Characterization of the Chicken Serotonin N-Acetyltransferase Gene

ACTIVATION VIA CLOCK GENE HETERODIMERE BOX INTERACTION*

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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 marked changes in melatonin 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, the retina, is 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 production 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 (SCN); 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).

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.

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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. 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 pWE15 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 plates and digested with HinIII. An ~6-kb fragment was gel-purified and subcloned into the phagemid vector pBluescript II SK(+) that has been cut with HinIII 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 (Watt 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 5′-CCAGTCCTTTGGCCGAGGACG (reverse, 5′-ACTCTCTTGAGCCATTATGCATATGC-3′) for bmal1, and CKF1 (forward, 5′-GGATCCTGGTCACGAAGGCGGCA-AG) (GenBank accession number AF144425) was subsequently 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′-TGGAGAGACAGACGGAGATGG-3′) and M4R1 (reverse, 5′-GGTTGAGAAGGCAGAGGAG-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 loaded from a pool of three pineal glands. RNA was extracted using Trizol according to the manufacturer’s instructions (Invitrogen). RNA samples were digested with the restriction enzyme EcoRI and the largest portion of the EcoRI fragment was blunted with Klenow fragment and subcloned into the pGEM-3Z vector (Promega). A luciferase vector, pGL3-Basic (Promega), was used as a positive control.

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**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′-ACAAGCTTGGGGCTTTTTTCCAGGAG-3′; reverse, 5′-ACTCTCTTGAGCCATTATGCATATGC-3′) for bmal1, and CKF1 (forward, 5′-GGATCCTGGTCACGAAGGCGGCA-AG) (GenBank accession number AF144425) was subsequently 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′-TGGAGAGACAGACGGAGATGG-3′) and M4R1 (reverse, 5′-GGTTGAGAAGGCAGAGGAG-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.

**Electrophoretic Mobility Shift Assay (EMSA)**—Pineal glands were dissected from chicks at nighttime (ZT 18) and quick-frozen on solid nitrogen. pineal glands (15–20 mg) were homogenized in 0.5 ml of 1× TNE buffer (20 mM Tris (pH 8.0), 1 mM EDTA, 0.5 mM PMSF, 1 mM Na3VO4) and centrifuged at 12,000 × g for 15 min. Supernatants were used for 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× TBE buffer (50 nM Tris (pH 8.3), 3 × 10−3 M glycine, 1× EDTA). Gels were dried; they were then imaged and analyzed using a STORM 860 PhosphorImager (Molecular Dynamics).

**Chickens were caged and allowed to acclimate for 7 days.**
The 5'-Flanking Region of the cAANAT Gene Contains an E Box—The full-length cDNA of the cAANAT was used to screen a chicken cosmid library. This identified genomic clone 2A, which was purified and digested with HindIII to release a ~6-kb insert, and was subsequently subcloned into pBlue-Script II SK (+). Nucleotide sequence analysis of the 5'-flanking region of the cAANAT gene (GenBank TM 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 (~1405 to ~1385), which could function as an Sp1-binding site (38) and/or AP-2-binding site (39), 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 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 cAANAT 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.

RESULTS

The 5'-Flanking Region of the cAANAT Gene Contains an E Box—the full-length cDNA of the cAANAT was used to screen a chicken cosmid library. This identified genomic clone 2A, which was purified and digested with HindIII to release a ~6-kb insert, and was subsequently subcloned into pBlue-Script II SK (+), Nucleotide sequence analysis of the 5'-flanking region of the cAANAT gene (GenBank TM 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 (~1405 to ~1385), which could function as an Sp1-binding site (38) and/or AP-2-binding site (39), 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 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).

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The cAANAT E Box Element Binds Protein and Is Required for High Level Reporter Activity of the cAANAT 5'-Flanking Region—To identify regulatory regions in the 5'-upstream portion of the cAANAT gene, a fusion gene construct pGL3-B HindIII 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 cAANAT 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. 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) were transfected with 200 ng of each expression plasmid, 100 ng of plasmid pGL3-TK, and 200 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).

