Published in final edited form as:

Brain Res. 2003 February 28; 964(2): 279–287.

A short half-life GFP mouse model for analysis of suprachiasmatic nucleus organization

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Abstract

Period1 (*Per1*) is one of several clock genes driving the oscillatory mechanisms that mediate circadian rhythmicity. *Per1* mRNA and protein are highly expressed in the suprachiasmatic nuclei, which contain oscillator cells that drive circadian rhythmicity in physiological and behavioral responses. We examined a transgenic mouse in which degradable green fluorescent protein (GFP) is driven by the *mPer1* gene promoter. This mouse expresses precise free-running rhythms and characteristic light induced phase shifts. GFP protein (reporting *Per1* mRNA) is expressed rhythmically as measured by either fluorescence or immunocytochemistry. In addition the animals show predicted rhythms of *Per1* mRNA, PER1 and PER2 proteins. The localization of GFP overlaps with that of *Per1* mRNA, PER1 and PER2 proteins. Together, these results suggest that GFP reports rhythmic *Per1* expression. A surprising finding is that, at their peak expression time GFP, *Per1* mRNA, PER1 and PER2 proteins are absent or not detectable in a subpopulation of SCN cells located in the core region of the nucleus.

Keywords

Circadian rhythm; Suprachiasmatic nucleus; Period gene; Green fluorescent protein; Transgenic

1. Introduction

Several converging lines of evidence indicate that the *Per1* gene is one of the key participants in the molecular feedback loop that drives SCN neuronal clock oscillations. Transcripts of *Per1* show a robust circadian rhythm in the SCN [20,22,23]. The negative and positive feedback elements which constitute the autofeedback loop of the system have been delineated [15]. Transcription of *Per1* is activated by the binding of the CLOCK–BMAL1 complex to the E-boxes in the promoter region of the *Per1* gene [6]. This activation is inhibited by PER1 protein and other negative elements [16]

Progress in understanding neural organization has been enhanced by our ability to visualize individual neurons and their activity. The development of jellyfish green fluorescent protein

(GFP) as a vital stain has made possible tremendous advances in neuronal imaging [3]. GFP confers a number of advantages over other cellular markers [4]. The pattern of expression is heritable, the cell is labeled in its entirety, the GFP protein can be placed under the control of other promoters and it can be mutated to be unstable, providing a tool for studying dynamic activity. Unlike luciferase, another commonly used reporter, the GFP chromophore is part of the protein and does not require delivery of exogenous substances to express its fluorescence.

GFP, like other transgenic reporters, also presents some disadvantages. While expression is similar among offspring of each transgenic founder, there is substantial variability in pattern of GFP expression among mice generated from the same construct [4]. Transcription factors in different cell types may recognize a transgene differently. Furthermore some chromatin adjacent to the integration site may silence the transgene, a phenomenon termed positive effect variegation. This results in different levels of GFP expression in different cell types [24]. Although the stability of GFP protein has contributed to its wide use as a cellular marker, it also presents some limitations and potential disadvantages. The long half-life of conventional GFP obviates its use in reporting the dynamics of gene promoters and whereas some reports find no toxicity [4,14], other reports indicate that accumulation of stable GFP induces cell damage or death [9,12].

We have been exploring the use of a *Per1::GFP* transgenic mouse, first described in Kuhlman et al. [10], to understand the organization of SCN pacemakers. In this animal, a degradable form of recombinant GFP is driven by the mouse *Per1* gene promoter, the half-life of GFP is about 2.1 h, allowing GFP intensity to report promoter dynamics on a circadian time scale and preventing accumulation of GFP. Previous results with this mouse model indicate that in acute slice preparations of the SCN, GFP fluorescence intensity differs at two time points, mid-day and mid-night, suggesting rhythmicity in GFP expression [10].

The first goal of the present study was to assess the usefulness of the *Per1::GFP* transgenic animal in understanding the organization of the SCN by determining the time course of GFP expression throughout the nucleus. GFP fluorescence was measured for 24 h in an acute SCN slice preparation taken from animals sacrificed at the onset of light in the morning, at zeitgeber time (ZT0). GFP immunoreactivity was measured in animals sacrificed at 4-h intervals in animals housed in light:dark (LD, indexing zeitgeber time, ZT) and in those housed in constant darkness (DD, indexing circadian time, CT), in order to compare GFP expression in vivo and in vitro. Circadian rhythms of GFP, *Per1* mRNA, PER1 and PER2 proteins were also determined. A surprising finding was that GFP was absent or not detected in a distinct region of the central SCN. Hence, the second goal was to examine the location of GFP, *Per1* mRNA, PER1 and PER2-immunoreactivity within the SCN. Our findings indicate that like GFP, *Per1* mRNA, PER1 and PER2 are absent in this region.

