Cell, Vol. 93, 929–937, June 12, 1998, Copyright ©1998 by Cell Press

# A Serum Shock Induces Circadian Gene Expression in Mammalian Tissue Culture Cells

**Aurélio Balsalobre, Francesca Damiola, and Ueli Schibler***
Département de Biologie Moléculaire, Sciences II
Université de Genève
30, Quai Ernest Ansermet
CH-1211 Genève-4
Switzerland

## Summary

**The treatment of cultured rat-1 fibroblasts or H35 hepatoma cells with high concentrations of serum induces the circadian expression of various genes whose transcription also oscillates in living animals. Oscillating genes include *rper1* and *rper2* (rat homologs of the *Drosophila* clock gene *period*), and the genes encoding the transcription factors Rev-Erbα, DBP, and TEF. In rat-1 fibroblasts, up to three consecutive daily oscillations with an average period length of 22.5 hr could be recorded. The temporal sequence of the various mRNA accumulation cycles is the same in cultured cells and in vivo. The serum shock of rat-1 fibroblasts also results in a transient stimulation of c-*fos* and *rper* expression and thus mimics light-induced immediate-early gene expression in the suprachiasmatic nucleus.**

## Introduction

Many physiological processes are subject to circadian regulation. In mammals, sleep-wake cycles, body temperature, heartbeat, blood pressure, endocrine secretion, renal activity, and liver metabolism are all under the control of a circadian pacemaker (Lavery and Schibler, 1993; Portaluppi et al., 1996; Rabinowitz, 1996; Hastings, 1997). Like any timing system, the circadian clock is made up of three components: an input pathway adjusting the time, a central oscillator generating the circadian signal, and an output pathway manifesting itself in circadian physiology and behavior. In most organisms, including mammals, the photoperiod (i.e., daily changes in light intensities) is thought to be the major environmental cue involved in circadian entrainment. Light signals are perceived by photoreceptor cells in the retina and transmitted to neurons of the suprachiasmatic nucleus (SCN) via the retinohypothalamic tract (Takahashi, 1995; Hastings, 1997). Based on ablation and transplantation experiments, the SCN is thought to contain the major mammalian pacemaker (Rusak and Zucker, 1979). Its oscillations appear to occur by cell-autonomous mechanisms (Takahashi, 1993): isolated SCN neurons kept in culture still display rhythmic firing frequencies (Welsh et al., 1995). Interestingly, different average period lengths have been measured for in vitro cultured SCN neurons from wild-type hamsters, and homo- or heterozygous tau mutant hamsters. These differences are qualitatively similar to the ones observed for the wheel-running activity of the donor animals (Liu et al., 1997).

Other neural tissues of higher organisms have been shown to possess circadian pacemakers. For example, hamster retina and primary chick pineal gland cells kept in tissue culture secrete melatonin in a circadian manner (Robertson and Takahashi, 1988; Tosini and Menaker, 1996). In case of pinealocytes, the cycles can even be phase-shifted by light. In birds and reptiles, the pineal gland is thought to play a crucial role in controlling cyclic outputs (Gwinner et al., 1997; Tosini and Menaker, 1998). Unlike SCN neurons, pinealocytes contain a light-sensitive pigment, pinopsin, and can directly reset their clock by nonocular light perception (Okano and Fukada, 1997).

The mechanisms employed by circadian output pathways are poorly understood but are likely to involve both nervous and humoral signals (Moore, 1992; Silver et al., 1996). The importance of the humoral output pathways is underscored by the observation that the blood levels of many hormones, including CRF, ACTH, glucocorticoids, thyroid hormones, and melatonin, oscillate according to a well-defined daily rhythm (Klein et al., 1997; Chrousos, 1998). Once entrained by the photoperiod, these rhythmic manifestations persist with a period length close to 24 hr in the absence of external time cues.

Genetic screens in the fungus *Neurospora* and the fruitfly *Drosophila* have revealed genes whose expression is essential in generating a 24 hr periodic signal. The *Neurospora* clock gene *frequency* (*frq*) is one such gene: mutations in it either abolish or change circadian periodicity (Dunlap, 1996). *Prd-4* and *wc-2* are two additional *Neurospora* genes required for the generation of circadian cycles (Dunlap, 1996; Crosthwaite et al., 1997). In *Drosophila*, two clock genes have been identified, *period* (*per*) and *timeless* (*tim*). Both are expressed in a circadian fashion and their products cycle with very similar phase (Myers et al., 1996; Rosbash et al., 1996; Young et al., 1996; Zeng et al., 1996). PER and TIM form stable heterodimers that are translocated from the cytoplasm to the nucleus several hours after synthesis (Rosato et al., 1997). It is believed that PER and TIM proteins repress transcription of their own genes, thereby establishing a negative feedback loop (Rosato et al., 1997). The master clock involved in driving the activity of circadian locomotor rhythms in *Drosophila* has been localized in pacemaker neurons in the head (Ewer et al., 1992; Kaneko et al., 1997). However, *per* expression oscillates in many different neuronal and nonneuronal tissues, including headless organ cultures (Plautz et al., 1997). Interestingly, *per* expression in these organ cultures can be phase-shifted by light. Thus, light can be sensed by still unknown nonocular mechanisms.

Putative mammalian clock genes have been isolated only recently. Takahashi and coworkers (Vitaterna et al., 1994) have identified a locus, *clock*, that is required for properly timed and persistent periodicity of locomotor activity in mice. *Clock* was subsequently isolated by positional cloning, and its protein product was identified as a PAS helix-loop-helix transcription factor (Antoch et

*To whom correspondence should be addressed.

al., 1997; King et al., 1997). Interestingly, the PAS domain of PER (originally identified in the three proteins **P**ER, **A**ryl hydrocarbon receptor, and **S**ingle-minded) is essential for the heterodimerization between the two *Drosophila* proteins PER and TIM. Very recently, the genes encoding two putative circadian regulators, mPER1 and mPER2 (mouse PER 1 and 2), have been isolated on the basis of their extensive sequence similarity to *Drosophila per* (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Sun et al., 1997; Tei et al., 1997).

