

Characterization of the Chicken Serotonin *N*-Acetyltransferase Gene

ACTIVATION VIA CLOCK GENE HETERODIMER/E BOX INTERACTION*

Received for publication, June 28, 2000, and in revised form, August 4, 2000
Published, JBC Papers in Press, August 7, 2000, DOI 10.1074/jbc.M005671200

Nelson W. Chong[‡], Marianne Bernard[§], and David C. Klein[¶]

From the Section on Neuroendocrinology, Laboratory of Developmental Neurobiology, NICHD, National Institutes of Health, Bethesda, Maryland 20892

The abundance of serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, AANAT) mRNA in the chicken pineal gland exhibits a circadian rhythm, which is translated into a circadian rhythm in melatonin production. Here we have started to elucidate the molecular basis of the circadian rhythm in chicken AANAT (cAANAT). The 5'-flanking region of the cAANAT gene was isolated and found to contain an E box DNA element that confers strong luciferase reporter activity. In transfection experiments using chicken pineal cells, an E box mutation dramatically decreased reporter activity. Northern blot analysis indicated that several putative clock genes (*bmal1*, *Clock*, and *MOP4*) are co-expressed in the chicken pineal gland. *bmal1* mRNA is expressed in a rhythmic manner in the chicken pineal gland, with peak levels at early subjective night, coincident with the increase in cAANAT expression. Co-transfection experiments in COS cells demonstrated that chicken BMAL1/CLOCK and human BMAL1/MOP4 heterodimers bound the AANAT E box element and enhanced transcription. These observations suggest that binding of clock gene heterodimers to the cAANAT E box is a critical element in the expression of the cAANAT gene *in vitro*.

Melatonin is a tryptophan-derived compound that is closely associated with vertebrate time keeping and circadian function. It is synthesized in the pineal gland of all vertebrates; circulating melatonin exhibits a daily rhythm, with markedly elevated levels occurring at night (1–3), hence the moniker “hormone of the night.” Circulating melatonin regulates seasonal changes in various aspects of physiology in photoperiodic species (4, 5) and has been implicated in the mechanisms that regulate circadian rhythms in some species of birds, reptiles, and mammals (2, 3, 6, 7). A second site of melatonin synthesis is the retina, where it probably acts locally as a paracrine signal to regulate various aspects of retinal physiology (8, 9).

The mechanisms that govern the rhythm in melatonin pro-

duction differ markedly among vertebrates (10, 11). In mammals, pineal melatonin production is elevated at night in response to sympathetic stimulation driven by a circadian clock in the suprachiasmatic nuclei (SCN¹; see Ref. 12). Light acts through the retina to modulate SCN stimulation of the pineal gland. In contrast to the mammalian pinealocyte, the avian pinealocyte is a self-contained melatonin rhythm-generating system; it has an internal clock and photodetectors (13–17). Similarly, a circadian clock is located in *Xenopus* retinal photoreceptor cells (8, 18, 19) and pike and zebrafish pinealocytes (20–22) where it regulates rhythmic synthesis of melatonin. Recent *in vitro* experiments have also demonstrated that these clock properties exist in mammalian retina (23, 24).

A critical regulatory element of all melatonin rhythm-generating systems is the penultimate enzyme in the serotonin → *N*-acetylserotonin → melatonin pathway, AANAT (EC 2.3.1.87). Large changes in the activity of this enzyme control large changes in the rate of production and circulating levels of melatonin. The regulatory mechanisms that control dynamic changes in melatonin production, including the circadian rhythm, act through AANAT, making this enzyme the molecular interface in vertebrates between photoneurochemical regulatory mechanisms and melatonin synthesis.

A variety of AANAT regulatory systems and strategies has evolved (10, 11). One of the most fascinating is the chicken pineal, in which the circadian rhythm in melatonin production in part reflects a circadian rhythm in AANAT mRNA (25). In this tissue there appears to be a close link between the circadian clock and the AANAT gene, as in the pike and zebrafish pineal gland (20, 22, 26). In these systems, a circadian rhythm in AANAT mRNA persists in constant lighting conditions in culture.

A central issue in the field of circadian biology is defining the molecular mechanisms underlying circadian clocks. Molecular components that comprise these pacemakers have been identified in a diverse set of organisms including the fruit fly *Drosophila melanogaster*, the mouse, and a fungus (27). A number of mammalian genes have been cloned recently that resemble the well studied circadian clock genes from the fruit fly (27), including *Clock*, *bmal1* (alternatively termed *MOP3*), *Period* (*Per*), and cryptochromes (*Cry*). In addition, another gene

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF144425, AF193070, AF193071, AF193072, AF205219.

[‡] Supported by the School of Biological Sciences, University of Surrey, UK. Present address: Centre for Chronobiology, School of Biological Sciences, University of Surrey, Guildford, Surrey GU2 7XH, UK.

[§] Present address: Laboratory of Neuroendocrinology, UMR CNRS 6558, 40 Ave. du Recteur Pineau, 86022 Poitiers Cedex, France.

[¶] To whom correspondence should be addressed: Bldg. 49, Rm. 6A80, NICHD, National Institutes of Health, Bethesda, MD 20892. Tel.: 301-496-6915; Fax: 301-480-3526; E-mail: Klein@helix.nih.gov.

¹ The abbreviations used are: SCN, suprachiasmatic nucleus; AVP, arginine vasopressin; AANAT, arylalkylamine *N*-acetyltransferase; cAANAT, chicken AANAT; bHLH-PAS, basic helix-loop-helix-PER-ARNT-SIM; *bmal1*, brain muscle ARNT-like protein-1; CRX, cone-rod homeobox containing protein; EMSA, electrophoretic mobility shift assay; kb, kilobase; MOP4, member of the PAS superfamily protein 4; *Per*, *Period*; PCR, polymerase chain reaction; TK, thymidine kinase; TSP, transcription start point; ZT, zeitgeber time; bp, base pair; Pipes, 1,4-piperazinediethanesulfonic acid.

named *MOP4* (alternatively termed *NPAS2*) has also been shown to interact with certain clock genes (28, 29). Based on these cross-species parallels, the mammalian genes were postulated to be components of an intracellular transcriptional/translational feedback loop (27, 30, 31). The exact mechanism whereby the clock genes interact is still a matter of debate, and it is likely that additional components remain to be discovered.

