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

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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 a circadian fashion and their products oscillate in many different neuronal and nonneuronal tissues. 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 nonneuronal 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
after an initial serum shock. The replaced after the first 2 hr with serum-free medium might also contain a circadian clock. In this paper, we before treatment of the cells with serum-rich medium.

Since the down-regulation of dbp suggests that cells of peripheral mammalian tissues levels of dbp daily levels about 4 hr earlier than the existence of upstream regulators governing circadian expression of its own gene, suggesting the ubiquitous expression of clock-related genes mper1, mper2, and clock is their ubiquitous spatial temporal pattern. 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 (Fonjalaz 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). 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.

**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 (Fonjalaz 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...
Circadian Gene Expression In Vitro

931

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 x 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, dbp, and rper2 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.

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 tef and actin mRNAs and rora 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.

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
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 rper1 and rper2 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 mRNAs 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 ror 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 of the SCN of some immediate-early genes, such as the ones encoding the AP-1 transcription factors c-Fos, FosB, and J unB, the Zinc-finger proteins NGFI-A/Zif268 and Egr-3, and the orphan receptor NGFI-B/Nur77 (Morris
These genes have previously been identified as immediate-early genes in serum-stimulated cells, hinting toward similar signal transduction pathways operating 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., 1997).

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

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 × 10⁵ 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.
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 length (22.5 hr) observed for serum-induced mRNA accumulation. 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 β-D arabinofuranoside or Ara-C, a potent inhibitor of DNA replication, did not influence serum-induced cyclic gene expression (data not shown).

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 circadian 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 τ, but with a different phase. Since each RNase protection analysis is performed with RNA from about 10⁶ 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.

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

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.
of diffusible signals, possibly driven by the SCN, may coordinate circadian timing in peripheral tissues. The importance of hormonal 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 immortality vectors differentially superinduce c-fos and c-jun by three distinct factors may involve the same immediate-early genes were linearized with a suitable restriction enzyme, and antisense 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 tef probe has been described (Schmidt and Schibler, 1995) and is complementary to mouse tef mRNA (+36 to +135). The rev-erba probe encompasses the region from +1131 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 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 [32P]-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 (∙’s)
The radioactive signals obtained in RNase protections for rev-erba, dbp, tef, per1, and per2 mRNAs were quantified by phosphor imaging and normalized to tef 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 ∙’s used for the calculation of the mean value were composed as follows: rev-erba (6), dbp (5), tef (6), per1 (2), and per2 (4).

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