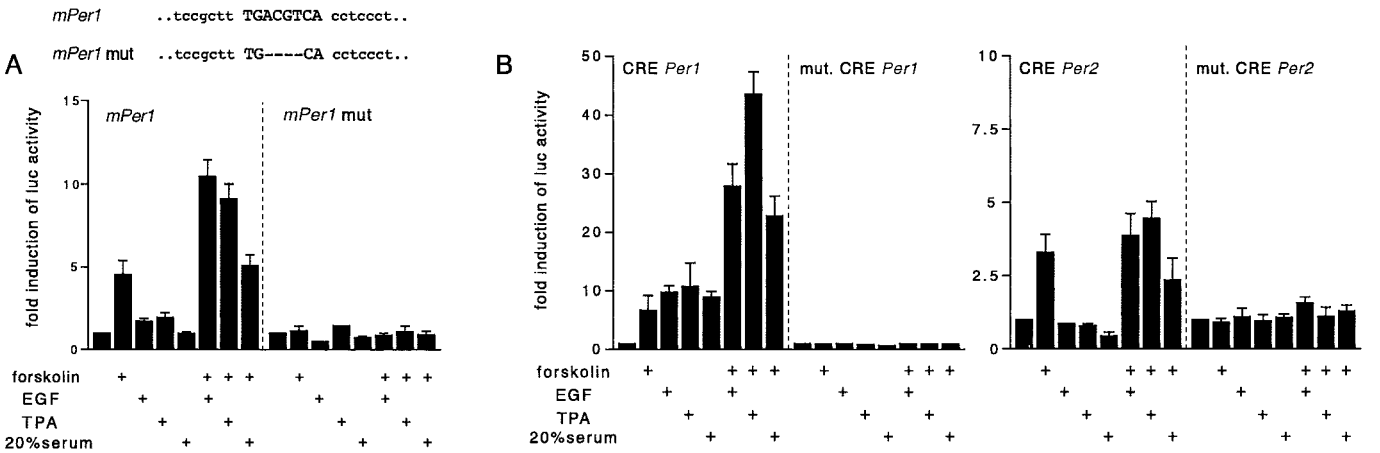

Fig. 4. Mutation within CRE abolishes the response of the promoter to cell-signaling stimulation. JEG-3 cells were transfected with a luciferase construct with either wild-type or CRE-mutated *mPer1* promoter (*A*), or with isolated wild-type or mutated CRE motifs of *mPer1* and *mPer2* promoters (*B*). Stimulation and luciferase assays were done as in Fig. 3.

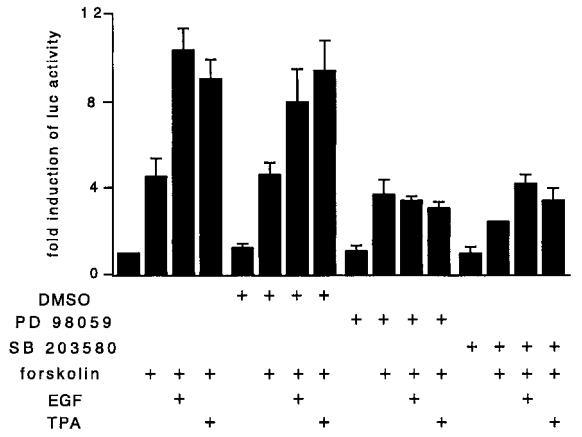

Fig. 5. Inhibitors of MAPK cascades abolish synergistic activation of the *mPer1* promoter by forskolin and EGF or TPA. Transfection, treatments, and luciferase analysis were conducted as in Fig. 3*B*, except that PD 98059 (30 μM) or SB 203580 (10 μM) or the vehicle was applied to the cells 1 h before the inducing treatments.

Discussion

The pathways that light uses to impinge on the clock molecular mechanism are still poorly understood. We reasoned that studying how the expression of clock genes is regulated by intracellular signaling systems would provide useful information in this respect. The *mPer* genes have been shown to respond differentially to light in the SCN (15, 16, 18, 19, 38). *mPer1* is induced quickly, with kinetics of an immediate-early gene; *mPer2* responds more slowly, reaching peak values 2–3 h after the light stimulus; *mPer3* is not light-responsive. Our results on the inducibility of the *mPer* promoters by various signaling agents establish an interesting parallel with light responsiveness. Whereas *mPer1* and *mPer2* contain a CRE, *mPer3* does not (Fig. 1). The absence of a CRE in the *mPer3* promoter is likely to be linked to its uninducibility by activation of the cAMP and MAPK pathways. Although the *mPer2* regulatory region contains a canonical and functional CRE, the promoter is poorly responsive, in sharp contrast to *mPer1* (Fig. 3). It seems that the *mPer2* CRE is functional, but that in the context of the whole promoter it becomes inactivated. This different activation potential of *mPer1* and *mPer2* CREs could account for the diverse induction kinetics of the two genes in the SCN of light-stimulated animals.