The mRNAs issued by mammalian *per* genes accumulate according to a robust circadian cycle in the SCN and, as shown in this paper, in the liver. Moreover, *mper1* and *mper2* expression is light-induced in the SCN of mice. This observation suggests that *mper1* and *mper2* belong to the class of immediate-early genes implicated in light-mediated phase shifting (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997). The genes specifying the transcription factors c-Fos, FosB, JunB, NGFI-A/Zif268, NGFI-B/Nur77, and Egr-3 are additional members of this class (Kornhauser et al., 1996; Morris et al., 1998).

A common feature of the putative circadian regulatory genes *mper1*, *mper2*, and *clock* is their ubiquitous spatial expression pattern (Sun et al., 1997; Tei et al., 1997). The same holds true for the transcription factors DBP, TEF, and Rev-Erbα, whose mRNAs accumulate with a robust circadian rhythm in most mouse or rat tissues (Fonjallaz et al., 1996; F. D., L. Lopez-Molina, N. Preitner, and U. S., unpublished data). DBP and TEF are two related leucine zipper proteins that can form homo- or heterodimers (Drolet et al., 1991). In peripheral mouse tissues such as liver, kidney, and lung, *dbp* and *tef* mRNAs cycle with the same phase (Lopez-Molina et al., 1997). *dbp* mRNA also oscillates in neurons of the SCN, but with a different phase: zenith and nadir levels are reached about 4 hr earlier than in peripheral tissues (Lopez-Molina et al., 1997). Mice homozygous for a *dbp* null allele display significant differences in circadian locomotor activity. However, since these mutant mice are still rhythmic under constant dark conditions, *dbp* is not a central component of the clock (Lopez-Molina et al., 1997). Moreover, DBP protein is not required for the circadian expression of its own gene, suggesting the existence of upstream regulators governing *dbp* transcription. Rev-Erbα, a nuclear orphan receptor, may fulfill such a role. As expected for an upstream regulator of *dbp* expression, *rev-erbα* mRNA reaches maximal daily levels about 4 hr earlier than *dbp* mRNA in both SCN neurons and liver cells (F. D., L. Lopez-Molina, N. Preitner, and U. S., unpublished data).

The ubiquitous expression of clock-related genes suggests that cells of peripheral mammalian tissues might also contain a circadian clock. In this paper, we demonstrate that immortalized rat fibroblasts that have been kept in cell culture for more than 25 years still harbor a clock capable of measuring time with astonishing precision. Circadian expression of *per1*, *per2*, *rev-erbα*, *dbp*, and *tef* can persist for at least three days in serum-free medium after an initial serum shock. The incubation of rat-1 fibroblasts with high concentrations of serum also induces c-*fos* and *per* expressions with kinetics similar to that observed for light-induced immediate-early gene expression in the SCN. Therefore, resetting of the circadian clock by light and serum factors might employ similar mechanisms.

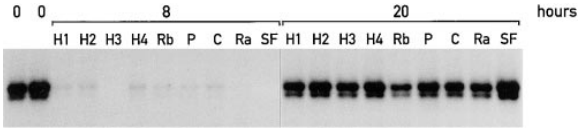


Figure 1. *tef* mRNA Expression in Rat-1 Fibroblasts Is Transiently Inhibited by Serum

Rat-1 cells were grown to confluence in a medium containing 5% fetal calf serum for 6 days. At time 0, cells were shifted to a medium containing 50% serum from different species: rabbit (Rb), pig (P), cow (C), rat (Ra), and horse (H1–H4 corresponding to four different batches). For one sample, the horse serum–rich medium was replaced 2 hr after its addition with serum-free medium (SF). At 8 and 20 hr after the addition of the serum-rich medium, whole-cell RNA was prepared from about $1.5 \times 10^7$ cells. Two independent samples for time = 0 were also prepared. The levels of *tef* mRNA were determined by RNase protection assays.

## Results

### High Concentrations of Sera from Different Animal Species Transiently Inhibit *tef* mRNA Expression in Rat-1 Fibroblasts

We have observed previously that the two PAR leucine zipper transcription factors TEF and DBP are expressed according to a robust circadian rhythm in many peripheral tissues (Fonjallaz et al., 1996). *tef* and *dbp* mRNAs also accumulate in cultured rat-1 fibroblasts, albeit at constant levels throughout the day (data not shown). We discovered, however, that the levels of these mRNAs markedly decreased 4–8 hr after feeding the cells with a medium rich in adult horse serum. No serum-induced changes in the accumulation of *tbp* mRNA (encoding TATA box binding protein) were observed, suggesting that the serum inhibition may be specific for transcripts with circadian accumulation in vivo (data not shown). We first wanted to examine whether this down-regulation can be elicited with serum from different individuals or species. Rat-1 fibroblasts were fed with media containing 50% of serum from horse (four different batches), rabbit, pig, rat, or cow, and whole-cell RNA was harvested 8 hr and 20 hr after the addition of the serum-rich medium. Figure 1 presents the *tef* mRNA accumulation determined by RNase protection experiments after incubation of cells with serum-rich medium. All tested sera were similarly efficient in inhibiting *tef* mRNA expression in rat-1 fibroblasts. Interestingly, this down-regulation was transient. Indeed, 20 hr after serum addition, the levels of *tef* mRNA were restored to the levels observed before treatment of the cells with serum-rich medium. Since the down-regulation of *tef* mRNA accumulation occurred immediately after addition of the serum-rich media, we examined whether a short treatment of serum would suffice to trigger a transient down-regulation of *tef* expression. To this end, the serum-rich medium was replaced after the first 2 hr with serum-free medium (lanes SF in Figure 1). It is clear from this experiment that a shock with the serum-rich medium is at least as

