Aging Affects the Distribution of the Circadian CLOCK Protein in Rat Hepatocytes

MANUELA MALATESTA,1,2 BEATRICE BALDELLI,1 SERAFINA BATTISTELLI,1 PATRIZIA FATTORETTI,2 AND CARLO BERTONI-FREDDARI2

1Istituto di Istologia ed Analisi di Laboratorio, University of Urbino “Carlo Bo,” Urbino, Italy
2INRCA, Ancona, Italy

KEY WORDS aging; cell nucleus; circadian rhythm; electron microscopy; liver; transcription factor

ABSTRACT Several biochemical, physiological, and behavioral processes exhibit cyclic oscillations of about 24 h, which have been defined as circadian rhythms. In mammals, the primary circadian pacemaker resides in the suprachiasmatic nuclei; however, cell-autonomous circadian oscillators occur also in extraneural tissues, including the liver. CLOCK protein is a transcription factor essential for normal circadian rhythms and recent studies have demonstrated that it undergoes intranuclear redistribution in hepatocytes, along the daily cycle. It is known that aging leads to a progressive deterioration of the circadian rhythm at the behavioral, physiological, and cellular levels; in addition, aging affects the organization of nuclear structural components involved in transcription and splicing. In this view, we carried out ultrastructural immunocytochemical analyses on hepatocytes of adult and old rats, so as to investigate possible qualitative and quantitative modifications of CLOCK protein, in relation to the aging process. Our observations demonstrated that most CLOCK protein was always located in the cell nucleus, where it accumulated on perichromatin fibrils (the sites of premRNA transcription and early splicing); in addition, CLOCK showed daily oscillations in the different nuclear compartments, but these oscillations differed significantly between adult and old animals. This unusual distribution of CLOCK protein during aging could be related to the prolonged diurnal activity of old animals and/or to altered nuclear pathways. Microsc. Res. Tech. 68:45–50, 2005. © 2005 Wiley-Liss, Inc.

INTRODUCTION

Several biochemical, physiological, and behavioral processes exhibit cyclic oscillations of about 24 h, which have been defined as circadian rhythms. In mammals, the primary circadian pacemaker resides in the suprachiasmatic nuclei (SCN), in the ventral part of the hypothalamus (Klein et al., 1991); however, extraneural tissues also contain their own cell-autonomous circadian oscillators, although with a phase delay with respect to the SCN rhythm (reviewed by Morse and Sassone-Corsi, 2002).

The oscillatory mechanism of the mammalian circadian clock depends on the protein products of several genes—e.g., period (per) 1, 2, and 3, cryptochrome (cry) 1 and 2, bmal1, clock—and on other molecular components involved in transcriptional regulation as well as in posttranscriptional and posttranslational modifications (reviewed by Dunlap, 1999; Harms et al., 2004; Reppert and Weaver, 2001). The clock gene is essential for normal circadian rhythms (Vitaterna et al., 1994) and CLOCK is a protein with a basic helix–loop–helix DNA-binding domain and a glutamine-rich transcription activation region (King et al., 1997), which are typical of the bHLH-PAS family of transcription factors.

The basic mechanism of the circadian clock, which is largely conserved during the evolution, relies on interlocking positive and negative transcriptional/translational feedback loops (reviewed by Ishida et al., 2001; Reppert and Weaver, 2001). In mammals, CLOCK and BMAL1 form a protein complex that activates the transcription of per and cry genes; as soon as PER and CRY proteins are translated, they enter the nucleus, where CRY acts as negative regulator by directly interacting with CLOCK and/or BMAL1 to inhibit transcription, while PER2 contributes to the rhythmic transcription of bmal1. The increased availability of BMAL1 presumably promotes heterodimerization of CLOCK and BMAL1, which is necessary for per and cry to be transcribed. The positive-feedback loop thereby promotes the negative-feedback loop, thus perpetuating the cycle (Reppert and Weaver, 2001). This fundamental oscillation elicits (through secondary pathways) additional cyclic events in other cellular targets.

