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Authors: Doi, Masao, Nakajima, Yoshito, Okano, Toshiyuki, and Fukada, Yoshitaka

Source: Zoological Science, 19(6) : 633-641

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.19.633

ZOOLOGICAL SCIENCE **19**: 633–641 (2002)

Light-Dependent Changes in the Chick Pineal Temperature and the Expression of *cHsp90α* gene: A Potential Contribution of *in vivo* Temperature Change to the Photic-Entrainment of the Chick Pineal Circadian Clock

Masao Doi†, Yoshito Nakajima†, Toshiyuki Okano and Yoshitaka Fukada*

Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan

ABSTRACT— The circadian clock is entrained to the diurnal alteration of environmental conditions such as light and temperature, but the molecular mechanism underlying the entrainment is not fully understood. In the present study, we employed a differential display-based screening for a set of genes that are induced by light in the chick pineal gland, a structure of the central clock entrainable to both light and temperature changes. We found that the level of the mRNA encoding chicken heat shock protein 90 α (cHSP90α) was rapidly elevated in the pineal gland within a 5-min exposure of chicks to light. Furthermore, the pineal *cHsp90α* mRNA was expressed rhythmically under both 12-hr light/12-hr dark (LD) cycles and constant dark (DD) conditions. The total amount of the pineal cHSP90α protein was, however, kept at nearly constant levels under LD cycles, and immunohistochemical analyses of the pineal cHSP90α showed invariable localization at the cytoplasm throughout the day. *In vivo* measurement of the chick pineal temperature demonstrated its light-dependent and time-of-day-dependent change, and the profile was very similar to that of the pineal *cHsp90α* mRNA level. These observations suggest that the *in vivo* temperature change regulates the expression of temperature-responsive genes including *cHsp90α* in the pineal gland. The temperature change may induce a phase-shift of the pineal clock, thereby facilitating its efficient entrainment to environmental LD cycles.

Key words: Hsp90α, circadian clock, differential display, chicken, pineal gland

INTRODUCTION

Daily rhythms in behavior and physiology are observed in a variety of orga nisms from bacteria to humans, and many of them are driven by endogenous oscillators called circadian clocks (Pittendrigh, 1993). The circadian clocks oscillate even in the absence of an external time cue (zeitgeber) with their period lengths slightly different from 24 hr. The circadian clocks are synchronized (entrained) to the environmental 24-hr cycles by several zeitgebers such as light and temperature. As a general feature of the entrainment, light and heat stimuli give a phase-dependent phase-shifting effect on the clock (Zimmerman *et al*., 1968; Pittendrigh, 1981; Francis and Sargent, 1979; Nakashima, 1987; Takahashi *et al*., 1989; Edery *et al*., 1994; Barrett and Takahashi, 1995; Ruby *et al*., 1999). It is conceivable that the clock systems have evolved so that the clock phase responds similarly to changes in light and temperature, because they are tightly linked to each other in nature. The circadian oscillation is cell-autonomous (Welsh *et al*., 1995; Nakahara *et al*., 1997), and the oscillatory mechanism within each cell is based on an autoregulatory feedback loop in which the products of clock genes inhibit their own transcription (Cermakian and Sassone-Corsi, 2000; Allada *et al*., 2001; Loros and Dunlap, 2001; Ripperger and Schibler, 2001; Williams and Sehgal, 2001; Reppert and Weaver, 2001). In the case of mice, essential components for the circadian oscillation in the central clock reside in individual neurons of the suprachiasmatic nucleus (SCN), where the products of clock genes (*mPer1, mPer2, mPer3, mClock, mBmal1, mBmal2, mCry1, mCry2, mCKIε*) seem to form interacting negative feedback loops (Shearman *et al*., 2000). Recent studies on clock systems in non-mammals such as chicken (Fukada and Okano, 2002), frog (Zhuang *et al*., 2000) and zebrafish (Cermakian *et al*., 2000) support the idea that the oscillatory mechanisms are conserved among

* Corresponding author: TEL: 81-3-5802-8871;
FAX: 81-3-5802-8871.
E-mail: sfukada@mail.ecc.u-tokyo.ac.jp
† These authors contributed equally to this work.

vertebrates.

In contrast to the accumulating knowledge of the oscillatory mechanism, less understood is the input pathway that entrains the circadian clock to environmental cycles of light or temperature. Among several clock-containing tissues of vertebrates, the chick pineal gland has been used as a prominent model for studies of the entrainment mechanisms (Takahashi *et al*., 1989). This is because the single pineal cell not only retains the clock oscillator but also is equipped with the input pathway for the entrainment to light and temperature cycles (Zatz *et al*., 1994; Barrett and Takahashi, 1995; Nakahara *et al*., 1997). In addition, recent molecular analyses of chick pineal clock genes (*cPer2, cPer3, cClock, cBmal1, cBmal2, cCry1, cCry2*) suggest that the chick pineal and the mouse SCN clock share a similar entrainment mechanism, in which phase-shifts of the clock are closely associated with changes (rise and fall) in the mRNA levels of *Per* genes (Maywood *et al*., 1999; Horikawa *et al*., 2000; Doi *et al*., 2001; Okano *et al*., 2001; Yamamoto *et al*., 2001; Pando and Sassone-Corsi, 2001). Thus, the chick pineal gland represents a good model system to explore the entrainment mechanisms conserved among vertebrate circadian clocks.