We have pursued the question of how the clock regulates *AANAT* expression in the chicken pineal gland by cloning and characterizing the 5'-flanking region of the *cAANAT* gene. Our data reveal that this region contains an E box element, which is closely associated with circadian gene expression in other systems (27, 30, 31). Our studies indicate that *cAANAT* transcription can be regulated via this DNA element, that homologs of mammalian clock genes, *Clock*, *bmal1*, and *MOP4*, are co-expressed in the chicken pineal gland and retina, and that *BMAL1/CLOCK* and *BMAL1/MOP4* heterodimers bind to the E box element and enhance transcription.

EXPERIMENTAL PROCEDURES

Library Screening—A chicken cosmid library subcloned into pWE 15 vector (Stratagene; a gift from Dr. Ignacio Rodriguez, NEI, National Institutes of Health) was screened with a random-primed ³²P-labeled full-length (1.4-kb) *cAANAT* cDNA probe. This identified four positive clones; the one (clone 76) with the largest 5'-flanking region was selected for further study. Plasmid DNA of clone 76 was prepared on Qiagen columns and digested with *Hind*III. An ~6-kb fragment was gel-purified and subcloned into the phagemid vector pBluescript II SK(+) that has been cut with *Hind*III and dephosphorylated. A positive clone (2A76) was selected and used for subsequent experiments.

Animals and Tissue Collection—One-day-old chicks (White Leghorn, Truslow Farms, Baltimore) were housed (for 10–11 days) in heated brooders on a 12-h light/12-h dark cycle (LD 12:12; lights on zeitgeber time (ZT) 0–12) with lights provided by cool white fluorescent tubes. Following this, the animals were released into either constant darkness (DD) or constant light (LL). Three chicks were sacrificed in DD every 4 h beginning at the second 24-h period. In LL experiments, three animals were sacrificed every 6 h. Dissection in “darkness” were performed under dim red light (Wratten number 92; <1 min from exposure to freezing of tissue). For cell culture experiments, animals were killed between ZT 6 and ZT 8.

Total RNA Isolation and S1 Nuclease Analysis—Total RNA was extracted using Trizol according to the manufacturer's instructions (Life Technologies, Inc.). The transcription start point(s) within the *cAANAT* gene was determined by S1 nuclease analysis (32), using total RNA from nighttime (ZT 18) chicken pineal gland and retina as templates. A ³²P-labeled single-stranded probe (481 nucleotides) was generated by asymmetric PCR using primers NAT 50 (5'-CATTACTTCT-GCTGACCTTCC-3') and NAT 27 (5'-TCCTCTCCAGCCTGATCCTG-3'; “driving” primer). The probe was gel-purified and added (60,000 cpm) to 30 μg of total RNA from pineal, retina, or transfer RNA (negative control), in 20 μl of S1 hybridization buffer (80% deionized formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl and 1 mM EDTA). The mixtures were denatured and hybridized overnight at 44 °C. Samples were then digested with 40 units of S1 nuclease at 37 °C for 60 min. After ethanol precipitation, samples were boiled and electrophoresed on a 6% sequencing gel. A dideoxy nucleotide sequencing reaction of clone 76, primed with a ³²P-end-labeled probe of NAT 27, was run in parallel for size comparison.

Electrophoretic Mobility Shift Assay (EMSA)—Pineal glands were dissected from chicks at nighttime (ZT 18) and quick-frozen on solid CO₂ dry ice. Crude cell extracts were prepared as described (32) and corrected for protein (33). Aliquots were stored at –80 °C. For the initial screening of the *cAANAT* 5'-flanking region, T4 polynucleotide kinase was used to end label PCR fragments (~80 to 300 bp) with ³²P and used as probes. Binding reactions were conducted for 20 min at room temperature using 30,000 cpm of radiolabeled probe (~2 fmol), 10 μg of cell extract, 2 μg of poly(dI-dC), in a reaction volume of 20 μl. Where indicated, unlabeled DNA (200-fold molar excess) was added to the binding reaction as competitors and incubated with the extract for 15 min on ice prior to the addition of labeled probe. Complexes were resolved by electrophoresis at 4 °C on a 5% nondenaturing acrylamide gel equilibrated in 1× TGE buffer (25 mM Tris (pH 8.3), 192 mM glycine, 1 mM EDTA). Gels were dried; they were then imaged and analyzed using a STORM 860 PhosphorImager (Molecular Dynamics).

Messenger RNA Analysis of Chicken Clock Genes—Partial cDNAs of chicken *bmal1*, *MOP4*, and *Clock* were isolated by degenerate-polymerase chain reaction (PCR) using chicken pineal cDNA as template and Ex-*Taq* DNA polymerase (Takara). Primers used were M3F1 (forward, 5'-AGAT(CT)GA(AG)AAGCGCGTCGGGA-3') and M3R1 (reverse, 5'-ACTCCTT(AG)AC(CT)TTG(GC)C(AT)AT(AG)TC-3') for *bmal1*, and CKF1 (forward, 5'-GATTCTT(CT)AC(GC)AA(AG)GGCCA-(AG)CAGTGGATATGG-3') and CKR2 (reverse, 5'-CTGCTGTTGTTG-(CT)TG(CT)GTTGCTG-3') for *MOP4* and *Clock*. PCR conditions were 94 °C for 5 min and then 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min. PCRs were size-fractionated, and DNA fragments with the predicted size (384 bp for *bmal1*, approximately 1.2 kb for *MOP4* and 490 bp for *Clock*) were gel-purified and subcloned into the pGEMT-Easy vector (Promega). Plasmid DNA were made from positive bacterial colonies and sequenced. Subsequently, specific internal primers for chicken *MOP4*, M4F1 (forward, 5'-TGGAGAGGAGACAG-GAGATG-3') and M4R1 (reverse, 5'-GGTTGAGAAGCAAGGAAG-3') were used to amplify a fragment (333 bp) using chicken pineal cDNA, subcloned, and sequenced to confirm authenticity. The full-length cDNA of chicken *bmal1* (GenBank™ accession number AF205219) and *Clock* (GenBank™ accession number AF144425) was subsequently cloned by screening a chicken pineal cDNA library using the partial cDNA.