In mammals, clock is constitutively expressed not only in the SCN but also in several peripheral tissues, including the liver (Bjarnason et al., 2001; King et al., 1997; Sun et al., 1997; Tei et al., 1997). Recent studies have demonstrated that CLOCK protein undergoes intranuclear redistribution in rodent hepatocytes, following both a daily cycle (Malatesta et al., 2003a) and a circannual cycle of euthermia-hibernation- arousal (Malatesta et al., 2003b). Moreover, it has been
recently reported (Davidson et al., 2004) that a circadian rhythm also exists in liver functions, which is not light- or CNS-dependent, and it is more likely needed to better regulate digestion and energy usage, in relation to the mealtime.

In the present study, ultrastructural immunocytochemical analyses have been carried out on hepatocytes from adult and old rats, so as to elucidate whether qualitative or quantitative modifications of CLOCK protein occur, in relation to the aging process. In fact, during aging, a progressive deterioration of the circadian rhythms is known to occur at different levels (behavioral, physiological, and cellular) (Asai et al., 2001; Aujard et al., 2001; Kolker et al., 2003, 2004). In addition, in rat hepatocytes, aging has been shown to affect the organization of nuclear structural components involved in transcription and splicing (Malatesta et al., 2003c, 2004).

**MATERIALS AND METHODS**

Twenty-four female Wistar rats from the INRCA breed (Ancona, Italy) were used. Twelve 9-month-aged (adult) and twelve 28-month-aged (old) animals received a standard diet ad libitum (Harlan Italy, Correzzano, Italy). The animals were exposed to a 12 h light-dark cycle. Eight rats (four from each group) were killed in the morning, at ZT4 (ZT, zeitgeber time; ZT0 = light on, ZT12 = light off), eight in the afternoon, at ZT12, and eight in the night, at ZT 22, after anesthetizing with an intraperitoneal injection of 2.2.2-tribromoethanol (20 mg/100 g body weight). To avoid phase shifts, at ZT22 the animals were anesthetized under red light.

For immuno-electron microscopical analyses, samples of liver were quickly removed and small fragments were fixed by immersion in 4% paraformaldehyde in 0.1 M Sörensen phosphate buffer at 4°C for 2 h. After washing in Sörensen buffer and in phosphate buffered saline (PBS), free aldehydes were blocked in 0.5 M NH4Cl in PBS at 4°C for 45 min. Following washing in PBS, the specimens were dehydrated through graded concentrations of ethanol and embedded in LRWhite resin. Ultrathin sections were placed on nickel grids coated with a Formvar-carbon layer and then processed for immunocytochemistry by using an antiserum antibody to the CLOCK protein (Alpha Diagnostic International, Inc.). Sections were floated for 3 min on normal goat serum (NGS) diluted 1:100 in PBS and then incubated for 17 h at 4°C with the primary antibody diluted 1:2 with PBS containing 0.1% bovine serum albumin (Fluka) and 0.05% Tween 20. After rinsing, sections were floated on NGS, and then reacted for 20 min at room temperature with the secondary 12-nm gold-conjugated antibody (Jackson ImmunoResearch) diluted 1:10 in PBS. Finally, the sections were rinsed and air dried. As controls, some grids were treated with the incubation mixture without the primary antibody, and then processed as described earlier. To clearly identify the nuclear structural constituents that contain ribonucleoproteins (RNPs), all immunolabeled sections were stained with the EDTA method (Bernhard, 1969). The specimens were observed in a Zeiss EM 902 electron microscope operating at 80 kV.

To assess the presence of the CLOCK protein quantitatively, the labeling density over some cellular compartments was evaluated on sections treated in the same immunolabeling experiment. The surface area of each compartment evaluated contained condensed chromatin, interchromatin granules (IG), interchromatin space devoid of IG (a nucleoplasmic region particularly rich in perichromatin fibrils (PF)), nucleoli, and cytoplasm, and was measured on 40 randomly selected electron micrographs (30,000×) from each animal group by using a computerized image analysis system (Image Pro-Plus for Windows 98). The percentage of nuclear area occupied by condensed chromatin was also calculated. For background evaluation, the resin outside the tissue was considered. The gold grains present over the investigated compartments were counted, and the labeling density was expressed as the number of gold grains per square micrometer. Statistical comparisons were performed by the one-way ANOVA test to evaluate the factor “time” for each cellular compartment considered, while the two-way ANOVA test was applied to evaluate the factors “age” and “cellular compartment” as well as the interaction term between the two factors. Statistical significance was set at *P* ≤ 0.05.