In order to find out the cellular pathways for the entrainment of the clock, it is a fruitful approach to investigate a set of genes that are induced by light or temperature change. In the present study, we aimed at extensive screening for light-induced genes in the chick pineal gland. To this end, the pineal glands were isolated from chicks that were exposed to a light stimulus at various circadian time (CT) points, and the pineal RNA preparations were subjected to differential display analyses. We found the light-dependent increase in the mRNA level of the pineal *cHsp90*α gene, which is known to be induced by a heat stimulus (Wolfe and Zatz, 1994). *In vivo* measurement of the chick pineal temperature revealed a light-dependent increase in the temperature, and its profile was similar to that of the *cHsp90*α mRNA level. Implications of the *in vivo* temperature change for the photic-entrainment of the pineal clock are discussed.

MATERIALS AND METHODS

Animals

Animals were treated in accordance with the guidelines of the University of Tokyo. Newly hatched chicks were purchased from a local supplier, and maintained under various light/dark conditions. The light was provided by fluorescent lamps to give a constant light intensity of ~300 lux at the level of chicks. The ambient temperature was kept at 28°C±0.5°C irrespective of light/dark conditions. Pineal glands were isolated from decapitated chicks, frozen in liquid nitrogen, and kept at –80°C until subsequent analyses. All of the procedures during the dark-period were performed under dim red light (>640nm).

Differential display-based cloning

Differential display analysis was performed as previously described (Doi *et al*., 2001). Briefly, total RNA was extracted from each pool of the isolated pineal glands by using guanidine thiocyanate (Takanaka *et al*., 1998), and it was treated with DNase I (Takara Shuzo, Kyoto, Japan) to digest contaminating genomic DNA. One micro gram of the total RNA was reverse transcribed by using ThermoScript (Invitrogen, CA, USA) with an anchored oligo (dT) primer (H-T_{11}A, H-T_{11}C or H-T_{11}G; RNAimage kit, GenHunter, TN, USA) at 55°C for 60min. One-hundredth of this reaction mixture was subjected to PCR in a reaction mixture (10 μl) composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of the dNTPs, 1unit AmpliTaq DNA polymerase (Applied Biosystems, Tokyo, Japan), 1 μM anchored oligo (dT) primer (H-T_{11}A, H-T_{11}C or H-T_{11}G), 1 μM H-AP primer (one of eighty kinds of H-AP primers; RNAimage kit, GenHunter). The amplification reaction was comprised of an initial denaturation step of 94°C for 5 min, and 30 cycles of 94°C for 30 sec, 36°C for 2 min and 72°C for 30 sec, with a final extension step of 72°C for 7 min. The PCR products were subjected to 7.5% native polyacrylamide gel electrophoresis (PAGE), stained with SYBR Green I (Molecular Probes, OR, USA) and the band intensities were quantified by FLA-2000 bioimage analyzer (Fuji Film, Tokyo, Japan). The PCR products selected were cut out from the gel and eluted by boiling in 50 μl of distilled water at 95°C for 5 min. An aliquot (2 μl) of the eluted DNA solution was subjected to the secondary PCR under the same conditions as those in the initial analysis. The reamplified DNA fragment was subcloned into pCR2.1 TOPO vector (Invitrogen) and sequenced.

Quantitative reverse transcription (RT)-PCR analysis

Quantitative RT-PCR analyses were performed as previously described (Hirota *et al*., 2001). Primer pairs used were as follows; for *cHsp90*α (GenBank accession number; X07265), 5'-GATGA TGAGC AGTAT GCTTG G-3' (forward) and 5'-TGGTC TTGTT GAGCT CTTCC-3' (reverse); for chicken TATA-box binding protein (*cTbp*, GenBank accession number; D83135), 5'-GTCGA ATATA ATCCC AAGCG-3' (forward) and 5'-TCTGC TCGAA CTTTA GCACC-3' (reverse). The optimal cycle numbers for quantitative analyses were 17 and 21 for *cHsp90*α and *cTbp*, respectively.

Immunoblot analysis

Each pool of the isolated pineal glands (n=5) was homogenized with 400 μl of ice-cold 20 mM Tris-HCl buffer (pH 7.4) containing 2.5 mM EDTA, 100 mM NaCl, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 0.1 mM phenylmethylsulfonyl fluoride, and the homogenate was centrifuged at 700 g for 10 min. These manipulations for the pineal glands isolated during the dark period were performed under dim red light (>640 nm). The supernatant contained almost all of the detectable cHSP90α protein expressed in the pineal gland (data not shown). An aliquot of the supernatant was subjected to sodium dodecyl sulfate (SDS)-PAGE (7.5% acrylamide) after boiling for 8 min in SDS-PAGE sample buffer (10 mM Tris-HCl, 6% [v/v] glycerol, 2% [w/v] SDS, 50 mM DTT, 2 mM EDTA, 0.002% [w/v] Coomassie Brilliant Blue, pH 6.8). Proteins in the gel were electrotransferred to a polyvinylidene difluoride membrane (Immobilon transfer membrane; Millipore Corp., MA, USA), which was then incubated for 1hr at 37°C in blocking solution I (10 mM sodium phosphate, 150 mM NaCl, 4% bovine serum albumin, pH 7.4), followed by incubation at 4°C overnight with HSP90α-specific antibody (final concentration, 400 ng/ml; StressGen Biotechnologies, Victoria, Canada) diluted in a reaction buffer (10 mM sodium phosphate, 150 mM NaCl, 0.1% bovine serum albumin, pH 7.4). After washing 3 times for 5 min each with the reaction buffer, the blot was incubated for 1 hr at 37°C with alkaline phosphatase-conjugated anti-rabbit IgG antibody (New England Biolabs, MA, USA) diluted in the reaction buffer (1:2,500 dilution). The immunoreactivities were visualized by using CDP-star detection system (New England Biolabs).