2. Materials and methods

2.1. Animals and housing

The *mPer1::d2EGFP* transgenic mouse was made with the B6C3F1 hybrid mouse as described in Kuhlman et al. [10]. The mice used in these studies were hemizygous for the d2EGFP transgene, which is an unstable form of GFP (we use hemizygotes in all our studies. In the few homozygotes we have processed, we do not detect any obvious difference in the amount of GFP expression). At about 3 months of age, animals were housed individually in translucent propylene cages (11.5×7.5×5 inches), in either a light–dark cycle (LD 12:12, *n*=33) or in constant darkness (DD, *n*=20) for 2 days (anatomical studies), or for 4–6 weeks (behavioral studies). The room was maintained at 21±1 °C. For animals in DD, a

white noise generator (91 spl) masked environmental noise and a dim red light (0.5–1 lux) allowed for animal maintenance. All animals were provided with food and water ad libitum and cared for in accordance with IACUC Animal Welfare regulations at Columbia University and University of Kentucky.

2.2. Measurement of circadian locomotor activity

For behavioral studies in constant darkness (DD) cages (35×21×18 cm) were equipped with a running wheel (diameter 12.7 cm). Locomotor activity was monitored continuously using a computer-based data acquisition system (Dataquest, Data Sciences, St. Paul, MN, USA). Analysis of rhythm amplitude, period, and profile was determined by using the Clocklab program in Matlab 5.3.1 from Mathworks (Natick, MA, USA).

For the phase shift studies, animals were housed individually in constant darkness in cages equipped with running wheels. Wheel running activity was monitored using Chronobiology Kit (Stanford) software. After reaching stable free-running conditions, mice were subjected to a 30-min light pulse (about 800 lux) at a given phase. Animals' free-running period (Tau) was calculated before and after light treatment (starting on the second cycle of activity onset following light treatment), using 6–10 cycles. The phase shift in response to light treatment was calculated as the difference in projected phase of activity onset prior to light treatment and phase of activity onset after light treatment. Phase of activity onset was determined using Chronobiology Kit by aligning a straight line to time of activity onset on a raster plot of activity (wheel revolutions per minute were binned at 2-min intervals) by eye, the slope of the line was set to Tau as calculated above. In some cases Tau varied slightly before and after light treatment (there was not a consistent direction), the two phase shift values were determined and values were averaged (for a given animal, the difference was less than 20 min). Animals were housed in a light-tight box, given animal to animal differences in period of free-running activity, animals received light treatment at different phases when they had reached a stable free-running period between treatments.

2.3. Conventional time-lapse imaging of SCN slices

Animals were housed in a 12-h light–dark cycle. Hypothalamic slices, 300 μ m in thickness, containing the SCN were taken from animals 3–5 weeks old and placed in a 35 °C heated either open or closed chamber (Warner Instruments) and perfused with Earle's balanced salts solution (Sigma) that had been supplemented with 2.2 g/l NaHCO₃, 0.8 g/l glucose, and 50 mg/l gentamicin. The solution was bubbled with 95% O₂/5% CO₂. Images of the SCN were captured every 30 min with the use of a narrow band GFP filter set (Chroma, Brattleboro, VT, USA) on either an upright microscope equipped with an Axon Imaging Workbench- (Axon, Foster City, CA, USA) controlled intensified CCD camera (Atto Instruments, Rockville, MD, USA) or an inverted microscope using IP Lab (Scanalytics, Fairfax, VA, USA) to control a cooled CCD camera (Roper Scientific, Trenton, NJ, USA). Using Axon Imaging Workbench or IP Lab, GFP fluorescence values were measured by delimiting areas inside and outside of the SCN using a region of interest tool and subtracting the non-SCN value from the SCN area value at each time point.