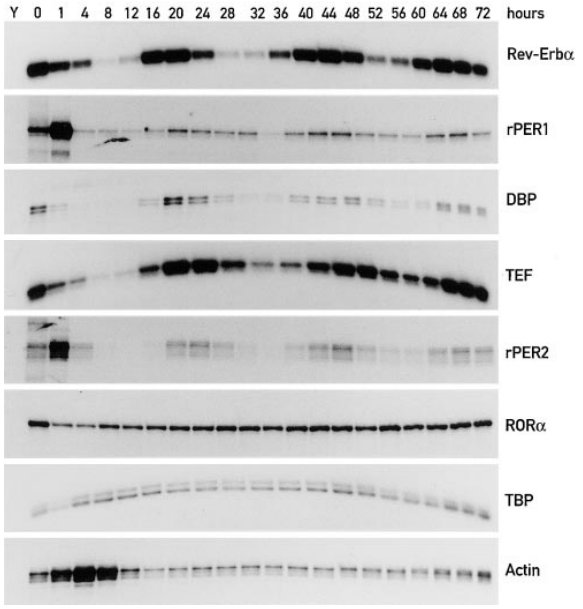


Figure 2. Accumulation of Various mRNAs in Serum-Shocked Rat-1 Fibroblasts

Rat-1 cells were grown to confluence in a medium containing 5% fetal calf serum. After having been kept for 6 days in the same medium, cells were shifted to a medium containing 50% adult horse serum and incubated for 2 hr, after which the serum-rich medium was replaced with serum-free medium. Whole-cell RNA was prepared from about $1.5 \times 10^7$ cells at the times shown on top of the figure after the serum shock, and the relative levels of the mRNAs indicated at the right side of the figure were determined by RNase protection assays. A *tbp* antisense RNA probe was included in each assay as a control for an mRNA with constitutive expression. The *tbp* mRNA results shown in this figure are those obtained in the assays containing probes for *rev-erbα* and *tef* mRNAs. Yeast RNA (Y) was used as negative control.

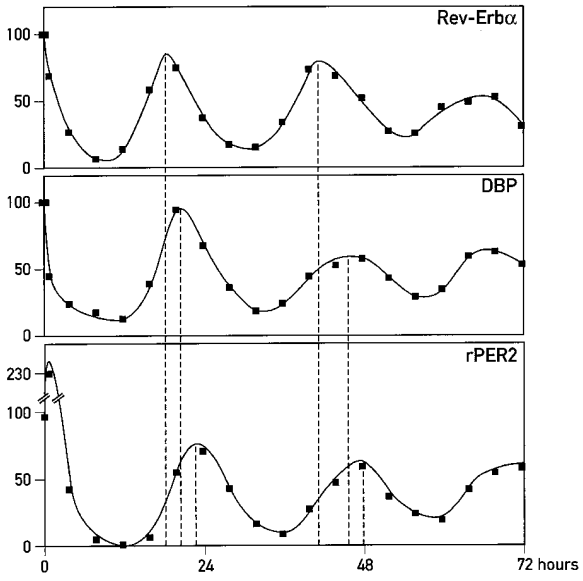


Figure 3. The Temporal Sequence of Cyclic mRNA Accumulations in Serum-Shocked Rat-1 Fibroblasts

The signals obtained in the RNase protection assays shown in Figure 2 for *rev-erbα*, *dbp*, and *rper2* mRNAs were quantified by phosphor imaging and normalized to the signals obtained for *tbp* mRNA. The normalized values obtained for each of the three mRNAs before induction (time 0) were set to 100.

efficient in inhibiting *tef* expression in rat-1 fibroblasts as a prolonged treatment with serum-rich medium. Moreover, in the absence of serum, the *tef* mRNA levels were restored to the same values as in its presence. Therefore, serum factors appear to be essential for the repression of *tef* expression but dispensable for the subsequent resynthesis of *tef* mRNA.

### A Serum Shock Induces Circadian Gene Expression in Cultured Rat-1 Fibroblasts

The observation that *dbp* and *tef* expression were oscillating after serum treatment was somewhat reminiscent of circadian gene expression. This prompted us to follow the mRNA accumulation profiles of additional genes with circadian expression for expanded time spans after the serum shock (see Experimental Procedures). We first demonstrated that several genes with known circadian expression were expressed in rat-1 fibroblasts (data not shown). Then, the mRNA accumulation profiles were recorded by RNase protection assays during the 72 hr following the serum shock (Figure 2). Surprisingly, all mRNAs known to follow circadian accumulation in the SCN and/or in the liver also oscillate in serum-shocked rat-1 fibroblasts. The expression of *rper1* and *rper2* (rat *per* 1 and 2) is first induced and then repressed. After 8 to 12 hr, the levels of these two mRNAs rise again and then assume circadian oscillations. Consistent with our preliminary results with *tef* and *dbp* mRNAs (Figure 1 and data not shown), the concentrations of the mRNAs encoding Rev-Erbα, DBP, and TEF gradually diminish until minimal values are reached at about 8 hr after serum treatment. After this time, the levels of these mRNAs rise again and then begin to fluctuate in a daily fashion like *rper1* and *rper2* mRNAs.

Importantly, the different mRNA accumulations cycle with different phases. As shown in Figures 2 and 3, the phase of *rev-erbα* mRNA oscillation precedes the one of *dbp* mRNA oscillation by about 3 hr and the one of *rper2* mRNA oscillation by about 6 hr. Moreover, the phases of *dbp*, *tef*, and *rper1* cyclic expression are very similar (Figure 2).

Transcripts that do not show circadian expression in vivo also do not cycle in vitro. This is demonstrated in Figure 2 for *tbp* and β-*actin* mRNAs and *rorα* mRNA, specifying a nuclear orphan receptor. As previously published (Elder et al., 1984), β-*actin* expression is transiently induced after serum treatment. However, β-*actin* mRNA does not fluctuate significantly at times later than 8 hr. Likewise, c-*fos* mRNA levels undergo a dramatic surge immediately after serum treatment (see below) but remain invariable at times later than 4 hr (data not shown).

The period lengths (τ's) of serum-induced mRNA accumulation cycles, such as the ones presented in Figure 2, were estimated graphically (see Experimental Procedures). All of the 23 estimated τ values varied between 20 and 27 hr, and the mean value +/− standard deviation was found to be 22.5 +/− 1.7 hr.