Preparation of tissue sections and immunofluorescence analysis

The isolated pineal glands were fixed with 4% (w/v) paraform-

aldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 4hr at 4°C. Tissues isolated during the dark period were fixed in complete darkness. The fixed tissues were incubated for cryoprotection in dehydrating solution I (10 mM sodium phosphate, 10% sucrose [w/v], 140mM NaCl, pH 7.2) at 4°C for 4 hr, and subsequently incubated in dehydrating solution II (10 mM sodium phosphate, 30% sucrose [w/v], 140 mM NaCl, pH 7.2) at 4°C overnight. Then, the tissues were embedded in a 2:1 (v/v) mixture of dehydrating solution II and OCT mounting medium (Sakura, Tokyo, Japan), frozen by using liquid nitrogen, and stored at –80°C until use. Finally, 10-μm-thick sections were cut out from the frozen tissues, mounted on gelatin-coated glass slides, and air-dried. The sections on glass slides were pretreated with blocking solution II (10 mM sodium phosphate, 1% bovine serum albumin, 3% horse normal serum, 3% goat normal serum, 0.05% Tween20, 140 mM NaCl, 1 mM $MgCl_2$, pH 7.4) for 30 min at room temperature, and then incubated with a mixture of rabbit anti-HSP90α antibody (final concentration, 1 μg/ml; StressGen Biotechnologies) and mouse anti-pinopsin antibody P9 (1:400 dilution; Okano *et al*., 1997) diluted in blocking solution II at 4°C overnight. After rinsing with phosphate-buffered saline buffer (10 mM sodium phosphate, 140 mM NaCl, 1 mM $MgCl_2$, pH 7.4), the sections were incubated with a mixture of fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody (final concentration, 1.2 μg/ml; Vector Laboratories, CA, USA) and Texas Red-conjugated anti-mouse IgG antibody (final concentration, 6 μg/ml; Vector Laboratories) diluted in blocking solution II at 4°C overnight. The sections were coverslipped with Vectashield mounting medium (Vector Laboratories) containing 4', 6-diamidino-2-phenylindole (DAPI) for nuclear staining.

***In vivo* measurement of the local temperature adjacent to the pineal gland**

The chick body temperature at a local region adjacent to the pineal gland was measured by using a small sensor tip of thermistor (globular shaped, ø 1.0 mm; 503ET-1, Ishizuka Electronics, Japan), which was connected to a digital monitor (DM1001C, Ishizuka Electronics, Japan) with a pair of electric wires (ø 0.8 mm, 1.0 m long) in a single silicon tube (1 mm in inside diameter, 2 mm in outside diameter; AS ONE, Tokyo, Japan). Fourteen-days-old chicks were subjected to the *in vivo* temperature measurement. The sensor tip was surgically placed in a brain position close to the pineal gland (1.0 mm anterior and 1.0 mm lateral to the protuberantia occipitalis interna, 1.0 mm ventral to the cortical surface), and the connecting tube was attached to the skull with the aid of dental cement (GC Corp., Tokyo, Japan) so that the sensor was fixed at the position. Activities of the chick were restricted by placing it in a small columnar arena (80 mm in diameter, 80 mm high) with a cover of a circular cloth loosely bound to the neck of the bird like a collar. Food and water were freely available, and the ambient temperature was monitored and kept at 28°C±0.5°C.

RESULTS

Identification of chick pineal *Hsp90α* as a light responsive gene

To explore the molecular mechanism underlying the phase-dependent phase-shifting effect of light on the circadian clock, we employed a differential display-based screening of the chick pineal genes that are induced by light in a phase-dependent manner. Newly hatched chicks were raised in LD cycles for 7days and transferred to DD on day 8. Then animals were exposed to a 90-min light stimulus from various CT points (CT0, CT14 or CT20) on day 8, and the pineal glands were collected from animals (30 chicks each) at two distinct time periods after the light onset (20–50 min and 60–90 min after the light onset). The control animals were maintained in the dark, and their pineal glands were collected at CT1.5, CT15.5 and CT21.5. This sampling schedule was chosen to identify various types of light-responsive genes such as those showing a CT-dependent light-induction and those induced rapidly or slowly after the light onset. We screened 5,699 bands of the PCR products and found 99 bands as light-induced products. As shown in Table 1, these products were classified into four types on the basis of the time point(s) when they were light-induced: (i) induced only at CT14 (early night), (ii) induced only at CT20 (the late night), (iii) induced both at CT14 and CT20 (night), but not at CT0 (morning), and (iv) induced at every time point tested. All the light-induced products showed higher band intensities at 60min than those at 20min after the light onset. We cloned three products, two from type (iv) (Fig. 1) and one from type (i) (Doi *et al*., 2001) because these three showed remarkable light induction. Sequencing analyses revealed that the latter one was derived from a message of *cE4bp4* gene (Doi *et al*., 2001). The sequences of the former two products (indicated by arrows in Fig. 1A and 1B) matched completely the *cHsp90α* cDNA sequence (GenBank accession no. X07265) within the 339 bases (+2513 to +2851; +1 indicates transcription initiation site) and within the 646 bases (+1650 to +2295), respectively. These results strongly suggest that the mRNA levels of the pineal *cHsp90α* increase profoundly in response to the light stimulus given at CT0, 14 and 20.