Northern blot analysis was performed as described (34). Unless indicated otherwise, a 20-μg sample of total RNA was prepared from a pool of three pineal glands or retinas and loaded on a 1.5% agarose-formaldehyde gel. RNA was then transferred to a nylon membrane (Nytran, Schleicher & Schuell) by passive capillary blotting with 20× SSC. The transferred RNA was cross-linked to the membrane in an UV Stratalinker (Stratagene; 120 mJ, 35 s). Blots were probed with random-primed ³²P-labeled cDNAs of chicken *bmal1* (1.9 kb), *MOP4* (1.2 kb), *Clock* (490 bp), and *cAANAT* (1.4-kb, see Ref. 35). Data were normalized for variations in RNA loading and transfer efficiency, by probing with a 2-kb human β-actin cDNA (CLONTECH). All probes were hybridized at 68 °C in QuikHyb (Stratagene), and the final wash was at 60 °C in 0.1× SSC, 0.1% SDS for 15 min. Hybridized blots were imaged and analyzed using a PhosphorImager. Transcript sizes were estimated by comparison with standard RNA markers (RNA Molecular Weight Marker I; Roche Molecular Biochemicals).

Generation of *cAANAT-Luciferase Reporter Constructs*—A luciferase reporter plasmid carrying the entire 5'-flanking region of the *cAANAT* gene was prepared using a *Hind*III/*Hind*III fragment generated from genomic clone 2A76 (35). This fragment was gel-purified and partially digested with the restriction enzyme *Nco*I; the largest portion of the digest (~4 kb; –3999 to +120) was gel-purified, blunt-ended with Klenow fragment, and subcloned into the *Sma*I site of the reporter vector pGL3-Basic (Promega). A series of 5'-deletions of the constructed plasmid (pGL3-Basic *Hind*III; –3999 to +120) was created using appropriate combinations of restriction enzymes, filled in, and religated; *Kpn*I and *Eco*RI for pGL3-B 3136 (–3136 to +120), *Kpn*I alone for pGL3-B 1981 (–1981 to +120), *Kpn*I and *Nco*I for pGL3-B 1633 (–1633 to +120), *Kpn*I and *Mlu*I for pGL3-B 1309 (–1309 to +120), and *Kpn*I and *Not*I for pGL3-B 870 (–870 to +120). In other cases, the DNA fragments were obtained by PCR with Ex *Taq* DNA polymerase (Takara) and gel-purified. The ends of these DNA fragments were blunted with Klenow fragment and subcloned into the *Sma*I site of pGL3-Basic. pGL3-B 484 (–484 to +120) and pGL3-B 217 (–217 to +120) were achieved with this strategy. To generate E box (CACGTG; –49 to –44) deletion mutations, *cAANAT-luciferase* reporter plasmids were linearized with *Pml*I and treated with Klenow at 37 °C for 30 min. The digested plasmids were religated and used to transform *Escherichia coli*. Bacterial colonies were picked and subjected to PCR using appropriate primers upstream and downstream to this E box. PCRs were then digested with *Pml*I; successful mutations were DNA fragments that were resistant to *Pml*I digestion. Plasmid DNA were isolated from these positives and sequenced. The mutation analyzed in the present study had a “G” deletion (CACGTG → CAC*TG). For pGL3-TK, the thymidine kinase (TK) minimal promoter was excised from the luciferase vector, PRL-TK (Promega), with *Hind*III and *Bgl*II and gel-purified. This DNA fragment (760 bp) was inserted into the pGL3-Basic vector that has been cut with the same restriction enzymes. pGL3-TKNATE box₄ was constructed using a sequence containing four 17-bp tandem repeats, each containing the E box with 7 bp of 5'- and 4 bp of 3'-flanking sequences (5'-ACTTCATCACGTGCTCC-3'). Supercoil plasmid DNAs was prepared on Qiagen columns. The correct orientations of all constructs were verified by restriction enzyme digestion and sequencing.

Clock Gene Expression Plasmids—Full-length coding regions of

chicken *bmal1* and *Clock* were PCR-amplified from the original excised plasmid DNA using Expand DNA polymerase (Roche Molecular Biochemicals) and ligated into pcDNA3.1 V5-His expression vector (Invitrogen). Correct orientation of each construct was verified by sequence analysis. Human expression plasmids of BMAL1 (MOP3) and MOP4 were a gift from Dr. John Hogenesch and Dr. Chris Bradfield (University of Wisconsin Medical School), and the arginine vasopressin (AVP) E box luciferase reporter constructs and mouse CLOCK expression plasmid were provided by Dr. Steven Reppert (Harvard Medical School).

Pineal Cell Culture and Transient Transfection—Chicks were killed by decapitation, and the pineal glands were removed and chilled in sterile complete culture medium. Complete medium had the following composition: modified McCoy's 5A medium (catalog number 12330-031, Life Technologies, Inc.) containing 25 mM Hepes buffer, L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin (Sigma), 2.5 μ g/ml amphotericin B (Sigma), and 10% heat-inactivated fetal bovine serum (Sigma). Primary cultures of dissociated chick pineal cells were prepared as described (36) with minor modifications. The dissociated cells were put through a cell strainer (70 μ m; Falcon) and pelleted. The supernatant was aspirated, and the pelleted cells were resuspended in complete culture medium and plated in two wells of a 6-well plate (Costar Corp) in 3 ml of culture medium per well. After 60 min, cells in suspension were harvested, collected, and resuspended in complete culture medium (0.5 \times 10⁶ cells/ml); and 0.4-ml samples were transferred to individual wells in Vitrogen (Collagen BioMedical)-coated 24-well plates (Costar Corp.). After 60 min, the culture media were changed to Opti-MEM (Life Technologies, Inc.), and pineal cells were transfected using LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer's instructions. Each reaction contained 1 μ g of plasmid with the promoter-luciferase construct, 4 μ l of Plus reagent, 1 μ l of LipofectAMINE, and 0.1 μ g of an internal control, PRL-TK (Promega). Plasmid DNAs and transfection reagents were diluted separately into equal volumes (50 μ l each) of Opti-MEM and mixed briefly. Following a further 3-h incubation, 1 ml of complete media was added to each well. Cells were harvested 48 h later and lysed in passive lysis buffer (Promega; 50 μ l per well). Lysates were immediately assayed for luciferase activity by the use of the Promega Dual Luciferase Assay system (catalog number E1960) according to the manufacturer's instructions. A 10- μ l sample of cell lysate was added to a 100- μ l volume of luciferin substrate; luminescence was measured with a Lumat LB 9507 luminometer (EG & G) set for a 2-s delay and 10-s integration. Relative luciferase activity was normalized to PRL-TK *Renilla* luciferase activity to correct for differences in transfection efficiency.

