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The Human *PER1* Gene is Inducible by Interleukin-6

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Abstract

The mammalian period (*Per*) genes which are components of the circadian clock are mainly regulated via an autoregulatory feedback loop. Here we show that a human *PER1* (*hPER1*) reporter gene activity is stimulated by interleukin-6 (IL-6), a member of the large cytokine gene family and an inducer of the acute phase reaction, in human hepatoma (HuH-7) cells. Our results confirm and extend the view that the *hPER1* promoter acts as a sensor for multiple signaling molecules thereby integrating different physiological parameters.

Index Entries: Circadian rhythm; chronobiology; Human *Per* genes; signaling pathways; clock resetting; hepatoma; acute phase.

Introduction

The circadian clock is a self-sustaining oscillator with a periodicity of approx 24 h and controls many physiological systems (Aschoff, 1981). On the molecular level, the oscillator consists of many genes which constitute an autoregulatory feedback loop (*see* Dunlap, 1999). Important components of the mammalian circadian clock are the period (*Per*) genes which are expressed in a diurnal manner in many tissues (Sun et al., 1997; Tei et al., 1997; Albrecht et al., 1997; Shearman et al., 1997; Zylka et al., 1998; Takumi et al., 1998; Field et al., 2000) and also in cell culture (Balsalobre et al., 1998). Because the intrinsic circadian rhythm of an organism is not exactly 24 h the clock needs to be reset periodically. In vivo, light induces *Per1* and *Per2* gene expression (Albrecht et al., 1997; Shearman et al., 1997; Takumi et al., 1998; Field et al., 2000; Shigeyoshi et al., 1997). In vitro, rhythmic *Per1* and *Per2* expression is inducible by high serum (Balsalobre et al., 1998), forskolin (Morgan et al., 1998; Yagita and Okamura, 2000; Akashi and Nishida, 2000; Motzkus et al., 2000) and PMA (Motzkus et al., 2000). In the central biological clock, the suprachiasmatic nucleus (SCN), circadian regulation of gene expression can be mediated by cAMP analogs (Gillette, 1996) through cAMP response elements (CREs; Obrietan et al., 1999).

Recent results indicate that expression of *Per1* is regulated in a species-dependent manner. Alternative usage of promoter elements can elicit different responses (Hida et al., 2000; Taruscio et al., 2000; Yamaguchi et al., 2000), but it has been shown previously that the reporter gene assay used here reflects endogenous *hPER1* expression (Motzkus et al., 2000).

One of the most prominent circadian rhythms is the sleep/wake cycle. This rhythm is, among other factors, depending on *Per*-genes as impressively shown recently for mutations in *Per2* that were correlated strongly with a human physiological state known as "advanced sleep phase syndrome" (Toh et al., 2001). Interleukin-6 has been shown to vary in a circadian manner in human urine and blood (Sothern et al., 1995). Moreover, patients with pathologically increased daytime sleepiness and fatigue display elevated daytime levels of IL-6 (Vgontzas

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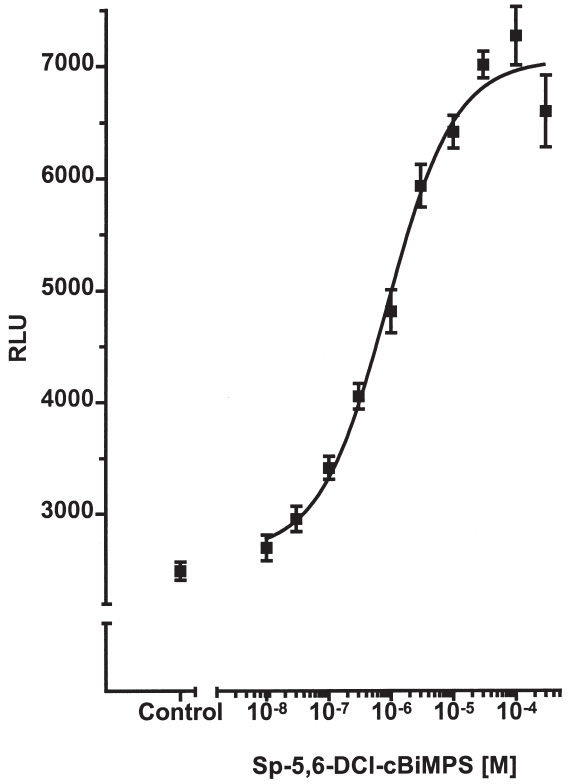