Table 1. Classification of PCR products in differential display analyses

	CT-dependent pattern of light-induction			
Type	CT0	CT14	CT20	Number of products
i	–	+	–	5
ii	–	–	+	3
iii	–	+	+	20
iv	+	+	+	71

+; induced –; not induced

Light-dependent and time-of-day-dependent expression of *cHsp90α* mRNA

A time course of the light-dependent *cHsp90α* induction was examined by quantitative RT-PCR analyses, in which the mRNA levels of the chick pineal *cHsp90α* were monitored during and after 60-min light exposure from CT20 (Fig. 2A). The *cHsp90α* mRNA level began to increase within 5min after the light onset, and at 90 min it reached a peak level ~4 fold higher than the basal level. Then, the *cHsp90α* mRNA level decreased gradually and reached to a level close to that of the control (dark-kept) animals within 3 hr after the light offset. To examine whether the pineal *cHsp90α* expression shows a CT-dependent light response,

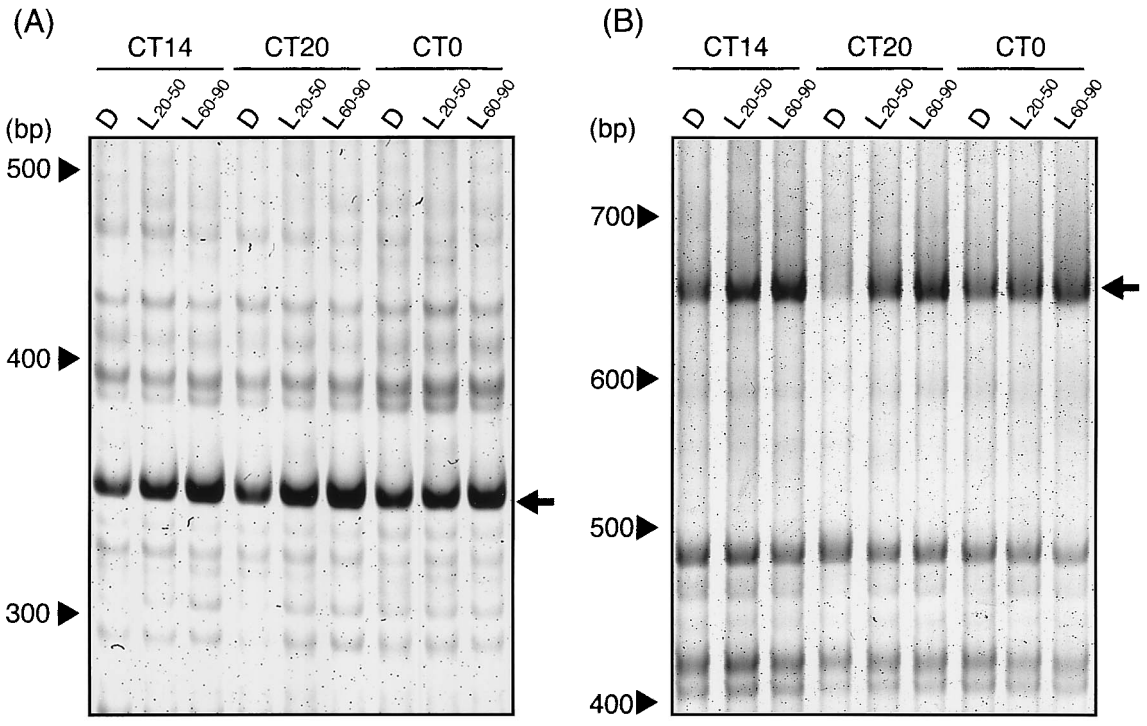

Fig. 1. Identification of chicken *Hsp90α* gene by differential display analyses. Chicks were exposed to light for 90 min from indicated CT points of a day, and their pineal glands were isolated at 20–50 min ($L_{20–50}$) or 60-90min ($L_{60–90}$) after the light onset. Control animals were kept in the dark (D). Amplification reactions were performed with a pair of H-T11C primer (5'-AAGCT TTTTT TTTTT C-3') and H-AP1 primer (5'-AAGCT TGATT GCC-3') in (A), and with a pair of H-T11G primer (5'-AAGCT TTTTT TTTTT G-3') and H-AP14 primer (5'-AAGCT TGGAG CTT-3') in (B). Arrows indicate the positions of the amplified DNA fragments derived from *cHsp90α* transcript.