2.4. Perfusion and immunocytochemistry

For immunocytochemistry, hemizygous mice housed in LD were sacrificed at ZT 2, 6, 10, 14, 18 and 22, those in DD were sacrificed at the projected CT 2, 6, 8, 10, 14, 18 and 22. Animals sacrificed in the dark were deeply anesthetized (pentobarbital: 200 mg/kg) under the dim red light illumination and their heads were completely covered with a light proof hood to prevent light from reaching the eyes. Mice were perfused intracardially with 50 ml 0.9% saline followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. Brains were post-fixed for 18–24 h at 4 °C, cryoprotected in 20% sucrose in 0.1 M

phosphate buffer overnight. The 40- μ m sections were processed free-floating. For single label immunocytochemistry, alternate or two of three sections were incubated with either rabbit polyclonal GFP (1:40 000, Molecular Probes), PER1 (1:5000, generous gift from Drs S.M. Reppert and D.R. Weaver, University of Massachusetts, MA, USA), or PER2 (1:5000, Alpha Diagnostic), or GFP, PER1 and *Per1* mRNA (in situ procedure below) and processed with a modified avidin–biotin–immunoperoxidase technique [11] using DAB as the chromogen. For double label immunocytochemistry, alternate sections were incubated in PER1 antibody, used at 1:50 000, amplified with biotinylated tyramide and stained with Cy3 (Jackson ImmunoResearch, West Grove, PA, USA). Sections were then incubated with the GFP antibody which was then labeled with Cy2. The sections were coverslipped with permount for DAB or Krystalon (EM Science, Gibbstown, NJ, USA) for Cy2–Cy3. In each immunocytochemistry run, sections from each time point were processed together to minimize variability attributable to handling conditions.

2.5. In situ hybridization

The *mPer1* cDNA fragment-containing vectors (the gift of Dr H. Okamura, Kobe University, Japan) were linearized with restriction enzymes and then used as templates for sense or anti-sense complementary RNA (cRNA) probes. Digoxigenin (Dig)-labeled probe for *mPer1* was made using Dig-UTP (Boehringer Mannheim, Mannheim, Germany) with a standard protocol for cRNA synthesis [23]. Sense *mPer1* cRNA probe revealed no specific hybridization signals in brain sections.

Every third coronal section (40 μ m) was processed simultaneously for in situ hybridization histochemistry. Tissue sections were processed with proteinase K (1 mg/ml, 0.1 M Tris buffer pH 8.0; 50 mM EDTA; 10 min) at 37 °C and 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The sections were then incubated in hybridization buffer [60% formide, 10% dextran sulfate, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.6 M NaCl, 0.2% *N*-laurylsarcosine, 500 mg/ml, 200 mg/ml tRNA, 1 $\times$ Denhardt's, 0.25% SDS and 10 mM dithiothreitol (DTT)] containing the Dig-labeled *mPer1* anti-sense cRNA probes for 16 h at 60 °C. After a high-stringency post-hybridization wash, sections were treated with RNase A, and then were further processed for immunodetection with a nucleic acid detection kit (Boehringer Mannheim). Sections were incubated in a solution containing nitroblue tetrazolium salt (0.34 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (0.18 mg/ml) (Boehringer Mannheim) for 16 h. The colorimetric reaction was stopped by immersing the sections in buffer 4 (10 mM Tris–HCl containing 1 mM EDTA, pH 8.0).

2.6. Immunocytochemical data analysis

Images of brain sections were captured using a CCD video camera (Sony XC77) attached to a light microscope (Olympus BH-2) using NIH Image (1.61). Three to five sections (depending whether alternate or every third sections were labeled, respectively), including rostral, mid, and caudal regions of the SCN, were analyzed per animal. Using the NIH Image program, measurements of relative optical density (ROD) were used to quantify the intensity of the *Per1* mRNA, GFP, PER1 and PER2 signals in the SCN. Non-specific staining (background) outside the SCN was subtracted from staining within the SCN. The staining for each section was expressed as a percentage of the maximum staining in that ICC run, and the staining for each animal was expressed as the average of its section percentages. One-way ANOVA was used to test for time of day effects.

3. Results

3.1. GFP transgenic mouse expresses precise circadian rhythms

As can be seen in Fig. 1 the circadian rhythms of the *Per1::GFP* mice are precise and have a high amplitude, with an average free-running period of 23.06 ± 0.11 h ($n=11$). The wheel running activity profile shows most of the running occurring at the beginning of subjective night, and a smaller bout at the end of subjective night. The phase response curve resembles that of normal mice with phase advances and delays to light pulses presented in late or early subjective night, and no phase shifting during mid-subjective day.