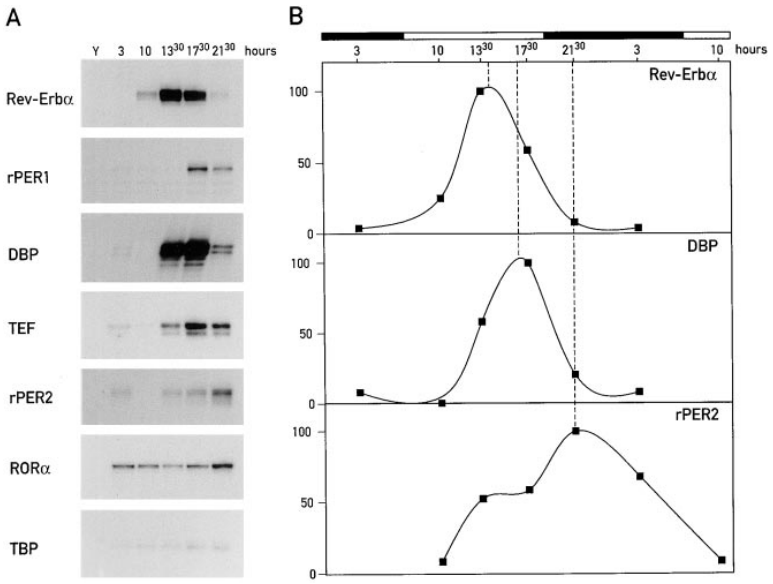


Figure 4. The Temporal Sequence of Cyclic mRNA Accumulations in the Liver Is Similar to the One in Serum-Shocked Rat-1 Fibroblasts

(A) Total RNAs were prepared from rat livers harvested at times indicated above the figure. The relative levels of different mRNAs were determined by RNase protection assays, with the antisense RNA probes indicated at the left side of the figure. Yeast RNA (Y) was used as a negative control.

(B) Signals obtained for *rev-erbα*, *dbp*, *rper2*, and *tbp* mRNAs were quantified by phosphor imaging. The values shown in the graphs are normalized to *tbp* mRNA. This was accomplished by dividing the signals obtained for the indicated mRNAs by the signals obtained for *tbp* mRNA, which is assumed to remain constant throughout the day. The highest ratios were arbitrarily set as 100 for each mRNA. On top of the figure, the light regimen (lights on 7 a.m., lights off 7 p.m.) of the animal house is indicated. Note that the sequence of cyclic mRNA accumulation profiles in the liver resemble those observed in serum-shocked rat-1 fibroblasts (Figure 3).

### The Temporal Sequence of Cyclic mRNA Expression in Rat-1 Fibroblasts Mimics the One Observed in the Liver

We were impressed by the similarity between the temporal sequence of cyclic mRNA accumulations in rat-1 fibroblasts and in the liver. Indeed, we have shown in Figure 3 that *rev-erbα* mRNA oscillation precedes the ones of *dbp* and *tef* mRNA. This is exactly what we have observed in the liver (F. D., L. Lopez-Molina, N. Preitner, and U. S., unpublished data; results shown below). We thus decided to extend this observation to *rper1* and *rper2* mRNA oscillations. While significant levels of *mper1* and *mper2* mRNAs have already been observed in most tissues, it has not yet been reported whether they also fluctuate during the day in peripheral tissues. Therefore, we recorded the *rper1* and *rper2* mRNA accumulation profiles in rat liver. Several other mRNAs with circadian or constitutive accumulation patterns were also included in this study. Figure 4A shows RNase protection experiments conducted with rat liver RNA prepared at different times during the day and various antisense RNA probes. The results of these experiments demonstrate that the levels of both *rper1* and *rper2* mRNAs, like those of Rev-Erbα, DBP, and TEF, fluctuate in a daily fashion in liver. *rev-erbα* mRNA reaches maximal levels at approximately 2 p.m. and is the transcript with the earliest peak accumulation among the RNAs tested. About 3 hr later (5 p.m.), *rper1*, *dbp*, and *tef* mRNAs attain zenith expression. The accumulation profiles of these three transcripts thus cycle with very similar phases in the liver as in rat-1 fibroblasts (Figure 2). Finally, *rper2* expression peaks at 9:30 p.m., and thus, the peak of *rper2* mRNA accumulation follows those of *rper1*, *dbp*, and *tef* mRNA accumulation by about 4 hr in the liver, similar to what was observed in rat-1 fibroblasts.

In contrast to these rhythmically expressed transcripts, *tbp* and *rorα* mRNAs show little if any circadian oscillation. The accumulation curves for *rev-erbα*, *dbp*, and *rper2* mRNAs are presented in Figure 4B and illustrate the different circadian phases observed for these transcripts.

### A Serum Shock Also Induces Cyclic Gene Expression in H35 Hepatoma Cells

Rat-1 fibroblasts were initially chosen for these experiments because they are relatively robust and proliferate with a short generation time (15 hr). We nevertheless wanted to extend this study to another rat cell line originating from a different tissue. To this end, H35 hepatoma cells were subjected to the same regimen of serum treatment, and the levels of several mRNAs were monitored as described above by RNase protection assays (Figure 5). The results obtained with H35 hepatoma cells for the mRNAs encoding Rev-Erbα, DBP, and rPER2 are qualitatively similar to those described above for rat-1 fibroblasts, although at most two consecutive mRNA accumulation cycles could be observed in H35 cells. Again, the relative phases observed in vivo are reproduced in vitro: *rev-erbα* mRNA oscillations precede *dbp* mRNA oscillations, which in turn precede *rper2* mRNA oscillations.

Nevertheless, some differences are evident between rat-1 and H35 cells. In H35 cells, *rper1* expression is also strongly induced by serum. However, after *rper1* mRNA levels have dropped to levels observed prior to stimulation, they remain relatively constant. An entirely unexpected result was revealed by the analysis of c-*fos* mRNA. In sharp contrast to rat-1 fibroblasts, c-*fos* expression is not induced by the serum shock in H35 cells. As one to two cycles of circadian expression could nevertheless be recorded for *rev-erbα*, *dbp*, and *rper2* (see Figure 5), c-*fos* induction may not be required for triggering the cyclic activity of these genes in H35 hepatoma cells (see Discussion).