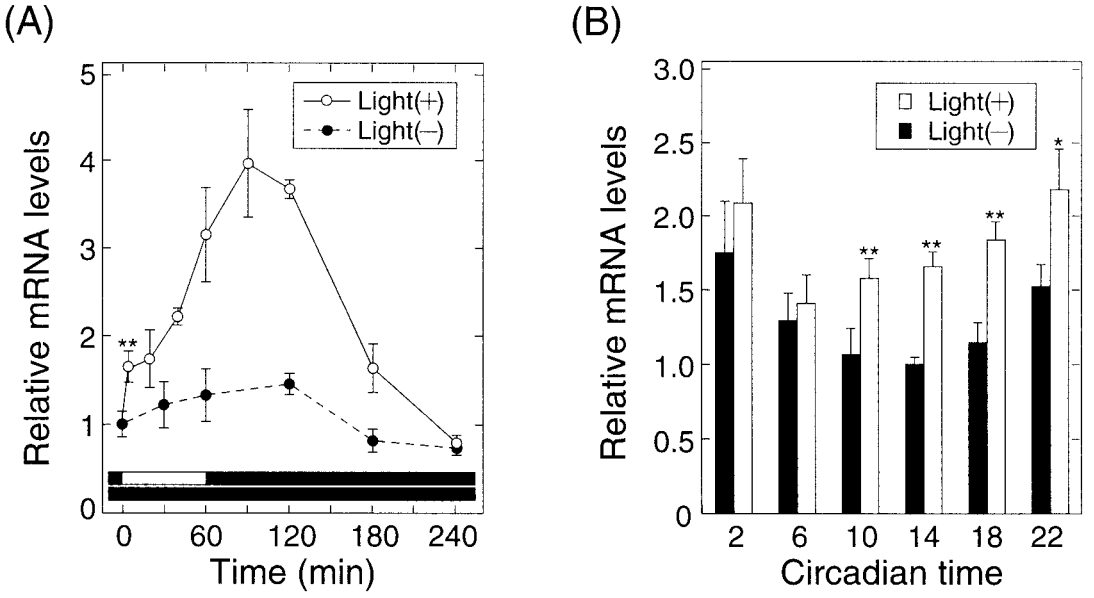

Fig. 2. Light-dependent changes in *cHsp90α* mRNA levels. One-day-old chicks were maintained in LD cycles for 8 days and then transferred to DD on day 9, when animals were exposed to light for 60min from each indicated time point [Light (+)] or they were kept in the dark for control [Light (–)]. (A) Time course of *cHsp90α* induction during and after light exposure at CT20-21. The pineal glands were isolated at indicated time points, and the relative mRNA levels of *cHsp90α* and *cTbp* in the isolated pineal glands were measured by RT-PCR. The band intensities of the amplified products of *cHsp90α* were normalized to those of *cTbp*, and the value at CT20 (0 min) was set to 1. $^{**}p<0.01$, compared with the value at CT20 (0 min) by Student's *t*-test. (B) Light-induction of *cHsp90α* at various CT points of a day. The pineal glands were isolated from light-exposed animals (open bars) or from dark-kept animals (solid bars) 60 min after indicated time point. Relative mRNA levels of *cHsp90α* and *cTbp* were measured by RT-PCR, and the value of dark-kept animals at CT14 was set to 1. All of the values are the mean±SEM from three independent experiments. $^{**}p<0.01$, $^{*}p<0.02$, compared with the value of dark-kept animals by Student's *t*-test.

chicks were exposed to a 60-min light stimulus at various CT points of a day (CT2, CT6, CT10, CT14, CT18 and CT22), and the level of the pineal *cHsp90α* mRNA was evaluated at 60 min after the light onset (Fig. 2B). The light stimuli given at CT10, CT14, CT18 and CT22 produced significant increases in the level of the pineal *cHsp90α* mRNA, but no significant effect was observed by light given at early morning to midday (CT2 and CT6) under the experimental conditions. In addition to the acute response to light, a daily change in the pineal *cHsp90α* mRNA level was examined. In a LD cycle (Fig. 3; on day 8), the pineal *cHsp90α* mRNA exhibited a profound daily fluctuation with a peak in the early morning, and a similar fluctuation with lower amplitude was observed in animals maintained in DD (Fig. 3; on day 9). These results indicate that the expression of *cHsp90α* is regulated not only by the external light but also by the circadian clock.

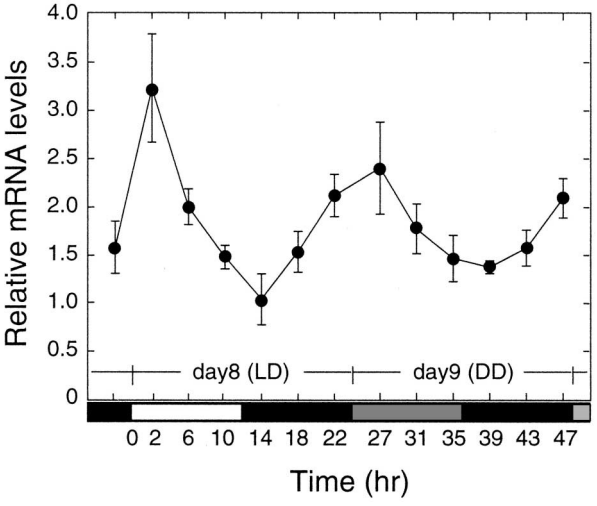

Fig. 3. Temporal changes in mRNA levels of *cHsp90α* in the chicken pineal gland. One-day-old chicks were maintained in LD cycles for 8 days and then transferred to DD. The pineal glands were isolated at indicated time points of day 7–8 in LD and day 9 in DD. Relative mRNA levels of *cHsp90α* and *cTbp* in the isolated pineal glands were evaluated by RT-PCR, and each value was expressed as described in Fig. 2 except that the minimal value of *cHsp90α* mRNA was set to 1. All of the values are the mean±SEM from three independent experiments.

Analyses of the chick pineal HSP90α protein

A possible change in cHSP90α protein level was examined by evaluating the total amount of HSP90α protein expressed in the chick pineal gland. In immunoblot analysis (Fig. 4), the expression of cHSP90α protein was detected in the pineal gland of chicks that were maintained in LD cycles at a constant room temperature, and the levels showed no significant change throughout the day in contrast to the profound fluctuation of its mRNA level.