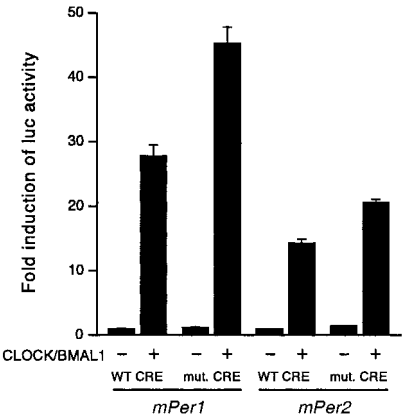

Fig. 6. Activation of *mPer1* or *mPer2* promoters by CLOCK/BMAL1 is unaffected by a CRE mutation. Cells were transfected with the wild-type or CRE-mutated *mPer1* or *mPer2* promoter constructs, and with CLOCK and BMAL1 expression plasmid or the empty vectors, and processed as in Fig. 3*A*.

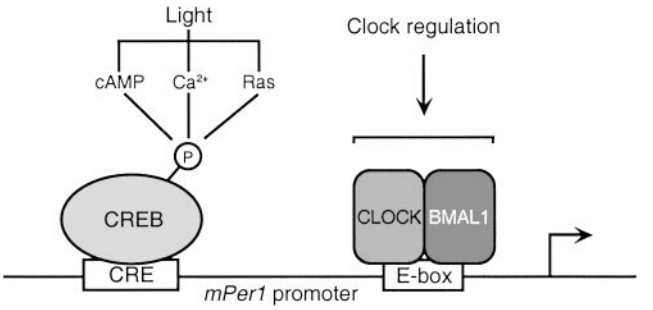

Fig. 7. Schematic representation of the *mPer1* promoter and its regulation. While the E- boxes (only one is shown here) are targets of the clock-controlled CLOCK/BMAL1 regulation, the CRE is essential for response to various signaling pathways.

One possibility is that newly synthesized factors are needed for the induction of *mPer2* in the SCN, which would explain its delayed kinetics of induction. These *de novo* synthesized proteins may be lacking in our cell-based system. Another aspect to take into account is the likely interactions that CREB may have with additional factors regulating the *mPer* promoters, including E-box-binding proteins, because these interactions may modulate responsiveness of the promoter. In this respect the combined presence of CREs and E-boxes in several promoters is noteworthy (53, 54). The CRE is often placed upstream from the E-boxes, suggesting a code of possible protein–protein interactions. Yet direct interactions of CREB with E-box-binding proteins have not been described. We have shown that CLOCK/BMAL1-dependent activation is independent of the CRE (Fig. 7).

The involvement of CREB in light-induced clock resetting is based on the observation that CREB becomes phosphorylated *in vivo* in response to photic stimuli (45), and *in vitro* after glutamate treatment (57). Furthermore, light exposure at night, when it causes phase shifts of overt rhythms, elicited a robust increase in CRE-mediated transcription in the retinorecipient part of the SCN (43). Here we have established a direct link between intracellular signaling, CRE-regulated transcription, and *mPer* gene expression. It is important to explore the role played by the CREB coactivator CBP (CREB-binding protein) in SCN neurons, because it has been shown that CBP itself is a target of signaling regulation (56).

CREB activation by phosphorylation at Ser-133 occurs in response to several signaling stimuli, including the ERK-MAPK pathway (58, 59). In the SCN, stimulation of these kinases occurs after a light pulse that would shift the clock (44) and contributes to activation of CREB by glutamate (43). A variety of treatments trigger expression of endogenous *Per1* and other clock and clock-controlled genes in mammalian cell lines (33–37, 60). Our results identify the cAMP and MAPK pathways, and their synergistic combination, as essential for CRE-mediated induction of *mPer1*. Future studies will need to focus on the important issue of how these signaling pathways are modulated within SCN neurons. Phosphorylation and dephosphorylation of specific nuclear substrates, including CREB and histone H3 tail (45, 50, 56), are essential in the fine tuning of transcription regulation.

This study provides further support to the hypothesis that light-induced resetting of the clock proceeds by activation of *Per* genes, namely *mPer1* (15, 16, 38, 39). It is still unclear how the same *mPer1* activation gives rise to different responses depending on the time in the circadian cycle (16, 50, 61). The changing phase relationships of clock genes and relative protein abundance likely play an important role in this respect (62). The possibility that synergistic action of signaling routes, as well as their known cross-talks, may occur at restricted temporal win-

dows will need to be taken into account to decipher the mechanisms of clock physiology.

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