### The Kinetics of *c-fos*, *rper1*, and *rper2* Induction in Serum-Shocked Rat-1 Fibroblasts

Circadian clocks can be reset by light. This light-induced phase-shifting is accompanied by the induction in the SCN of some immediate-early genes, such as the ones encoding the AP-1 transcription factors c-Fos, FosB, and JunB, the Zinc-finger proteins NGFI-A/Zif268 and Egr-3, and the orphan receptor NGFI-B/Nur77 (Morris

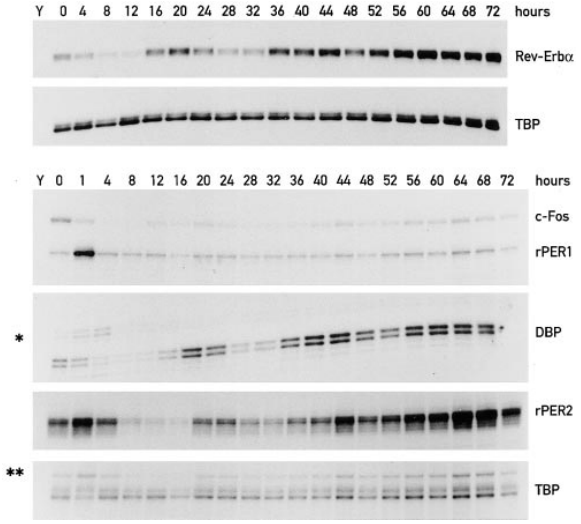


Figure 5. Accumulation of mRNAs in Serum-Shocked H35 Hepatoma Cells

Rat H35 hepatoma cells were grown close to confluence and shifted to a serum-rich medium, as described for rat-1 fibroblasts in Figure 2. The levels of the mRNAs indicated at the right side of the figure were determined at different times (top of the figure) after serum shock, as described in Figures 1–4. For *rev-erbα* mRNA (and *tbp* mRNA obtained in the same assays), the 1 hr time point is omitted. The signals marked with * or ** represent minor signals derived from the actin probes or rper2 probes, which were included in the same assays. Note that the accumulation of c-*fos* mRNA does not increase after the serum shock.

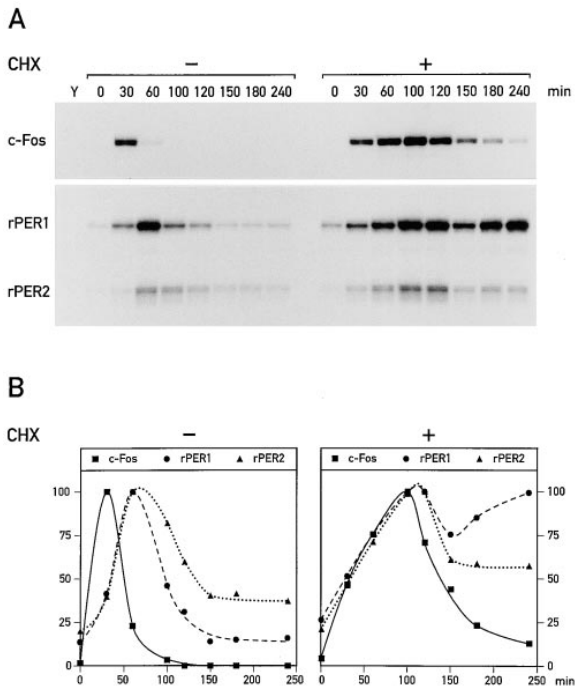


Figure 6. *per1* and *per2* Are Serum-Induced Immediate-Early Genes

(A) Rat-1 cells were grown to confluence in a medium containing 5% fetal calf serum. After having been kept for 6 days in the same medium, cells were shifted to a medium containing 50% adult horse serum, and RNAs were prepared from about $1.5 \times 10^7$ cells at the indicated times after addition of the serum-rich medium. The levels of c-*fos*, *rper1*, *rper2*, and *tbp* mRNAs were determined by RNase protection assays (data not shown for *tbp*). CHX stands for cycloheximide (10 μM final concentration). In the experiment displayed in the right panel, CHX was added 1 hr before addition of the serum-rich medium. The autoradiographs were exposed for 30 min (c-Fos) and 16 hr (*rper1* and *rper2*). Yeast RNA (Y) was used as a negative control.

(B) The signals obtained in the RNase protection experiments shown in (A) were quantified by phosphor imaging and normalized for the signal obtained with an antisense probe for *tbp* mRNA, which was not induced by serum (data not shown). The highest values were arbitrarily set as 100 for each mRNA species. Therefore, the profiles cannot be compared to one another in absolute terms but, rather, reflect the relative kinetics of mRNA accumulation after serum induction. The time after serum induction is given in minutes.

et al., 1998). These genes have previously been identified as immediate-early genes in serum-stimulated cells, hinting toward similar signal transduction pathways operative in light-induced SCN neurons and serum-stimulated cells.

In recent reports, the putative circadian regulatory genes *mper1* and *mper2* have also been shown to belong to the class of light-induced genes in the SCN (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997). We have shown herein that serum also triggers the expression of *rper1* and *rper2* in rat-1 fibroblasts and H35 hepatoma cells (see Figures 2 and 5, 1 hr time points). We were thus interested to determine the relative kinetics of induction of *per* and c-*fos* genes in serum-stimulated cells, as it has been done in the SCN of light-induced animals. Furthermore, we investigated whether *rper* mRNA induction requires ongoing protein synthesis to confirm that *per1* and *per2* are indeed immediate-early genes in serum-stimulated rat-1 fibroblasts. The accumulation profiles of c-*fos*, *rper1*, and *rper2* mRNAs were recorded by RNase protection assays at 30 min intervals during 4 hr after the serum shock. As shown in Figures 6A and 6B (left panels), the levels of all three mRNAs strongly increase as a result of serum treatment. Similar to the results obtained in the SCN after light induction, the c-*fos* mRNA concentration reaches a maximal value as early as 30 min after the serum shock and then sharply drops to levels observed prior to stimulation. The kinetics of both the increase and the decrease of c-*fos* mRNA levels suggest a half-life of less than 10 min for this transcript, an observation consistent with previous reports (Rahmsdorf et al., 1987). The levels of both *rper1* and *rper2* mRNAs rise and fall with considerably slower kinetics, maximal values being reached about 60 and 75 min, respectively, after the serum shock. In conclusion, the sequence of activation of c-*fos*, *rper1*, and *rper2* is similar in serum-induced cells and in the SCN of light-stimulated animals.