Although the total amount of the cHSP90α protein accumulated in the whole pineal tissue was apparently constant (Fig. 4), the level of the cHSP90α protein may diurnally fluctuate within a subset of the pineal cells. To test this,

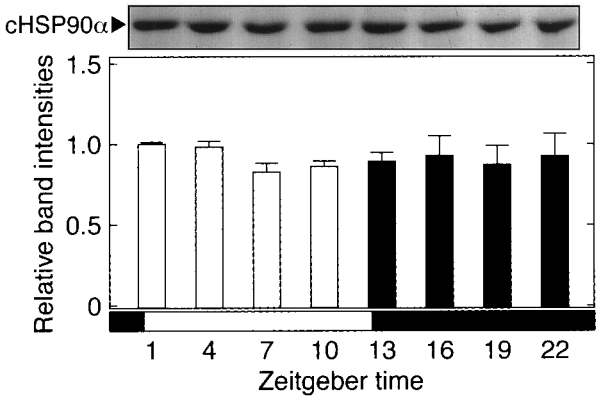

Fig. 4. Immunoblot analysis of the temporal change in the chick pineal HSP90α protein level. One-day-old chicks were maintained in LD cycles for 8 days and their pineal glands were isolated at indicated time points of day 8 (from 5 chicks for each time point). Equal amount of proteins (16 μg) of the pineal homogenate were immunoblotted with anti-HSP90α antibody (Upper). The band densities were quantified by densitometry and the value of ZT1 was set to 1 (Lower). All of the values are the mean±SEM from three independent experiments.

localization of cHSP90α protein in the chick pineal gland was investigated by immunohistochemical analyses (Figs. 5 and 6). Examination of a possible colocalization of cHSP90α with pinopsin, a pineal specific photoreceptive molecule (Okano *et al*., 1994) revealed cHSP90α immunoreactivity in almost all the pineal cells including the follicular pinealocytes (indicated by solid arrowheads in Fig. 5) whose rudimentary outer segments showed pinopsin immunoreactivity (indicated by open arrowheads in Fig. 5). This suggests cHSP90α protein expression in the photosensitive clock cells in the pineal gland (Takahashi *et al*., 1989; Okano *et al*., 1994; Bernard *et al*., 1997). Pineal cHSP90α proteins were localized mainly in the cytosol and diffusely in the nucleus in the follicular and parafollicular pinealocytes (indicated by arrows in Fig. 5). The immunohistochemical analysis of the temporal change in the localization demonstrated that cHSP90α immunoreactivities were kept at a nearly constant level in immunopositive pineal cells of the pineal glands isolated at ZT1, ZT7, ZT13 and ZT19 (Fig. 6). At every ZT point tested, the subcellular localization of cHSP90a protein was mainly cytoplasmic as observed at ZT6 (Fig. 5A). These observations indicate that not only the total amount of the pineal cHSP90α protein but also its subcellular localization in each pineal cell is kept almost constant all day long in LD cycles.

Light-dependent and time-of-day-dependent changes in the *in vivo* temperature of the pineal gland

Considering that the expression of heat shock proteins is generally responsive to a temperature change, we assumed that the changes in the mRNA level of the pineal *cHsp90α* (Figs. 2 and 3) might be ascribed to the changes in the *in vivo* temperature of the pineal gland (designated T_P). To test this, T_P was measured continuously with the aid