For co-transfection experiments, COS-7 cells (grown to ~50% confluency in Dulbecco's modified Eagle's medium) were transfected with 200 ng of each expression plasmid, 100 ng of pGL3-TKNATE box₄, and 20 ng of PRL-TK (internal control) and pTarget (Promega) or pcDNA3.1 to keep the amount of DNA per transfection constant. Cells were harvested 48 h post-transfection and assayed for luciferase activity as described above. Co-transfection experiments using AVP E box reporter constructs were done essentially as described (37).

RESULTS

The 5'-Flanking Region of the cANAT Gene Contains an E Box—The full-length cDNA of the cANAT was used to screen a chicken cosmid library. This identified genomic clone 2A, which was purified and digested with *Hind*III to release a ~6-kb insert, and was subsequently subcloned into pBlue-Script II SK(+). Nucleotide sequence analysis of the 5'-flanking region of cANAT gene (GenBankTM accession number AF193072) revealed that it contained several possible regulatory elements (Fig. 1A). A putative TATA box (TATAA) occurs at position -25 upstream of the transcription start point (TSP). A GC-rich region is present (-1403 to -1385), which could function as an Sp1-binding (38) and/or AP-2-binding (39) site, and an A/T-rich region occurs at position -468 to -422, which contains eight repeats of -TTATT- as the core sequence; the A/T-rich elements may act as binding sites for MEF-2 transcription factors (40, 41). In addition, this region could also provide binding sites for the photoreceptor-specific transcription factor CRX (Cone-rod homeobox containing protein (42)). Of special interest was the present finding of an E box element (CACGTG) at position -49 to -44. E box elements are well

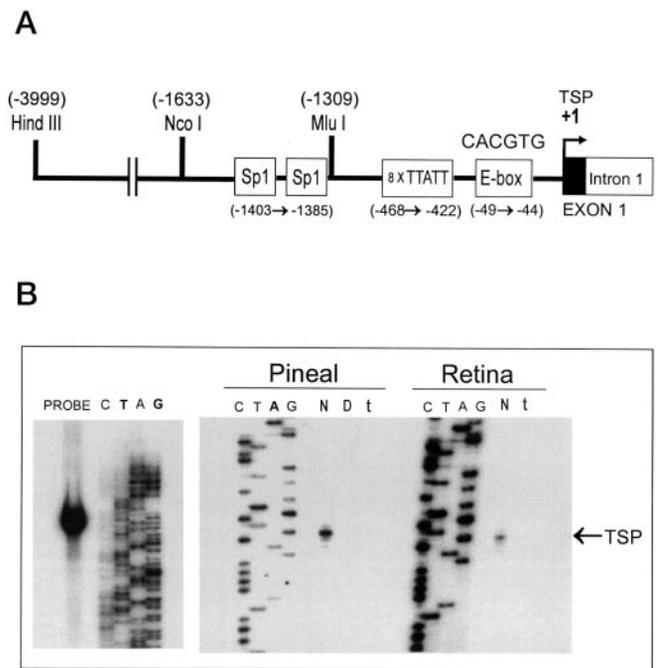


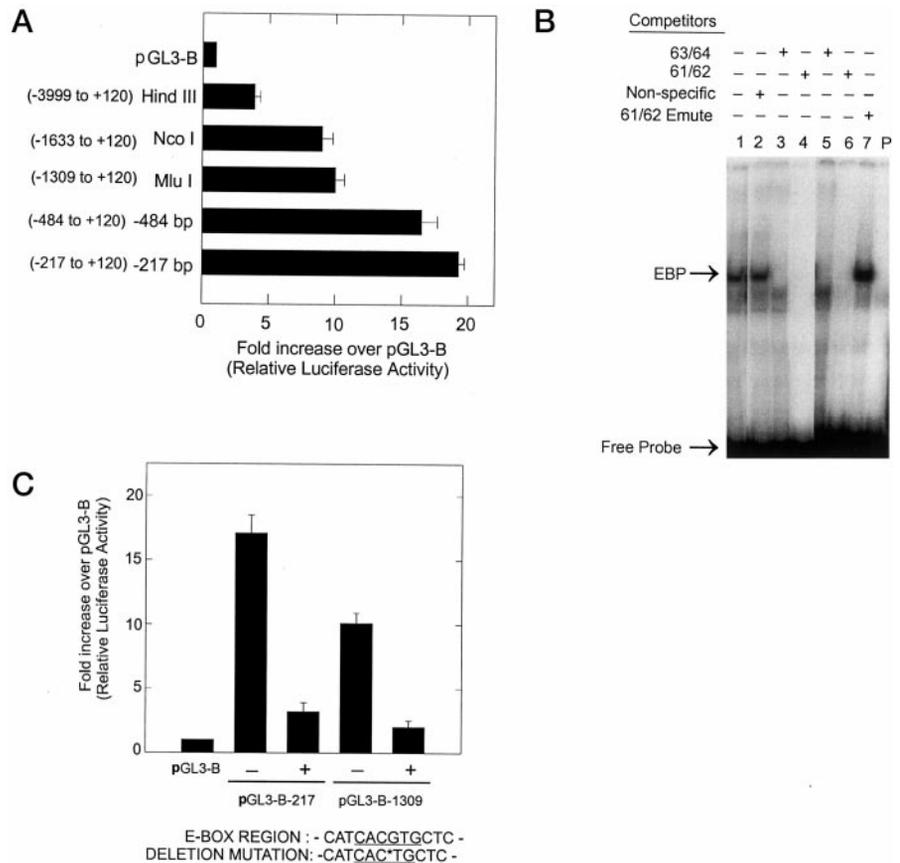
FIG. 1. Structural analysis of the cANAT promoter. A, schematic representation of a partial restriction map of the 5'-flanking region of the cANAT gene showing unique restriction enzyme sites. Potential regulatory elements are boxed between position -1633 and -1, relative to the transcription start point (TSP). B, mapping of the TSP of the cANAT gene by S1 nuclease analysis. A ³²P-labeled single-stranded probe (481 nucleotides) was generated by asymmetric PCR using primers NAT 50 and 27 (see "Experimental Procedures"). A dideoxynucleotide sequencing reaction of clone 76, primed with a ³²P-end-labeled probe of NAT 27, was run in parallel and served as a molecular weight standard (CTAG). N, nighttime; D, daytime; t, transfer RNA control.