Fig. 1. Dose-dependent induction of *hPER1-luc* in HUH-7 human hepatoma cells. Stimulation of *hPER1-luc* by the specific PKA activator Sp-5,6-DCl-cBiMPS. Shown are the means ± SD of $n = 8$ experiments. The effect of Sp-5,6-DCl-cBiMPS is significant already at 30 n*M* (ANOVA with Bonferroni Post-test ($p \leq 0.05$ basal vs 30 n*M* Sp-5, 6-DCl-cBiMPS).

et al., 1999). We present here, that beside its known regulation by PKA- and PKC-activators (Morgan et al., 1998; Yagita and Okamura, 2000; Akashi and Nishida, 2000; Motzkus et al., 2000) *hPER1* is also inducible by IL-6 thereby adding a previously undescribed pathway to *hPER1* regulation.

Material and Methods

Materials

Sp-5,6-DCl-cBiMPS was purchased from BioLog Lifescience Institute, Bremen, Germany. IL-6 was from TEBU, Frankfurt, Germany. Transfection reagents (Effectene) were from Qiagen, Hilden, Germany.

Cell Culture

Human hepatoma (HuH-7) cells were cultured in Delbeco's Modified Eagle Medium (Life Technologies), 10% FCS (Whittaker), penicillin/streptomycin (100 U/mL), and 2 m*M* L-glutamine at 37°C in 5% CO_2 atmosphere.

Construction of the hPER1-promoter Luciferase Reporter Gene

The *hPER1* reporter gene was constructed as described (Motzkus et al., 2000) and will be referred to as *hPER1-luc*. Putative regulatory elements on the promoter we used in the construct were analyzed *in silico* using Transfac 4.0 (Wingender et al., 2000).

Transfection, Stimulation, and Luciferase Reporter-Gene Assays

Transfection was done as described (Motzkus et al., 2000). In brief, 40,000 cells per well were plated onto 96-whitewell plates (Corning Costar, Bodenheim, Germany), incubated for 16 h, stimulated for 4 h in 25 µL total volume, lysed by adding 25 µL Steady Glo solution (Promega, Heidelberg, Germany) per well and 20 min shaking at 250 U/min. Light emission at 562 nm was measured in a microtiter plate luminometer (Lumistar; BMG).

Statistics

Data were analyzed using MS Excel and GraphPad Prism (3.0 for Windows; GraphPad Prism Software Inc., San Diego, CA). Data are expressed as the means (± SD) and were compared using ANOVA with subsequent Bonferroni tests for multiple comparisons with $p \leq 0.05$ as the criterion of significance.

Results

As suggested by previous experiments using forskolin as an activator of adenylate cyclase and inducer of intracellular cAMP, we found a dose-dependent induction of *hPER1-luc* in HUH-7 human hepatoma cells after application of the specific PKA activator Sp-5,6-DCl-cBiMPS and IL-6 (Figs. 1 and 2). When cells were treated with fixed doses of Sp-5,6-DCl-cBiMPS together with increasing doses of IL-6 the activation of *hPER1-luc* activity was additive (Fig. 2). In this context, we identified potential IL-6 responsive regions in the human Per1 promoter (Fig. 3). However, we could not find an effect of IL-6 on the phosphorylation of p42/44 mitogen-activated protein kinase (MAPK) or the phosphorylation of calcium/cAMP-responsive element binding protein (CREB), components that have previously been shown to participate in the PKA/PKC pathways leading to *hPER1-luc* activation in human hepatoma cells (data not shown).