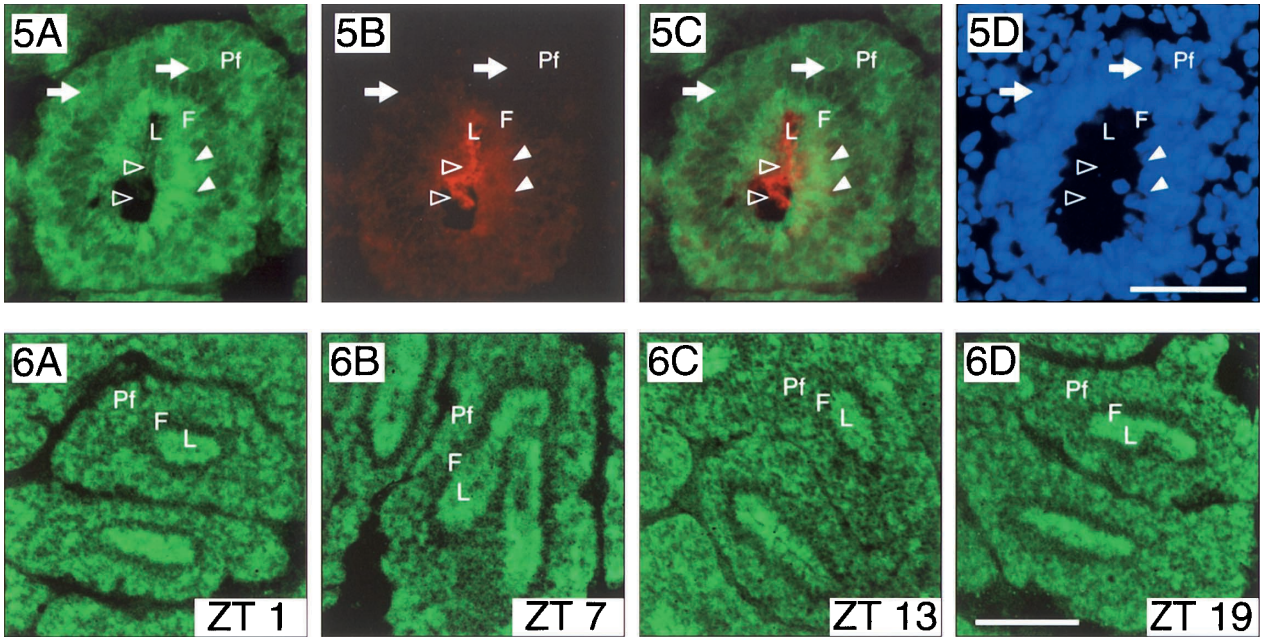

Fig. 5. Localization of the chick pineal HSP90α protein. One-day-old chicks were maintained in LD cycles for 8 days and tissue sections derived from the pineal gland isolated at ZT6 of day 8 were double-labeled with anti-HSP90α antibody (A, *green*) and anti-pinopsin antibody P9 (B, *red*). The nucleus was detected by staining with DAPI (D, *blue*). Panel C is a merged image of A and B, demonstrating the distinctive distribution of HSP90α and pinopsin in the follicular pinealocytes. Solid arrowheads indicate the region of inner segments of the follicular pinealocytes, which were immunopositive to anti-HSP90α. Open arrowheads indicate rudimentary outer segments of the follicular pinealocytes, which were immunopositive to anti-pinopsin antibody. Arrows indicate the parafollicular pinealocytes, which were immunopositive to anti-HSP90α but immunonegative to anti-pinopsin. L, follicular lumen; F, follicular zone; Pf, parafollicular zone. Scale bar=50 μm

Fig. 6. Immunohistochemical analysis of the temporal changes in the chick pineal HSP90α protein level. One-day-old chicks were maintained in LD cycles for 8 days and their pineal glands were isolated at ZT1 (A), ZT7 (B), ZT13 (C) and ZT19 (D) on day 8. The pineal sections were stained with anti-HSP90α antibody. L, follicular lumen; F, follicular zone; Pf, parafollicular zone. Scale bar=100 μm

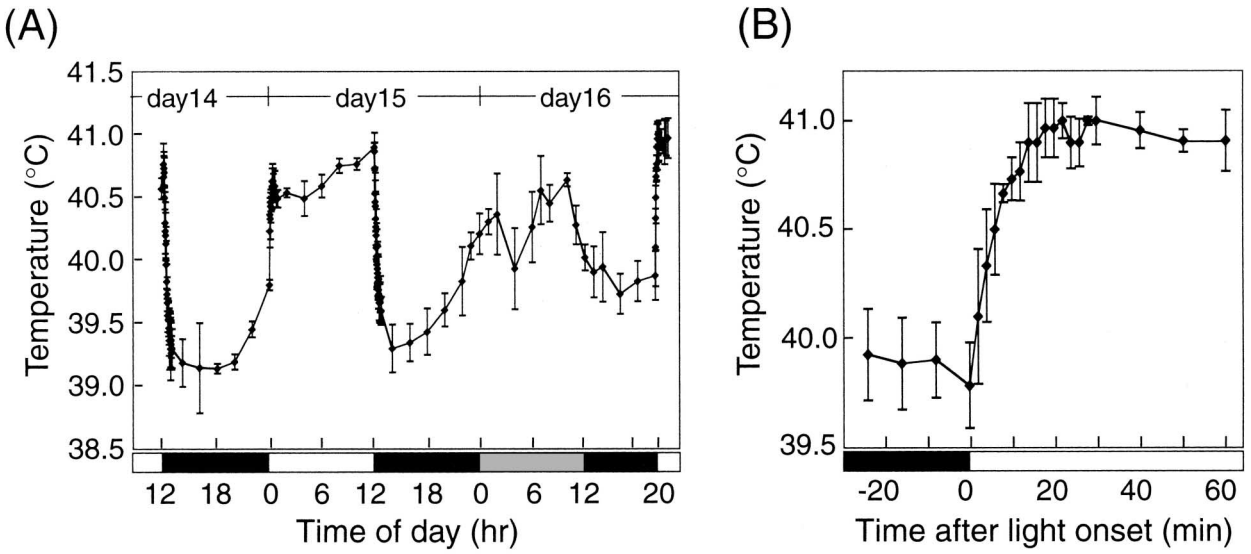

Fig. 7. Light-dependent and time-of-day-dependent changes in the temperature of the chick brain close to the pineal gland. One-day-old chicks were maintained in LD cycles for 15 days and then kept in constant dark. The animals were exposed to light late at night of day 16 (time of day=20). A sensor tip of thermistor was placed in a brain position adjacent to the pineal gland at the beginning of day 14 (see Materials and Methods), and the temperature was measured continuously from the light offset on day 14. (A) A temporal profile of the pineal temperature from day 14 to day 16. (B) A time course of the pineal temperature change before and after the light exposure on day 16. All of the values are the mean±SEM from the temperature data of three chicks.

of a thermometer fixed at a brain position close to the pineal gland of the chick (see Materials and Methods). When chicks were maintained in LD cycles, T_P exhibited an overt diurnal fluctuation. As shown in Fig. 7A, T_P rapidly decreased and reached trough levels of 39.2–39.4°C within 1 hr after the light offset (on day 14 and 15). Then T_P gradually increased during the late dark period. After the light onset (on day 15), T_P was rapidly elevated and kept at 40.5-40.9°C during the light period. T_P also exhibited a diurnal fluctuation in constant dark condition (on day 16) albeit with a lower peak/trough ratio (Fig. 7A). In response to light exposure during the nighttime (on day 16), T_P rapidly increased by 1.1°C from 39.9°C to 41.0°C (Fig. 7A). Notably, T_P began to increase within 2min after the light onset, and at 20 min it reached a plateau of 40.9–41.0°C (Fig. 7B). The light-dependent and time-of-day-dependent changes of the pineal temperature (Figs. 7A and 7B) indicate a possible association of the expression profile of the pineal *cHsp90α* mRNA with the temperature change of the pineal gland.