The delayed expression of *rper* genes could reflect a longer half-life for *rper* mRNAs with regard to c-*fos* mRNA and/or different mechanisms of induction for c-*fos* and *rper* genes. Conceivably, *rper1* and *rper2* induction, in contrast to c-*fos* activation, may require the synthesis of immediate-early transcription factors and may therefore be retarded. The serum shock was thus repeated with cells that had been pretreated with cycloheximide for 1 hr. As shown in Figure 6A and 6B (right panels), inhibition of translation does not prevent the induction of c-*fos*, *rper1*, and *rper2* mRNAs, suggesting that all three transcripts are products of immediate-early

genes. Inhibition of protein synthesis does, however, affect the kinetics of immediate-early gene expression. In the case of c-*fos* mRNA, both the surge and the fall of accumulation are about 4-fold slower in the presence of cycloheximide than in its absence. Quite different results are observed for *rper* mRNAs. Inhibition of protein synthesis has a rather marginal effect on the rising phase of *rper1* and *rper2* mRNA accumulation (compare left and right panels of Figure 6B). However, it delays the down-regulation of *rper2* expression and prevents the down-regulation of *rper1* expression for at least 4 hr, the largest interval examined.

In control experiments (data not shown), we have examined the effect of cycloheximide alone on the accumulation of c-*fos* and *rper* mRNAs. The addition of cycloheximide leads to a 6-fold increase of c-*fos* mRNA levels, but to only slightly higher accumulations of *rper1* mRNA (2-fold) and *rper2* mRNA (1.3-fold). The higher accumulation of c-*fos* mRNA in cycloheximide-treated cells had been explained by several mechanisms, including a longer half-life of c-*fos* mRNA and the absence of autorepression by c-Fos protein (Edwards and Mahadevan, 1992; Zinck et al., 1995). As c-*fos* mRNA levels decrease close to values observed prior to stimulation in the presence of cycloheximide (Figure 6), c-*fos* transcription appears to be down-regulated even in the absence of protein synthesis. Therefore, the difference between the c-*fos* mRNA accumulation in the absence and presence of cycloheximide may mainly reflect an increased mRNA stability in cycloheximide-treated cells. For *rper* mRNAs, this difference cannot be attributed solely to different mRNA half-lives. In particular, in the case of *rper1* mRNA, the down-regulation following serum induction is likely to require repression of *rper1* mRNA transcription by an immediate-early gene product (see Discussion).

## Discussion

### Serum Induces Circadian Gene Expression in Cultured Cells

The addition of a serum-rich medium to cultured rat-1 fibroblasts or H35 hepatoma cells triggers the rhythmic expression of several genes whose activity also oscillates in vivo. On the basis of several criteria, we believe that the observed oscillations are driven by a circadian timing system. First, to date, only mRNAs with circadian accumulation in vivo have been found to cycle after the serum shock. Furthermore, the average period length ($\tau$) of the mRNA accumulation cycles in vitro is 22.5 $+/-$ 1.7 hr, well within the range of $\tau$'s accepted for circadian periods. Second, of the two putative rat homologs of the *Drosophila* clock gene *period*, at least one, *rper2*, cycles with robust amplitude in both cell types tested. Third, the relative phases of the different mRNA accumulation profiles are faithfully reproduced in vitro. Finally, the serum shock elicits an induction of *rper* expression similar to that observed in the SCNs of light-induced animals.

Interestingly, many of the light-induced genes in the SCN are also immediate-early genes after serum stimulation of growth-arrested cells. Therefore, these genes may not only play a role in resetting the circadian clock, but they may also be implicated in cell cycle regulation. Nevertheless, we do not believe that cell cycle progression is relevant to the oscillating gene expression observed in our experiments. First, the generation time of rat-1 cells (15 hr) is considerably shorter than the period length (22.5 hr) observed for serum-induced mRNA accumulation. Second, circadian gene expression proceeds for 3 days in serum-free medium, which prohibits cellular proliferation. Third, cytosine $\beta$-D arabinofuranoside or Ara-C, a potent inhibitor of DNA replication, did not influence serum-induced cyclic gene expression (data not shown).

On the basis of our results with fibroblasts and hepatoma cells, it appears that peripheral tissues contain a clock capable of measuring time with impressive precision. One can thus hypothesize that many circadian outputs might be controlled by peripheral clocks, which may themselves be synchronized by the central clock (see below).

Our studies were inspired in part by the resemblance of light-induced gene expression in the SCN and serum-induced gene expression in cultured cells. Even the relative kinetics of c-*fos*, *per1*, and *per2* immediate-early gene expression turned out to be similar in SCN neurons and rat-1 fibroblasts. On the basis of our results with cycloheximide-treated cells, we would predict that light-induced immediate-early gene expression in SCN neurons does not require protein synthesis either. The fast kinetics of c-*fos* and *mper1* induction in the SCN is in keeping with this conjecture.

Surprisingly, c-*fos* transcription in H35 hepatoma cells is diminished rather than increased by serum. However, serum does elicit at least one daily cycle of circadian gene expression in these cells. Therefore, c-*fos* induction may be dispensable for the activation for circadian rhythmicity in vitro. From the results presented in Figure 5, it appears that c-*fos* mRNA accumulation oscillates with a low amplitude in H35 hepatoma cells. However, such a cycle could not be observed in rat-1 fibroblasts (data not shown).

Interestingly, disruption of the c-*fos* gene in mice also had only minor consequences for circadian behavior (Honrado et al., 1996). Mice homozygous for a c-*fos* null allele still display rhythmic locomotor activity and can be phase-shifted by light, albeit with a somewhat attenuated response. Thus, while c-*fos* may augment the amplitude of light-induced phase-shifting, it is not absolutely required for photic entrainment.