defined recognition sites for basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS)-binding domains (43–46) and have been proposed to mediate clock-regulated gene expression in other systems (37, 47).

The TSP was identified using S1 nuclease protection analysis of total RNA isolated from nighttime and daytime chicken pineal glands. A major protected product was detected using the nighttime tissue indicating that the cANAT gene is likely to be transcribed from a single TSP located at the "G" residue (designated +1) in the sense strand of the gene (Fig. 1B). A similar protected product was also obtained when total RNA from nighttime chicken retina was used.

The cANAT E Box Element Binds Protein and Is Required for High Level Reporter Activity of the cANAT 5'-Flanking Region—To identify regulatory regions in the 5'-upstream portion of the cANAT gene, a fusion gene construct pGL3-B *Hind*III was made, containing a segment from -3999 to +120 (relative to the TSP). This was fused to the promoterless luciferase gene in pGL3-Basic. Deletion constructs of the 5'-flanking region of the cANAT gene were transfected into primary chick pineal cells (Fig. 2A). Luciferase activity was normalized to the level of *Renilla* luciferase activity by the co-transfected plasmid pRL-TK. Progressive deletions from the 5'-end increased reporter expression, the highest activity being pGL3-B 217 (-217 to +120). These results suggest either a negative element(s) resides within the region between position -3999 to -217, and/or an enhancer element(s) is located within the shorter fragment (-217 to +120). By taking into account the location of the putative TATA box, it appeared likely that the enhancer element(s) resides between position -217 and -25. Examination of this small fragment of the cANAT 5'-flanking region (217 bp) revealed several potential recognition elements

FIG. 2. Functional analysis of the cANAT promoter. *A*, nested fragments of the cANAT promoter were generated either by 5'-nested deletion or PCR, ligated to the firefly luciferase gene (*pGL3-B*), and transfected into primary chicken pineal cells as described (see "Experimental Procedures"). Cells were harvested after 48 h and assayed for luciferase. Numbers represent position relative to the transcription start point (TSP). Firefly luciferase activity was corrected relative to the *Renilla* luciferase activity. Assays were done in duplicate, and each value is the mean \pm S.E. ($n = 3$). *B*, detection of cANAT E box-specific DNA-binding protein in chicken pineal extracts. Electrophoretic mobility shift assay was carried out using a radiolabeled promoter fragment containing the E box DNA element (-217 to -1). Lane 1, control lane containing the labeled probe with no competitor DNA. Lane 2, nonspecific DNA. Lanes 3 and 5, competitor 63/64 (-56 to -37). Lanes 4 and 6, competitor 61/62 (-217 to -1). Lane 7, competitor 61/62 with E box mutation (*Emute*, see *C*). *P*, no cell extracts; *EBP*, E box-binding protein(s). This experiment was repeated with a different batch of cell extracts and produced similar results. *C*, the effect of a single nucleotide deletion E box mutation on cANAT promoter activity in chicken pineal cells *in vitro*. Numbers represent position relative to the TSP. Each value is the mean \pm S.E. of three replicates for a single assay. Similar results were found in a replicate assay.



for DNA-binding proteins, including the E box.

Putative cis-acting element(s) involved in cANAT transcription were identified using EMSA. DNA fragments (~80–300 bp) of the 5'-flanking region (from -1700 to -1), which were amplified using reverse transcriptase PCR and end-labeled with ^{32}P , were used as probes. Initial screening showed that three small stretches were capable of recruiting DNA-binding proteins. These include the E box at position -49 to -44, two tandem Sp1-binding elements (-1403 to -1385; data not shown), and the A/T-rich region (-468 to -422; data not shown). An end-labeled probe containing the E box (-217 to -1) formed a single major complex with cell extracts of chicken pineal glands (Fig. 2*B*); similar results were obtained with retina extracts (data not shown) in EMSA. This binding was effectively competed by a 200-fold molar excess of nonradiolabeled DNA (lanes 4 and 6) or with a 20-bp oligonucleotide that contains the cANAT E box sequence (lanes 3 and 5). The ability to compete was abolished when the core sequence of the E box element was mutated (CACGTG to CAC*TG; lane 7). Unrelated DNA had no effect on the shifted band (lane 2). These observations indicate that the E box element in the cANAT 5'-flanking region is a target for a sequence-specific DNA-binding factor(s).

To further define the functional elements responsible for cANAT transcription, a single nucleotide deletion was introduced in the core sequence of the E box element in two cANAT reporter constructs (pGL3B-217 and pGL3B-1309). These mutated constructs were transiently transfected into primary chicken pineal cells. For both constructs, the E box mutation resulted in 85–90% loss in transcriptional activity (Fig. 2*C*). These results demonstrated that the E box element could account for the majority, if not all, of the reporter activity within this 1309-bp 5'-flanking region.