DISCUSSION

The differential display-based screening of light-induced genes in the chick pineal gland resulted in identification of the light-induced products, which were classified into four types on the basis of the CT-dependent pattern of the light-induction (Table 1). It is most probable that the pineal gland has a gene expression system discriminating the circadian time when the chick is exposed to a light stimulus (Doi *et al*., 2001). Here we showed that the two products with remarkable light-responsiveness at every CT point tested were derived from a message of *cHsp90α* gene (Fig. 1), and demonstrated that the level of the pineal *cHsp90α* mRNA changed in light-dependent and time-of-day-dependent manners (Figs. 2 and 3). Monitoring the *in vivo* temperature of the pineal gland demonstrated a close link between the profiles of the pineal temperature and the *cHsp90α* mRNA level (Figs. 2, 3 and 7). Although it is unclear whether the *in vivo* temperature change of 1–2°C (Fig. 7) is responsible for the *cHsp90α* expression, the data presented here strongly suggest that the gene expression mechanism sensitive to a temperature change operates in the light-dependent and time-of-day-dependent manners *in vivo*.

Contrary to the mRNA level exhibiting pronounced diurnal changes (Fig. 3), the total amount of the pineal cHSP90α protein was kept at nearly constant level under the LD cycle condition (Figs. 4 and 6). Such a constant level expression of HSP90α protein has been seen in several rabbit tissues such as cerebellum, retina, thymus and testis (Quraishi and Brown, 1995), where HSP90α is an abundant protein constituting ~2% of the total cellular protein under unstressed conditions (Lai *et al*., 1984). Very interestingly, the total amount of HSP90α protein expressed in these tissues showed no significant change even after a heat stimulus that elevated the rate of its protein synthesis (Clark and Brown, 1982). On the other hand, a heat-stimulus-dependent increase in the total amount of the HSP90α protein was observed in tissues such as muscle, heart and kidney, showing a low-level expression of HSP90α protein under unstressed conditions (Quraishi and Brown, 1995). These observations support an idea that the high level expression of HSP90α protein in certain tissues would mask the upregulation of its protein synthesis therein. This idea may account for the constant level of cHSP90α protein in the chick pineal gland (Figs. 4 and 6), although alternative explanations are also possible. For example, the rates of synthesis and degradation of cHSP90α protein may be balanced, or alternatively, the amount of the pineal cHSP90α protein is regulated at the translational level, not at the transcriptional level.

The constant expression level of cHSP90α protein implies invariable cHSP90α activities in the pineal gland, excluding a possible function of cHSP90α as a zeitnehmer (Roenneberg *et al*., 1999) or a state variable in the pineal circadian clock. However, considering the fact that some of clock proteins are expressed at constant levels in the clock-containing tissues (Ripperger *et al*., 2000; Kloss *et al*., 2001; Chilov *et al*., 2001), it is still possible for cHSP90α protein to participate in the chick pineal clock system. Indeed, human HSP90α is known to interact with BMAL1 and NPAS2 (Hogenesch *et al*., 1997), both of which play pivotal roles in the vertebrate circadian clock system (Bunger *et al*., 2000; Reick *et al*., 2001). A potential involvement of the cHSP90α protein in the circadian oscillation and/or in the light-entrainment is also suggested by colocalization of a photoreceptive molecule pinopsin and cHSP90α protein in the follicular pinealocyte (Fig. 5) that contains both the circadian oscillator and the photic-input pathway (Okano *et al*., 1997; Bernard *et al*., 1997; Nakahara *et al*., 1997).

In previous studies, the effect of temperature on the pineal clock was examined in *in vitro* experiments with cultured chick pineal cells, and it was clearly demonstrated that a transient increase of temperature from 36.7°C to 40.0°C (Zatz *et al*., 1994) or from 37.0°C to 39.5°C (Barrett and Takahashi, 1995) induces a phase-shift of the clock in a CT-dependent manner. Such a temperature-dependent phase-shift is observed in fungi (Francis and Sargent, 1979; Nakashima, 1987), insects (Zimmerman *et al*., 1968; Edery *et al*., 1994), reptiles (Underwood, 1985), and mammals (Ruby *et al*., 1999). In the present study, we demonstrated that the *in vivo* temperature of the chick pineal gland fluctuated between 39.2°C and 40.9°C under LD cycles (Fig. 7A), and that it rapidly increased from 39.9°C to 41.0°C upon receiving a light stimulus (Fig. 7B). Although it is difficult to examine whether the *in vivo* temperature increase in the dark can also induce a phase-shift of the pineal clock, the *in vivo* temperature increase would likely contribute to the phase-shift of the pineal clock. Then it is conceivable that the cooperative effects of light and temperature signals would allow an efficient entrainment of the pineal clock to environmental LD cycles. Further analyses of the heat-responsive pathways in the clock structures might be impor-

tant to know the molecular mechanisms underlying the phase-shift of the clock and the temperature compensation, both of which are functionally prerequisite for the circadian clock.

ACKNOWLEDGEMENTS

We thank Dr. K. Kokame at National Cardiovascular Center Research Institute (Osaka, Suita, Japan) for technical advice in the differential display analysis. This work was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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(Received January 25, 2002 / Accepted April 1, 2002)