3.2. GFP rhythms in vitro

Hypothalamic slices containing the SCN were taken between ZT0.25 and ZT1, and time-lapse imaging of the *Per1*-driven GFP fluorescence in the SCN was monitored for 24 h. The in vitro GFP relative fluorescence intensity in the SCN rises during the subjective day, exhibits a broad peak after mid-day (around ZT 7.0 based on the waveform average, $n=4$), and falls during the subjective night (Fig. 2A). Fig. 2B illustrates, for one recording, the change in GFP fluorescence in the SCN compared to background, among images captured at ZT1.5, ZT7.5, and ZT15.5. Early in the day, the SCN has detectable levels of GFP fluorescence that become more intense during the course of the day as the fluorescence peaks in the middle of the subjective day. Following the peak, GFP fluorescence in the SCN decreases during the subjective night.

3.3. GFP rhythm in vivo

In tissue taken from animals housed in LD, a circadian rhythm in intensity of GFP-ir staining was seen (ANOVA for LD, $F(5)=4.8$, $P=0.004$), with a peak around ZT 10 and a trough at ZT 22. With the 4-h sampling interval, the timing of the GFP-ir rhythm is similar to that of the fluorescence rhythm described in Fig. 2.

Fig. 3 shows rhythmic expression of *Per1* mRNA, GFP, and PER1 protein from the same animals and PER2-ir in a different group, in animals held in DD. The left panel shows quantification of the data, and the right panels, photomicrographs depicting distinct high and low points of immunoreactivity.

In animals kept in DD for 2 days, circadian rhythms are seen in *Per1* mRNA ($F(6)=41.2$, $P=0.0001$), GFP ($F(6)=22$, $P=0.0003$), and PER1 protein ($F(6)=23.5$, $P=0.0003$). *Per1* mRNA appears to peak at around CT6–8, GFP and PER1 at about ZT10 (all quantified in serial sections from the same animals). PER2-ir measured in a different group of animals also shows a circadian rhythm ($F(5)=15.3$, $P=0.002$) peaking around CT 14 and with a trough at CT 02. There is a small population of residual *Per1* mRNA cells seen at CT 18 (see Fig. 3), similar to that previously reported for PER1 protein by Field et al. [5]. In some animals depending on the precise plane of section, GFP, PER1 and PER2 proteins are also seen in this region.

3.4. Distribution and localization of GFP *Per1* mRNA, PER1 and PER2

Double-label ICC for GFP and PER1 at the time of high expression reveals that both proteins have the same regional distribution within the SCN, and are co-localized in many of the same cells. The distribution of *Per1* mRNA and PER2-ir at their peak expression times is also similar. Fig. 4 shows the regional distribution of GFP, PER1, *Per1* mRNA and PER2-ir in five levels of the SCN, from rostral to caudal. All three proteins and *Per1* mRNA expression are seen throughout the rostral SCN (levels 1 and 2). An interesting finding is that neither are expressed throughout the SCN. At the 3rd level, they are absent in a small 'hole' in the middle of the SCN. At the 4th level, most cells are concentrated within the

medial SCN, the lateral SCN is almost devoid of *Per1* mRNA GFP, PER1 or PER2-ir. We also noted that not all GFP and PER1-ir cells are double-labeled. Some cells contain GFP alone, and some cells contain PER1 alone.

Fig. 5 shows GFP immunoreactivity in the caudal SCN at the time of peak expression, CT 10. Double-labeled staining for GFP and the nuclear stain bis-benzimide shows that the region devoid of GFP is a cell-rich zone of the SCN.

4. Discussion

The *Per1::GFP* transgenic mouse free-runs in constant conditions with a with very precise onset times (Fig. 1A), a high amplitude rhythm (Fig. 1B) and an activity profile similar to the B6C3F1 strain [21]. Its phase advances and delay in response to light pulses are also similar to that reported for one of its parent strain C57BL/6 mice [17]. This indicates that the *Per1::GFP* transgene does not adversely affect circadian behavior, and that there are no toxic effects of the transgene.