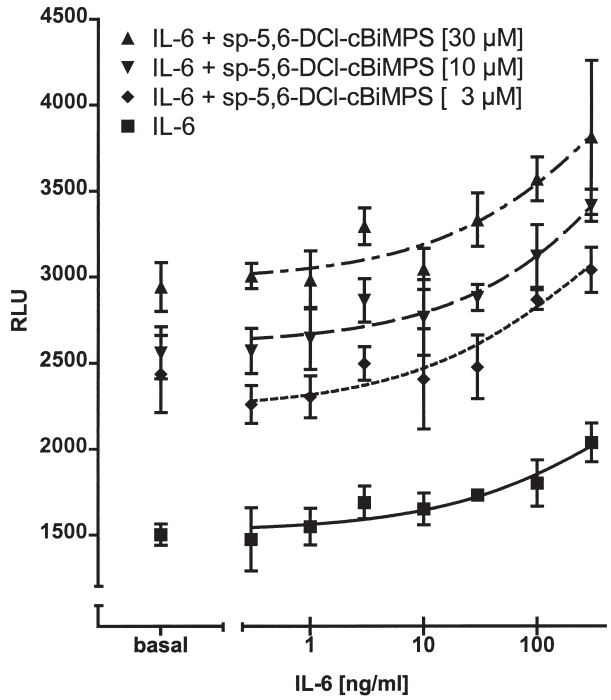

Fig. 2. Effect of increasing doses of Sp-5,6-DCl-cBiMPS and IL-6 on *hPER1-luc* activity in HUH-7 cells. Cells were treated with Sp-5,6-DCl-cBiMPS alone (basal), IL-6 alone or treated with fixed doses of *Sp*-5,6-DCl-cBiMPS as indicated in the legend plus increasing concentrations of IL-6 as indicated on the bottom (right side). Shown are the means ± SD of $n = 4$ experiments. The EC_{50}-values for IL-6 do not vary with the Sp-5,6-DCl-cBiMPS concentration added (mean EC_{50} = 7.5 ± 1.0 ng/mL).