**RESULTS**

Electron microscopic analysis of immunolabeled rat hepatocytes revealed that the CLOCK protein generally showed a similar intracellular distribution in all animals. In detail, the labeling was concentrated in the nucleolus, where it was mostly located on the PF, while the condensed chromatin showed a weak signal, especially close to the perichromatin region (Fig. 1a). The perichromatin granules (PG) and the IG were almost devoid of gold grains (Figs. 1a and 1c). The nucleolus was significantly labeled on its dense fibrillar component (DFC) (Fig. 2). In the cytoplasm, the labeling was mostly located over the rough endoplasmic reticulum (Fig. 1b).

The morphometrical evaluation of the percentage of nuclear area occupied by condensed chromatin revealed similar values in adult and old rats (16.12 ± 0.74 vs. 17.05 ± 0.96). The quantitative evaluation of the intracellular distribution of CLOCK protein (Fig. 3) confirmed the aforementioned observations: in all animals the highest labeling was found in the interchromatin space devoid of IG (a nuclear region particularly rich in PF), followed by the nucleolus, the cytoplasm, and the condensed chromatin. As for the IG, in adult animals, they showed labeling densities not significantly different from that of background evaluated over the resin, whereas in old animals, the signal over IG was significantly higher than that of the background at ZT4 and ZT12.

Significant differences in the daily distribution of antiCLOCK immunolabeling were found between adult and old rats. In adult rats, the antiCLOCK labeling significantly peaked at ZT4 in the interchromatin space devoid of IG, nucleolus, and cytoplasm (condensed chromatin and IG values did not change during the daily cycle) (Table 1). Conversely, in old rats, the labeling densities of all cellular compartments were high at ZT4 and ZT12 and drastically decreased at ZT22 (Table 1). Moreover, the labeling densities over all cellular compartments obtained at ZT4 and ZT12 in old rats were significantly higher than...
the respective ones found in adult rats, whereas no difference was found between the two animal groups at ZT22 (Table 2).

Quantitative evaluation of background labeling on resin showed that it was negligible (0.06 ± 0.02 grains/μm²) and significantly lower than the labeling levels found in the cellular compartments considered (with the exception of IG in adult rats in all daily phases and in old rats at ZT22).

**DISCUSSION**

Our observations on hepatocytes from adult and old rats in different phases of the daily cycle demonstrated that: (a) most CLOCK protein is always located in the cell nucleus, where it accumulates on PF; (b) CLOCK protein shows daily oscillations in the different nuclear compartments; and (c) these oscillations differ between adult and old animals.

CLOCK is a bHLH-PAS transcription factor, which heterodimerizes with BMAL1 to enhance transcription through E box elements (Reppert and Weaver, 2001). Accordingly, CLOCK protein is preferentially associated with PF, which represent the structural counterpart of transcription and early splicing of premRNA (Fakan, 1994), and occurs—although in lower amounts—also in the nucleolar DFC, where transcription and early splicing of rRNA take place (Biggiogera et al., 2001; Cmarko et al., 2000; Fakan and Puvion, 1980; Jimenez-Garcia et al., 1993). On the other hand, CLOCK protein is absent from the PG, storage and/or transport sites of already-spliced mRNA or premRNA (Bauren and Wieslander, 1994; Fakan et al., 1984; Vazquez-Nin et al., 1990) and, generally, from the IG.
which is involved in presplicing complex storage and/or assembly (reviewed by Puvion and Puvion-Dutilleul, 1996; Spector, 1996). The presence of some CLOCK protein over the condensed chromatin could indicate a binding to nontranscribing DNA; however, the absence of a clear-cut borderline between condensed chromatin and perichromatin region could induce to include hardly recognizable PF in the chromatin area. This intranuclear distribution is quite similar to that previously observed in mouse hepatocytes (Malatesta et al., 2003a) and confirms that CLOCK protein usually does not accumulate in nuclear constituents that are different or distant from its functional sites. Finally, the antiCLOCK signal occurring in the cytoplasm probably reveals synthesized molecules before entering the nucleus to play their functional role.