4.1. GFP expression is rhythmic

The rhythm in GFP expression in the SCN, reflecting *Per1* promoter activity, is similar in animals taken from LD or DD conditions and shows a peak around ZT and CT 7–10 and a trough around ZT and CT 22–24. The rhythm is similar in vitro, in cultured slices taken from animals all sacrificed at ZT0–1 (Fig. 2) and in vivo, in animals sacrificed at different time points (Fig. 3). Dynamics of *Per1* gene expression tested in the same animals in DD show that *Per1* mRNA expression peaks first, then, the increase in GFP expression precedes that of PER1 protein, although peak expression time is similar for both.

In summary, the data on intensity of the GFP signal, whether measured by fluorescence or by immunocytochemistry (ICC) indicates a true circadian rhythm of GFP expression. The results establish the usefulness of the *Per1::GFP* transgenic mouse as a tool to analyze circadian gene dynamics in the SCN. The *mPer1* promoter sequences driving the short half-life GFP transgene contain three E-box elements and a CRE, and our data indicate that the dynamics of the reporter faithfully reflect the influence of transcription factors on these elements. The rhythmic patterns of *Period* genes expression in the transgenic *Per1::GFP* mouse is similar to what has been previously reported in other mice [5,18,25].

4.2. GFP distribution is topographically organized

As can be seen in Fig. 4, the overall distribution of *Per1* mRNA, PER1, PER2 and GFP is similar from the rostral to the caudal aspect of the SCN: most cells colocalize PER1 and GFP, although with different intensities of staining for each protein. The occurrence of some single-labeled GFP and single-labeled PER1 cells is difficult to interpret. Possibly, these cells really don't express both proteins. Alternatively, one of the proteins may be undetectable, as suggested by different staining intensities among cells for each protein. GFP and PER 1 proteins likely have a different time course of expression, with GFP accumulating faster than PER1. Another possible explanation is that, depending on the geometry of sectioning, single staining profiles may be observed since PER1 is a nuclear antigen, whereas GFP is in both compartments.

A surprising finding in this study was the absence of *Per1*-driven *Per1* mRNA, GFP, PER1 and PER2 expression in some regions of the SCN. Specifically, cells of the mid-ventrolateral and caudo-lateral SCN (Fig. 4, 3rd and 4th level, respectively) do not express these mRNAs or proteins at any ZT or CT time examined. Nevertheless, this region is a neuron-rich zone of the SCN (Fig. 5). Previous work suggested that at its peak, *mPer1* is expressed in all parts of the SCN, although an area devoid of *Per1* expression would be

difficult to detect with the autoradiographic methods used [19]. Furthermore most studies of mouse SCN, using ICC or digoxigenin labeling of PER proteins or *Per* mRNA, respectively, focus largely on the rostral to mid region of the nucleus (approximately levels 2–3 in Fig. 4). Importantly, at their peak *Per1* mRNA, PER1 and PER2 proteins are not detected in parts of the mid to caudal SCN. At times of lowest expression, a few residual *Per1* mRNA, PER1, PER2 and GFP labeled cells are seen in this region. These neurons account for a small fraction of the cells labeled with bis-benzimide (Fig. 5). Consistent with our observation, the occurrence of PER1 and PER2 protein has previously been reported in this zone [5,8]. It is not known whether these cells are rhythmic, in antiphase with the larger population, or whether they constitutively express *Per* genes.

It has been suggested that the organization of the mouse SCN differs from that of the rat and hamster in that light induced c-Fos is distributed extensively throughout the full extent of the SCN [2]. Other studies show that the mouse SCN can be delineated into a core and a shell according to its chemoarchitecture, and that the RHT terminates bilaterally over much of the SCN core [1]. Hastings et al. [8] showed that in the mouse SCN, rhythmic PER1 expression occurs mostly in vasopressin neurons, while the residual *Per1* seen at ZT0–2 is not in vasopressin cells. The present results support the notion of a universal organization in the mammalian SCN. We have previously shown a highly topographical and compartmentalized expression of *Per1*, *Per2*, and *Per3* mRNAs in the hamster SCN [7]. The rostral and dorsomedial vasopressin-rich regions are characterized by rhythmic expression of *Per1* and *Per2* mRNA. In contrast, in the caudal region marked by calbindin-immunoreactive cells, rhythmic expression is not detected. In the rat SCN, where clear markers for distinct SCN zones are not available, rhythms of *Per1* and *Per2* mRNA are strong in the dorsomedial and weak in the ventrolateral SCN [23], and the percentage of neurons showing rhythmic firing rate is much larger in the dorsomedial region than in the ventrolateral region [13].