Circadian Clock Genes Are Expressed in the Chicken Pineal Gland—The hypothesis that cANAT expression is driven by

BMAL1/CLOCK, as in mammals (27), was examined by determining whether these transcription factors are expressed in the chicken pineal gland. Partial cDNAs encoding *bmal1*, *MOP4*, and *Clock* were isolated from chicken pineal mRNA using degenerate primers. The isolated *bmal1* fragment (384 bp; covering the bHLH and part of the PAS A region, GenBankTM accession number AF193070) shared 83% identity with other *bmal1* homologs from different species (28, 48, 50, 51). Certain regions of the isolated chicken *MOP4* cDNA (~1.2 kb) shared 80–83% identity with other published *MOP4* sequences (49, 52). The cloned *MOP4* sequence also had some degree of homology to CLOCK. *MOP4* has been reported to be a homolog of CLOCK, and their proteins share a high level of sequence identity in the bHLH and PAS domains (28, 53). Moreover, they both share BMAL1 as a common dimeric partner (27, 28, 30, 50, 54, 55). Specific primers for chicken *MOP4* were subsequently synthesized and used in PCR with chicken pineal cDNA as template. This produced a single product of the predicted size (333 bp, GenBankTM accession number AF193071), and its authenticity was confirmed by sequencing. The isolated fragment of chicken *Clock* (490 bp) shares 87% identity with the mouse CLOCK at the amino acid level (amino acids 358–520). The full-length cDNA clone of chicken CLOCK shares 85% identity (with 5.5% strongly similar and 5.1% weakly similar) to the mouse CLOCK amino acid sequence (MegAlign, Lasergene program, DNASTar) with percentage identity for bHLH, PAS A, and PAS B domains of 100, 92 (96% strongly similar), and 100, respectively. In addition, a high conservation of the polyglutamine-rich region near the C terminus was present in the chicken CLOCK protein.

Specific probes were generated for chicken clock genes and used to identify their mRNA transcripts using Northern blot analysis at high stringency (Fig. 3). The *bmal1* probe hybridized to two fragments in polyadenylated RNA from chicken pineal gland (ZT 9), a major transcript size of 2.6 kb and a

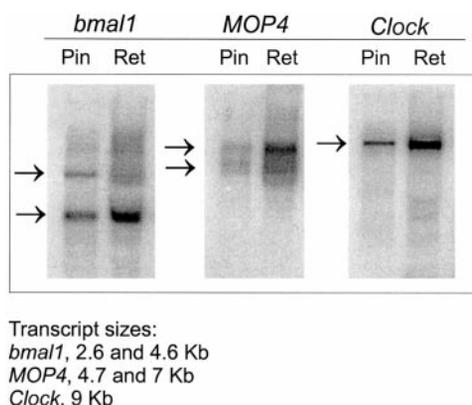


FIG. 3. Northern blot analysis showing co-expression of chicken *bmal1*, *MOP4*, and *Clock* mRNAs in pineal gland and retina. 1.5 μ g of pineal poly(A)⁺ RNA and 20 μ g of retinal total RNA were loaded. The blots were repeated with similar results on independently obtained samples. The arrows are pointing to the transcripts; the largest one is on top. *Pin*, pineal gland; *Ret*, retina.

minor one at 4.6 kb. The *MOP4* probe hybridized to two transcripts (4.7 and 7 kb) with apparent similar mRNA abundance (Fig. 3). *MOP4* mRNA expression was weak, as has previously been reported for mammalian tissues (28, 49, 52). The *Clock* probe hybridized to one major transcript, approximately 9 kb, in chicken pineal and retinal RNA, although minor transcripts may also be present in the retina (Fig. 3; see Ref. 56). This establishes that the *bmal1*, *Clock*, and *MOP4* genes are expressed in the chicken pineal gland and retina.

Daily Rhythms in Circadian Clock Genes—The existence of daily rhythms in *bmal1* mRNA was examined in RNA prepared from pineal tissue. cANAT mRNA was also examined to provide an internal marker of functional rhythmicity (25, 35). The marked 24-h rhythm in pineal cANAT was evident, and high levels occurred at ZT 18 (Fig. 4A). Pineal *bmal1* mRNA levels changed on a 24-h basis in an L:D cycle with a 4-fold increase at ZT 13–16 (Fig. 4A). This rhythmic pattern persisted in animals maintained in DD (Fig. 4B), indicating that these changes are controlled by an endogenous clock. A rhythm in *bmal1* mRNA was also found to exist in the retina in LL, with a similar profile of expression.² *MOP4* mRNA changed in a rhythmic manner in the chicken retina in LL, with peak levels at early subjective night.² However, pineal *MOP4* mRNA levels in poly(A)⁺ RNA failed to exhibit a detectable rhythm in DD or LL.

Chicken pineal *Clock* mRNA did not exhibit robust rhythm in L:D (Fig. 5). However, cycling of *Clock* mRNA, or its gene product, cannot be ruled out as there was a small amplitude (~25%) in mRNA expression, with apparent levels peaking at the light-dark transition (Fig. 5). This is consistent with observations of small (~20–80%) amplitude rhythms in *Clock* mRNA in chicken and rat retina (56, 57).

Transcription Factors That Interact and Transactivate the cANAT E Box—The role of clock genes in cANAT transcription was studied following strategy that has been used previously to demonstrate clock gene regulation of transcription, in which a reporter plasmid containing four copies of an E box element in tandem (28, 29, 31, 37) is co-transfected with putative regulatory bHLH-PAS transcription factors to determine their influence on transcription.

To examine the functionality of the cANAT E box, we constructed and used a reporter plasmid that contained four copies of the chicken E box element (17 bp with 5'- and 3'-flanking sequences) in tandem, upstream of a TK promoter-