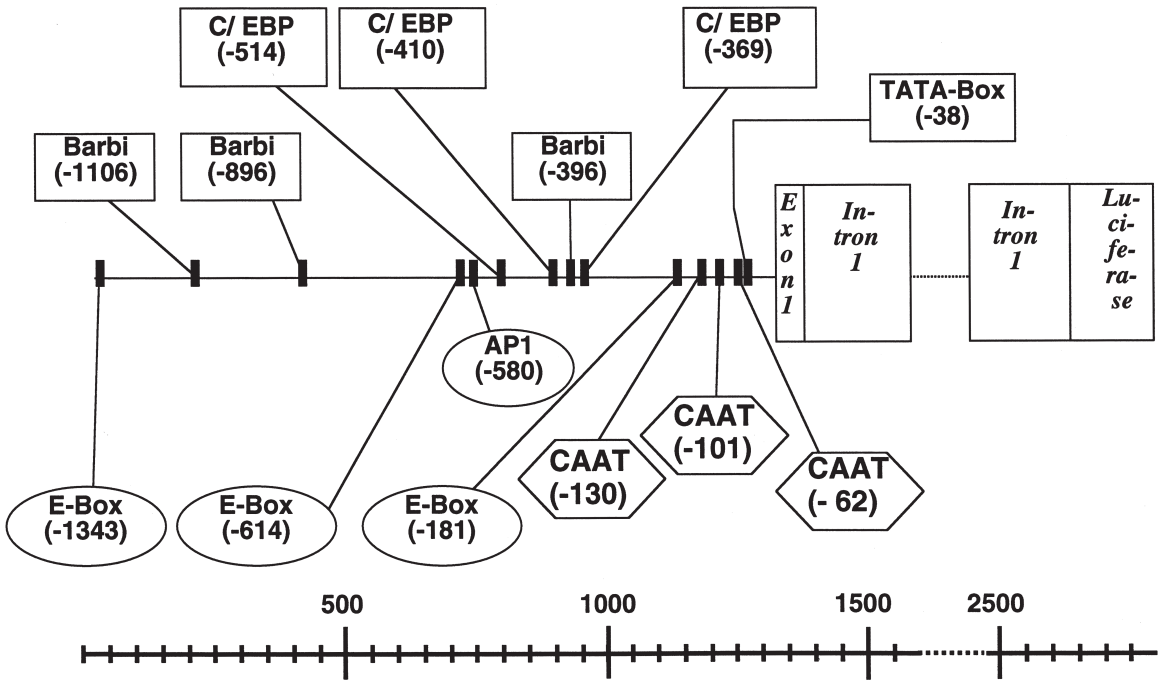

Fig. 3. Location of putative regulatory elements in the promoter part of the hPer1-luc reporter. We found three putative E-boxes, three C/EBP-, three CAAT-, and one AP-1-like element. To our knowledge the C/EBP-elements are possible mediators of the IL-6 effect.

Discussion

Beside its well-described regulation by the components of the autoregulatory feedback loop (Dunlap, 1999) the mammalian *Per1* gene has previously been shown to respond to activation of PKA and PKC pathways (Yagita and Okamura, 2000; Balsalobre et al., 2000; Motzkus et al., 2000). Both the PKC activator PMA and the cAMP analog *Sp*-5,6-DCl-cBiMPS induce *hPER1-luc* activity in a dose dependent manner (Figs. 1 and 2; Motzkus et al., 2000). These effects are potentiated by coapplication of both PKA and PKC activators (Motzkus et al., 2000). However, the *hPER1* promoter is not exclusively activated through the PKA and PKC pathway. It has been described that elevated intracellular calcium and glucocorticoids induce Per1 mRNA levels in the rat-1 fibroblast cell line (Balsalobre et al., 2000). Here we show that the cytokine IL-6 is capable of inducing *hPER1-luc* activity and that this induction is additive to *Sp*-5,6-DCl-cBiMPS. This indicates that the IL-6 effect is independent of or at least not mediated by PKA. Possible mediators of the IL-6 effect are the CCAAT/enhancer binding protein-beta (C/EBP-beta) response elements present in the *hPER1* promoter sequence that we identified using the program TRANSFAC (Wingender et al., 2000). C/EBP-elements are reportedly involved in the IL-6 signal transduction pathway (Akira et al., 1990). Beside these sequences we found three putative E-boxes, elements that characterize many genes which are expressed in a circadian manner, as well as three CAAT-boxes and one AP-1-like element. The latter element is presumably involved in the mediation of the PMA/PKC effects.

The findings described here may be interesting on the background of the described connection between disturbed IL-6 levels and sleep problems. Importantly, patients with pathologically increased daytime sleepiness and fatigue have elevated levels of IL-6 (Vgontzas et al., 1999). Our data may provide one possible way how disturbed IL-6 levels influence an important component of day/night regulation, hPer1, and hence sleep.

In conclusion, we show here that beside stimulators acting through the PKA and PKC pathways also the cytokine IL-6 is capable of regulating the human *Per1* promoter in human hepatoma cells. However, MAPK and CREB pathways seem to be of no or minor importance in human hepatoma unlike the situation in the human neuroblastoma SHSY-5Y cell line (Schumann et al., 1999). There is increasing evidence that beside the well established autoregulatory feedback mechanism for *Per1* gene regulation time resolved expression of hPer1 is also modulated by several diffusible molecules like hormones and glucocorticoids (Balsalobre et al., 2000) and, as shown here, cytokines. Thus the *Per1* promoter may serve as a sensor and integrator of physiological changes in an organism in order to adjust the central but also peripheral clocks like the liver to environmental challenges, but may also be disturbed by pathologically changed hormone levels as shown here for the acute phase mediator IL-6.

Acknowledgments

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