In summary, the present results reveal that the molecular organization of clock genes in the mouse SCN is similar to that of the hamster, with a specific zone of rhythmic expression of clock genes. The mouse is becoming increasingly important for the behavioral and molecular analyses of the circadian system. The present results indicate that the degradable GFP construct in the *Per1::GFP* transgenic mouse is an excellent tool for this analysis.

Acknowledgments

We are grateful to Dr Michael Lehman for his advice on this manuscript. This study was supported by NIH Grants NS-37919 to RS, MH63341 and EY09256 to DGM.

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Fig. 1.

(A) Locomotor activity of a *Per1::GFP* transgenic mouse in DD shows a very strong and precise rhythm. To facilitate inspection of the rhythms, the daily activity is plotted twice on a 48-h horizontal time scale (labeled 0–48 h at the top). In this animal, light pulses (white stars) presented at the end and at the beginning of the subjective night induced a phase advance of 1.95 circadian hours and a phase delay of 0.93 circadian hour. (B) The periodogram taken from day 1 to day 15 (11–14 to 11–28 included) depicts a strong amplitude rhythm with a period of 22.9 h, the line near the bottom of the plot indicates the amplitude significance level, $P=0.01$. (C) The phase response curve (PRC) to a 30-min light pulse given at specific time points (x-axis) shows phase delays and advances at the beginning and end of night, and very little effect during the middle of the day. Seven animals represented by different symbols, each received several light pulses at different times. (D) Mean wheel running activity profile for 10 *Per1::GFP* mice. The mice are not active during subjective day. Most of the wheel running occurs at the beginning of the subjective night, with a secondary peak at the end of subjective night.

Fig. 2.

(A) *Per1*-driven GFP fluorescence cycles in the in vitro SCN. The heavy line shows the average of normalized fluorescence from four individual recordings, depicted by the differing symbols (○ ▲ ☆ -), as it changes during the course of one cycle. The gray line indicates the background staining of the outside of the SCN (right y-axis). (B) An image captured at ZT1.5 shows the low level of fluorescence intensity in the SCN at the beginning of the subjective day. By ZT7.5, the fluorescence intensity peaks and then begins to decline to a lower fluorescence intensity level in the SCN while the autofluorescence outside of the SCN begins to rise as demonstrated in the image captured at ZT15.5. OC: optic chiasm; 3V: third ventricle. Scale bar: 100 μ m.

Fig. 3. *Per1* mRNA, GFP, PER1 and PER2 are rhythmic in the SCN. Left: bar graph depicting the rhythmic circadian pattern of *Per1* mRNA, GFP, PER1 (in the same animals) and PER2 (in a different group) immunoreactivity in the SCN in DD. Right: photomicrographs showing the difference in levels of GFP immunoreactivity at two times in DD.

Fig. 4.

Photomicrographs showing the regional distribution of *Per1* mRNA (CT6), GFP, PER1 (CT10) and PER2-ir (CT14) at five levels of the SCN from rostral to caudal. In the first column, the sections were stained with digoxigenin. The next three columns show the same double-labeled sections. In the second column, the sections were photographed with a GFP filter (Chroma, # 41020 HQ). In the third column they were photographed with a Texas red filter (Chroma, # 96109 C). The fourth column is an overlay of the first two columns showing the double-labeled cells. The fourth section shows PER2 labeled with Cy3. The distribution of all proteins and mRNA overlaps. Note the paucity of immunoreactivity in the central and lateral SCN at the 3rd and 4th levels (arrows). The bottom row is a high power view of GFP and PER1 in the medial SCN (white box on column 4, level 3). Most cells are double labeled, although with different intensities for each protein. A few cells seem to contain either GFP or PER1 alone (white arrows). This may be due to a difference in the timing of accumulation of both proteins (see Section 4). * Indicates a staining artifact (level 2).

Fig. 5. Photomicrographs showing the regional distribution of GFP in the mid to caudal SCN (levels 3 and 4 in Fig. 4) at the time of peak expression, CT 10. Double-staining for bis-benzimide shows that the regions devoid of GFP is a cell-rich zone of the SCN.