Genetic loss-of-function and gain-of-function experiments will be required to evaluate whether *per1* and *per2* play essential roles in generating circadian oscillations in mammals. Nevertheless, the sequence similarity of these genes to *Drosophila per* (Sun et al., 1997; Tei et al., 1997) and their circadian expression and induction by light in the SCN (Sun et al., 1997; Tei et al., 1997) all suggest circadian functions for these two genes. The immediate induction and the circadian expression of *per1* and *per2* in cultured cells, reported in this paper, further support their potential roles as central clock components. In the presence of cycloheximide, a potent inhibitor of translation, the levels of *rper1* and *rper2* mRNAs are still induced by a serum shock, suggesting

that the serum-activated transcription of these genes does not require protein synthesis. Interestingly, however, the down-regulation of these mRNAs does appear to necessitate translation. Conceivably, the proteins required for the repression of *rper* genes include rPER1 and/or rPER2 themselves. Such a negative feedback loop is likely to be operative in *Drosophila*, as constitutive ectopic expression of PER protein abolishes the circadian cycling of endogenous *per* mRNA accumulation (Zeng et al., 1994).

In contrast to *rper1* and *rper2*, the other examined genes with circadian expression were not induced as an immediate result of serum treatment. Rather, the levels of *rev-erbα*, *dbp*, and *tef* mRNAs gradually decrease within the first 8 hr after exposure to a high serum concentration. Therefore, the serum-triggered induction of immediate-early genes such as *rper1* and *rper2* may also result in repression of clock-controlled genes such as *rev-erbα*, *dbp*, and *tef*. We assume that after the degradation of immediate-early repressors, circadian cycles may restart in all cells in a synchronous fashion. A hypothetical model reflecting these speculations is presented in Figure 7A. This model does not demand that all genes with circadian expression are directly under the control of intrinsic clock genes, such as *per1* and *per2*. In the case of *dbp*, for example, it may be sufficient to drive the cyclic expression of the putative upstream regulatory gene *rev-erbα* (F. D., L. Lopez-Molina, N. Preitner, and U. S., unpublished data).

Rhythmic gene expression elicited by serum treatment could be the result of either a synchronization of already existing cycles in desynchronized cells, or an induction of oscillations in arrhythmic cells. The cartoons displayed in Figure 7B illustrate the difference between these models. In the synchronization model, the expression of, for example, *rev-erbα* would cycle in each cell with a similar $\tau$, but with a different phase. Since each RNase protection analysis is performed with RNA from about $10^6$ cells, the oscillations in individual cells would not be revealed by such biochemical assays. If, indeed, serum synchronized preexisting cycles in desynchronized cells, it would have to induce phase shifts of up to 12 hr. In the induction model presented in the upper panel of Figure 7B, the expression of *rev-erbα* would be constitutive and similar in all cells. The cycles would then start in each cell as a result of the serum shock.

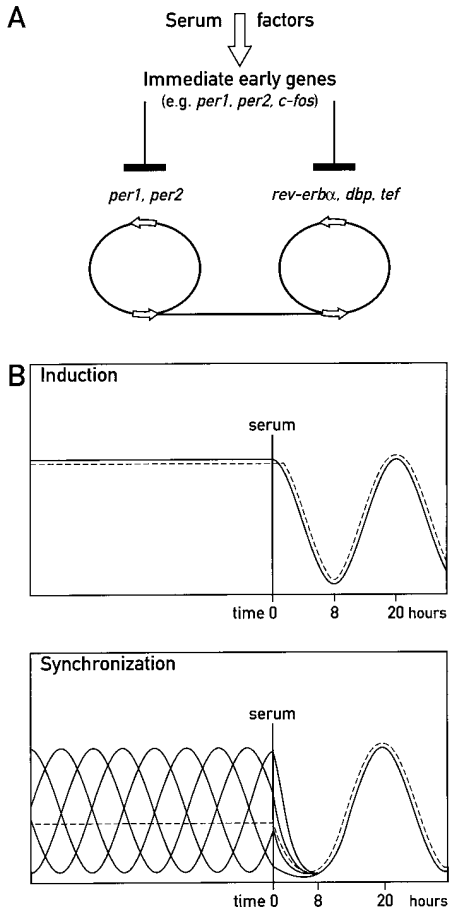


Figure 7. Hypothetical Models for Serum-Induced Circadian Gene Expression

(A) Resetting of the circadian clock by serum factors. Signaling molecules in the serum induce the expression of immediate-early genes, such as c-*fos*, *per1*, and *per2*. The regulatory proteins encoded by these genes repress both clock genes such as *per1* and *per2*, and other genes with circadian expression such as *rev-erbα*, *dbp*, and *tef*. Repression by immediate-early genes may be accomplished by direct or indirect mechanisms. After their burst of synthesis, immediate-early gene products decay, resulting in derepression. As a consequence, clock gene products could again accumulate, until they repress the expression of their own genes, and that of additional genes with circadian expression. This would lead to the synchronous transcription cycles observed in this paper.

(B) Induction or synchronization of circadian transcription cycles? The mechanisms proposed in (A) could either induce circadian gene expression in arrhythmic cells (top panel) or synchronize preexisting circadian cycles of desynchronized cells (bottom panel). The expected mRNA accumulation curves determined by biochemical approaches before and after serum induction are depicted as dotted lines. Discrimination between the two models would require the temporal recording of gene expression in single cells before and after treatment with serum-rich medium.