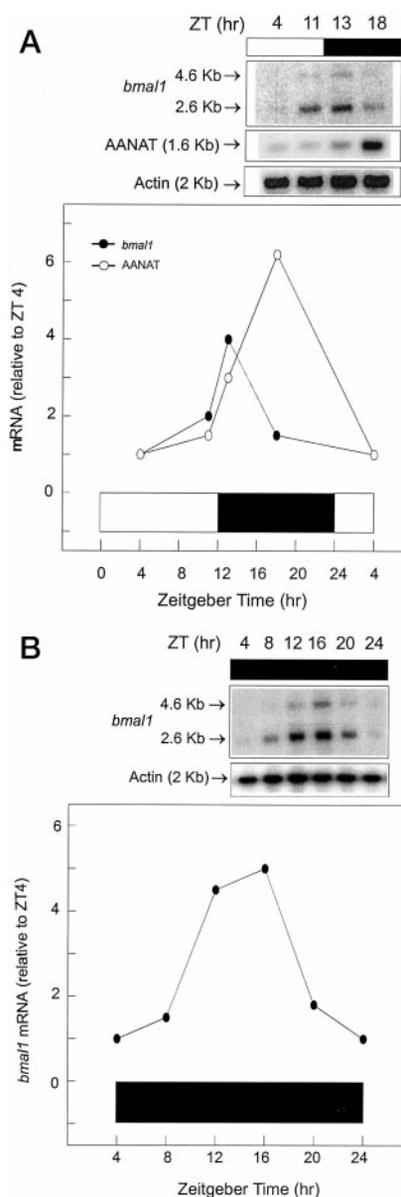


FIG. 4. Rhythmic expression of *bmal1* mRNA in the chicken pineal gland. A, light-dark profile of *bmal1* and AANAT mRNAs. The light cycle is indicated by the open and filled bars. Only the 2.6-kb *bmal1* transcript was quantified. Data at ZT 4 are double-plotted. B, temporal profile of *bmal1* mRNA studied in constant darkness. Under both lighting conditions, *bmal1* mRNA clearly demonstrates a rhythmic variation in abundance with peak levels at ZT 12–16. All experiments were repeated with similar results on independently obtained samples. The abundance of *bmal1* and AANAT transcripts has been normalized to actin mRNA.

luciferase reporter. Co-transfection of the reporter plasmid into COS-7 cells with chicken BMAL1 and CLOCK enhanced transcription 7-fold (Fig. 6); co-transfecting human BMAL1 and MOP4 also enhanced transcription (5.3-fold, see Fig. 6). In contrast, transfections with BMAL1, CLOCK, or MOP4 alone failed to drive transcription over control levels. In control experiments, transcription of the AVP E box reporter was enhanced 11-fold following co-transfection with mouse CLOCK and human BMAL1; E box mutations of this AVP reporter decreased enhancement by ~85% to 1.8-fold. Transfection with BMAL1 or CLOCK alone failed to drive transcription of the AVP E box reporter (data not shown). These results show that chicken BMAL1/CLOCK and BMAL1/MOP4 heterodimeric partners can activate cANAT transcription in the chicken pineal gland *in vitro*.

² N. W. Chong and D. C. Klein, unpublished results.

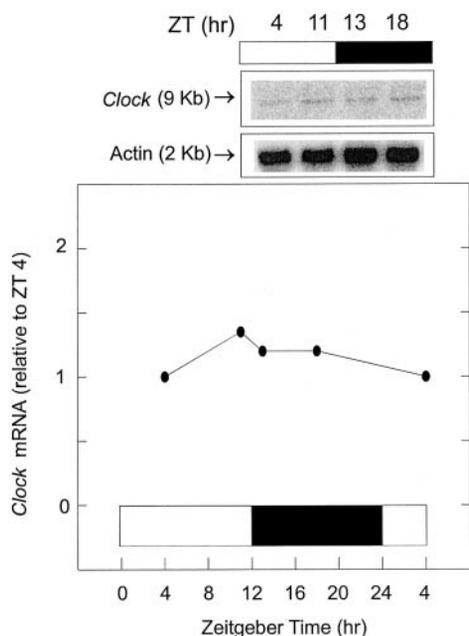


FIG. 5. The *Clock* transcript does not cycle. A Northern blot of chicken pineal RNA, taken at the indicated times and probed with *Clock*, is shown. The transcript migrates at ~9 kb and shows no robust cycling in this and one other separate experiment. The abundance of the *Clock* transcript was normalized to actin mRNA. Data at ZT 4 are double-plotted.

DISCUSSION

We report here the isolation and characterization of the 5'-flanking region for the cAANAT gene. The results of this study indicate that AANAT mRNA levels in the chicken pineal gland are regulated by an E box enhancer. These findings can be organized into four groupings. The first points to the E box as having regulatory function. The second has determined that the chicken pineal gland and retina express genes encoding three important clock-related transcription factors. The third provides evidence of rhythmic expression of the transcription factor *bmal1*. The fourth provides evidence that cAANAT transcription can be activated through the interaction of these transcription factors. These four sets of advances will be discussed sequentially below.

The cAANAT E Box—E box elements (27, 30, 31, 43–46) appear to mediate clock-regulated expression of several genes (37, 47). Data presented here indicate that an E box sequence mediates expression of cAANAT. Specifically, this includes the presence of a perfect E box sequence in the cAANAT promoter and the essential nature of this E box for binding pineal proteins and for full reporter activity of the 1309 bp 5'-flanking region. In addition, mutation of this E box blocks function. It is of interest that a functional E box element has also been identified in the rat AANAT gene (58) and that it functions in the context of the rat retina, which is reported to have endogenous clock function, but not in the rat pineal, which is not known to have a functional clock.

Clock Genes in the Chicken Pineal Gland and Retina—E box sequences are thought to bind heterodimeric complexes composed of combinations of CLOCK, BMAL1, and MOP4 proteins (27, 29, 30, 55). Gene expression is turned on as a result of this E box/transcription factor interaction. In the studies presented here, Northern blot analysis data indicates all three genes are expressed in the chicken pineal gland and retina. This makes it appear likely that the E box sequence in the cAANAT gene can mediate activation by a heterodimeric complex of two of these gene products.