It should be underlined that the antiCLOCK labeling, although highly specific, was quite weak in all the cellular compartments considered. This could indicate that a low amount of CLOCK molecules are able to control the intracellular circadian rhythms, probably by just binding to the site of RNA synthesis, thereby initiating a cascade of events.

Fig. 2. Detail of a hepatocyte nucleus from an old rat at ZT12; immunolabeling with antiCLOCK antibody. In the nucleolus, CLOCK protein is mainly located on the DFC (arrows). Scale bar = 0.25 μm.

Fig. 3. The histograms show the mean values ± standard error (SE) of antiCLOCK labeling densities (gold grains/μm²) over various cellular compartments of adult and old rats in different phases of the circadian cycle.
pression can be modulated by the cellular redox state (Kolker et al., 2003, 2004) and that aging is accompanied by a circadian clock (Davidson et al., 2004; Kornmann et al., 2001; Villeponteau, 1997). As for the accumulation of CLOCK protein in IG, where transcription and splicing factors are usually stored and/or assembled (Puvion and Puvion-Dutilleul, 1996; Spector, 1996), it suggested by various authors (reviewed by Imai and Kitano, 1998; Villeponteau, 1997). As for the accumulation of CLOCK protein in IG, where transcription and splicing factors are usually stored and/or assembled (Puvion and Puvion-Dutilleul, 1996; Spector, 1996), it could indicate altered intranuclear transport mechanisms; alternatively, an imbalance between CLOCK synthesis and utilization could be hypothesized, with a consequent accumulation of excessing protein in the IG. In this view, further studies are in course at present to elucidate whether CLOCK protein undergoes similar modifications in other tissues of old rats, especially in the CNS. In parallel, other molecular components of the intracellular circadian clock are under investigation, to understand their possible involvement in the aging process.

**ACKNOWLEDGMENTS**

The authors are particularly grateful to Mr. M. Solazzi for his skillful technical assistance and to Prof. D. Formenti for his kind help in statistical analysis.

**REFERENCES**


Kim EY, Bae K, Ng FS, Glossop NR, Hardin PE, Edery I. 2002. Dro-

Jimenez-Garcia LF, Segura-Valdez MD, Ochs RL, Echeverria OM,

Harms E, Kivimae S, Young MW, Saez L. 2004. Posttranscriptional and

Fakan S, Leser G, Martin TE. 1984. Ultrastructural distribution of

Fakan S, Puvion E. 1980. The ultrastructural visualization of nucleo-

Fakan S. 1994. Perichromatin fibrils are in situ forms of nascent tran-


Davidson AJ, Castanon-Cervantes O, Stephan FK. 2004. Daily oscill-

Kolker DE, Fukuyama H, Huang DS, Takahashi JS, Horton TH,

Kolker DE, Vitaterna MH, Fruechte EM, Takahashi JS, Turek FW.


Malatesta M, Baldelli B, Marcheggiani F, Gazzanelli G. 2003a. Immuno-

malatesta M, Baldelli B, Marcheggiani F, Gazzanelli G. 2003b. Fine distribution of clock protein in hepatocytes of hibernating dor-


Morse D, Sassone-Corsi P. 2002. Time after time: inputs to and out-


Cmarko D, Verschure PJ, Rothblum LI, Hernandez-Verdun D, Amal-}

Cmarko D, Verschure PJ, Rothblum LI, Hernandez-Verdun D, Amal-}

Cmarko D, Verschure PJ, Rothblum LI, Hernandez-Verdun D, Amal-}

Cmarko D, Verschure PJ, Rothblum LI, Hernandez-Verdun D, Amal-}

Cmarko D, Verschure PJ, Rothblum LI, Hernandez-Verdun D, Amal-}

Cmarko D, Verschure PJ, Rothblum LI, Hernandez-Verdun D, Amal-}

Cmarko D, Verschure PJ, Rothblum LI, Hernandez-Verdun D, Amal-}