## How Is Circadian Gene Expression Coordinated in Peripheral Tissues?

*dbp* mRNA oscillates with a different phase in the SCN and in peripheral tissues. In mouse, zenith and nadir levels of this mRNA in SCN neurons forerun those in hepatocytes by about 4 hr (Lopez-Molina et al., 1997). The same holds true for *rev-erbα* mRNA whose cyclic oscillation precedes that of *dbp* mRNA (F. Damiola, L. Lopez-Molina, N. Preitner, and U. S., unpublished data). We did not monitor *per1* and *per2* mRNAs accumulation in the SCN, but the comparison of published data for the SCN and our own results for liver suggest that the cyclic expression of these putative clock genes also runs ahead in the SCN as compared to peripheral tissues. Thus, the relative temporal sequence of daily mRNA accumulation cycles is *rev-erbα* → *per1*/*dbp*/*tef* → per2 in the SCN, peripheral organs, and synchronized cultured cells. The delayed oscillation in the periphery as compared to the SCN may reflect the utilization of different entrainment mechanisms. Electrical signals from the retina are probably the major entrainment input for SCN neurons, while the cyclic secretion

of diffusible signals, possibly driven by the SCN, may coordinate circadian timing in peripheral tissues. The importance of humoral signaling by the SCN has already been demonstrated by transplantation experiments: circadian locomotor activity could be rescued in SCN-lesioned hamsters by implants of fetal SCN tissue encapsulated into porous plastic (Silver et al., 1996). Our demonstration that circadian cycles can be entrained by serum components and that entrainment by light and serum may involve the same immediate-early genes underscores the importance of chemical signal transduction for the coordination of circadian gene expression. We wish to emphasize, however, that all examined sera from adult animals were capable of triggering at least one round of circadian oscillation in rat-1 cells. The tested sera included both commercial sera from different species (horse, cow, rabbit, and pig) as well as rat sera that we harvested at 4 hr intervals around the clock (Figure 1 and data not shown). Conceivably, it may be the difference in daily levels of blood-borne signaling factors, rather than their absolute concentration, that synchronizes peripheral clocks. In our experimental serum shock protocol, we generated a large concentration difference in blood-borne factors simply by increasing the serum concentration. In the animal, the circadian secretion of such factors would produce similar differences. Regardless of how clocks are reset in peripheral tissues, the observation that commonly used immortalized cells contain endogenous clocks which have very similar properties to the SCN should have a major impact on future studies in this field. The availability of such in vitro systems should greatly facilitate the molecular dissection of the circadian clock.

## Experimental Procedures

### Cell Culture and Serum Shock Procedures

Rat-1 fibroblasts and H35 hepatoma cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal calf serum (GIBCO) and a mixture of penicillin-streptomycin-glutamine (PSG from GIBCO #10378-016). The serum shock was done as follows: Approximately $5 \times 10^5$ cells/10 cm petri dish were plated 6–7 days before the experiment. Under these conditions, the cells reach confluence after about 4 days. At time = 0, the medium was exchanged with serum-rich medium (DMEM + PSG, supplemented with 50% horse serum [GIBCO]), and after 2 hr this medium was replaced with serum-free DMEM + PSG. At the indicated times, the petri dishes were washed twice with ice-cold PBS, frozen on a layer of liquid nitrogen, and kept at −70°C until the extraction of whole-cell RNA (Chomczynski and Sacchi, 1987). When indicated, cycloheximide (10 μM, final concentration) was added to the medium 1 hr before the serum shock. At time = 0, the medium was exchanged with serum-rich medium containing cycloheximide at 10 μM. In the experiments shown in Figures 1 and 6, the serum-rich medium was kept for the entire experiment, unless otherwise indicated.

### Cloning of the *rper* Probes

Whole-cell RNA from rat-1 cells was reverse-transcribed into cDNA using random hexamers. Fragments of *rper1* (position 653–801 of *mper1*) and *rper2* (position 165–297 of *mper2*) were PCR-amplified using the following oligonucleotides: 5′-GGGAGCTCAAACTTCGAC TGCCACCAGAGC-3′ and 5′-CAGAGCTCACCCTCCTCCAGACTCC-3′ for *per1*, and 5′-CTCCCCAAGTCCCACCAGTC-3′ and 5′-CGTCCCG TGGAGCAGTTCTC-3′ for *per2*. These fragments were then cloned into Bluescript and sequenced to verify their identity and orientation.

### RNase Protection Experiments

The ribonuclease protection assays were performed exactly as described previously (Schmidt and Schibler, 1995). The *rper1* and *rper2* probes were produced from the two fragments described above. The *dbp* and *tef* probes are complementary to rat *dbp* mRNA (+1126 to +1221) and rat *tef* mRNA (+598 to +693) (Fonjallaz et al., 1996). The *tbp* probe has been described (Schmidt and Schibler, 1995) and is complementary to mouse *tbp* mRNA (+36 to +135). The *rev-erbα* probe encompasses the region from +1313 to +1484 of the rat mRNA, and the *rorα* probe spans the region from +1 to +412 of the mouse mRNA (F. D., L. Lopez-Molina, N. Preitner, and U. S., unpublished data). The β-actin probe is complementary to mouse β-*actin* mRNA (+630 to +812). The c-*fos* probe is complementary to the rat c-*fos* mRNA (+287 to +437). In all cases, the plasmids were linearized with a suitable restriction enzyme, and antisense RNA probes were prepared by in vitro transcription of the linearized templates with T7 or T3 RNA polymerase using $^{32}$P-labeled UTP. Autoradiography was performed with an intensifying screen (FUJI) at −70°C for 1–3 days, unless indicated otherwise. Signals were quantified using a Bio-Rad phosphorimager (GS-363). The data were analyzed using Molecular Analyst software version 1.2 (Bio-Rad).

### Evaluation of the Period Lengths (τ's)

The radioactive signals obtained in RNase protections for *rev-erbα*, *dbp*, *tef*, *per1*, and *per2* mRNAs were quantified by phosphor imaging and normalized to *tbp* mRNA signals, assuming that these remain constant. We then plotted the data from three independent experiments and measured the time span between the centers of two consecutive peaks. The 23 τ's used for the calculation of the mean value were composed as follows: *rev-erbα* (6), *dbp* (5), *tef* (6), *per1* (2), and *per2* (4).

### Acknowledgments

We thank Erich Nigg and George Fey for rat-1 and H35 cells, respectively. We are indebted to Steve Brown and Juergen Ripperger for their critical comments on the manuscript, to Nicolas Roggli for preparing the illustrations, and to Olivier Schaad for his help with computers and software. This work was supported by the Swiss National Science Foundation and the State of Geneva.

Received April 3, 1998; revised May 4, 1998.

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