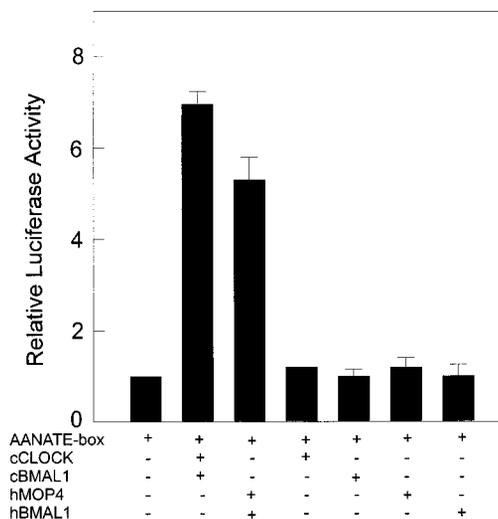


FIG. 6. *Clock* gene heterodimers activate cAANAT gene transcription via an E box (CACGTG). *In vivo* interaction of chicken BMAL1 (*cBMAL1*) with chicken CLOCK (*cCLOCK*) and human MOP4 (*hMOP4*) with human BMAL1 (*hBMAL1*), COS-7 cells were transfected with the cAANATE box luciferase reporter with each expression plasmid as indicated. Cells were harvested 48 h post-transfection. Assays were done in triplicate, and the experiment was repeated with similar results. Each value is the mean \pm S.E. of three replicates for a single assay.

Activation of cAANAT by Clock Gene Dimers—As indicated above, it is thought that heteromeric dimers containing combinations of BMAL1, MOP4, and CLOCK activate clock-related genes through interactions with the E box. In the current report, evidence was obtained that indicate cAANAT is regulated through an E box by a heterodimeric complex of BMAL1 with either CLOCK or MOP4. This combination has also been found to activate expression of the mouse AVP and *Per1* gene through an E box (29, 37). Although the co-transfection strategy has been used extensively in characterizing the interactions between putative clock genes, it must be emphasized that this approach uses artificial *in vitro* systems that may not fully reconstruct the features of the natural *in vivo* system. Factors that form heterodimers with proven clock genes *in vitro* may not be co-expressed with those genes *in vivo*. This appears to be the case for mammalian MOP4 where *MOP4* mRNA is undetectable in the SCN (59). This is not the case in the chicken, where *MOP4* mRNA is expressed in both the pineal gland and retina and therefore may play a role in avian clock function. It should be added that the molecular organization of the clock appears to be more complex than originally proposed, and it is not unlikely that the number of proteins involved in clock function will also increase (31).

At present, the role of the CRX-binding sites in cAANAT transcription is uncertain. Interactions between proteins that bind to separate promoter elements are likely to require DNA bending and the correct orientation of transcription factor binding to allow juxtaposition of the molecular surfaces that mediate the interaction (60–62). Although the distance between the CRX sites and the E box is ~300–380 bp, these poly(dA)-poly(dT) tracts could act as potential DNA bend sites (63, 64). By taking into account that full-length dimer proteins bend DNA by ~25–30% (65), it is conceivable that CRX and clock gene heterodimers may act in a cooperative manner to regulate cAANAT transcription.

Several studies have shown that DNA-binding elements such as CRX, and a similar site called photoreceptor consensus element, play a pivotal role in directing cell-specific expression of genes (66). Since the mutation of the cAANAT E box eliminated 90% of reporter activity (Fig. 2C), it seems likely that the

major function of the cAANAT CRX site is to confer tissue-specific expression of cAANAT. In support of this, CRX-binding sites have been identified in promoter regions of several pineal gland- and retina-specific genes in rat, including AANAT (67). CRX was able to transactivate these sites and enhance promoter activity using a reporter assay. In addition, the expression of pineal gland and retina AANAT mRNA is greatly reduced in Crx-deficient mice, and the photo-entrainment component in these Crx-deficient mice was attenuated (68).

Rhythmic Expression of Clock Genes in the Pineal Gland—A current theory of the molecular basis of clock-regulated gene expression is that rhythmic expression reflects rhythmic changes in the abundance of the appropriate heterodimeric complexes (27). This appears to occur in the chicken pineal gland, based on the analysis of mRNA encoding *bmal1*, a robust rhythm in *bmal1* mRNA occurs in DD. At present, the rhythmic expression in *MOP4* mRNA in chicken pineal is uncertain. Accordingly, it appears likely that a rhythm in the BMAL1/MOP4 or BMAL1/CLOCK heterodimers occurs and is the essential perturbing factor that drives the rhythm in cAANAT. It is assumed that changes in mRNA are translated into changes in protein. It will be important to confirm this in the chicken pineal gland, retina, and in other systems by direct analysis of proteins. Collectively, these studies provide evidence that circadian changes in cAANAT mRNA may reflect a direct link to the circadian clock that involves interaction between the cAANAT E box and a heterodimeric complex, which is likely to be BMAL1/CLOCK or BMAL1/MOP4. The proposed model of clock-driven cAANAT expression presented here does not address the issue of negative factors, such as *Per* and *Cry* genes, which turn off expression (29, 31, 37). It is not clear whether these are directly involved in turning off cAANAT or whether this is only a reflection of rhythmic changes in clock gene dimers.

In conclusion, our data suggest that BMAL1/CLOCK and BMAL1/MOP4 heterodimers can regulate cAANAT mRNA expression. This is of special interest because it supports the hypothesis that there is a functional molecular link between the synthesis of pineal melatonin and clock function. As indicated above, this link appears to exist in the rat retina, as well. Future research on the cAANAT system might provide important new insights into the circadian clock within the chicken pineal gland, how it is linked to output genes that control or modulate rhythms in melatonin physiology and behavior, and the basis of molecular differences among species in the links between the clock and output genes. The direct clock-AANAT mRNA link in the pineal gland has special utility because the chicken pinealocyte is used routinely as a model system of clock function; it is especially attractive because it is easily removed, and the population of pinealocytes is relatively homogenous and the output signal, melatonin, is easily detectable. Based on this, and the highly conserved nature of clock mechanisms and molecules, it is reasonable to consider that the chicken pineal gland might serve as a useful tool for the identification and development of drugs that alter clock function in man.

Acknowledgments—We thank Drs. J. Hogenesch and C. Bradfield for generously providing the human expression constructs of BMAL1 (MOP3) and MOP4; Dr. S. Reppert for the arginine vasopressin E box reporter constructs and mouse CLOCK expression plasmid; and Dr. I. Rodriguez for the chicken cosmid library. We gratefully acknowledge Dr. Michael Iuvone for providing some of the tissue samples that were used in this study. We also express our appreciation to Dr. Ruben Baler for valuable discussion and for providing us his manuscript (Chen and Baler (58)) prior to publication.

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