FIG. 1. Structural analysis of the cAANAT promoter. A, schematic representation of a partial restriction map of the 5'-flanking region of the cAANAT 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 cAANAT 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.
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**FIG. 2. Functional analysis of the cAANAT promoter.** A, nested fragments of the cAANAT 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 ± S.E. (n = 3). B, detection of cAANAT 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 cAANAT promoter activity in chicken pineal cells in vitro. Numbers represent position relative to the TSP. Each value is the mean ± 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 cAANAT 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-labelled with 32P, 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 Spi-binding elements (−1403 to −1385; data not shown), and the A/T-rich region (−468 to −422; data not shown). An end-labelled probe containing the E box (−217 to −1) formed a single major complex with cell extracts of chicken pineal glands (Fig. 2B); similar results were obtained with retina extracts (data not shown) in EMSA. This binding was effectively competed by a 200-fold molar excess of nonradiolabelled DNA (lanes 4 and 6) or with a 20-bp oligonucleotide that contains the cAANAT 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 cAANAT 5’-flanking region is a target for a sequence-specific DNA-binding factor(s).

To further define the functional elements responsible for cAANAT transcription, a single nucleotide deletion was introduced in the core sequence of the E box element in two cAANAT reporter constructs (pGL3B-217 and pGL3B-1309). The 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. 2C). 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 cAANAT 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, 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) shared 87% identity with the mouse CLOCK at the amino acid level (amino acids 358–520). The full-length cDNA clone 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
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. cAANAT mRNA was also examined to provide an internal marker of functional rhythmicity (25, 35). The marked 24-h rhythm in pineal cAANAT 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 cAANAT E Box—The role of clock genes in cAANAT 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 cAANAT 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-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 of the AVP E box reporter (data not shown). These results show that chicken BMAL1/CLOCK and BMAL1/MOP4 heterodimeric partners can activate cAANAT transcription in the chicken pineal gland in vitro.

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

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 bind 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 characteristic of chicken serotonin N-acetyltransferase gene.

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.

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 cAANAT 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 ± S.E. of three replicates for a single assay.
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 homodimeric 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 AANAT 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 pine 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 homogeneous 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.

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REFERENCES

1. Klein, D. C. (1985) CIBA Found. Symp. 117, 38–56
2. Binkley, S. (1988) The Pineal: Endocrine and Neuroendocrine Function, Prentice-Hall, Englewood Cliffs, NJ
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55. Green, C. B. (1998) Trends Cell Biol. 8, 342–343
56. Larkin, P., Baehr, W., and Semple-Rowland, S. L. (1999) Mol. Brain Res. 70, 253–263
57. Namihira, M., Honma, S., Abe, H., Tanahashi, Y., Ikeda, M., and Honma, K.-I. (1999) Neurosci. Lett. 271, 1–4
58. Chen, W., and Baler, R. (2000) Mol. Brain Res., in press
59. Shearman, L. P., Zylka, M. J., Reppert, S. M., and Weaver, D. R. (1999) Neuroscience 89, 387–397
60. Giese, K., Kingsley, C., Kirshner, J. R., and Grosschedl, R. (1995) Genes Dev. 9, 995–1008
61. Robertson, L. M., Kerppola, T. K., Vendrell, M., Luk, D., Smyne, R. J., Bocchiaro, C., Morgan, J. I., and Curran, T. (1995) Neuron 14, 241–252
62. Kim, T. K., and Maniatis, T. (1997) Mol. Cell 1, 119–129
63. Wada-Kiyama, Y., and Kiyama, R. (1999) J. Biol. Chem. 270, 12439–12445
64. Wada-Kiyama, Y., Kuwabara, K., Sakuma, Y., Onishi, Y., Trifonov, E. N., and Kiyama, R. (1999) FEBS Lett. 444, 117–124
65. Kerppola, T. K., and Curran, T. (1991) Science 254, 1210–1214
66. Mani, S. S., Besharse, J. C., and Knox, R. E. (1999) J. Biol. Chem. 274, 15590–15597
67. Li, X., Chen, S., Wang, Q., Zack, D. J., Snyder, S. H., and Borjigin, J. (1998) Proc. Natl. Acad. Sci. U. S. A. 97, 1876–1881
68. Furukawa, T., Morrow, E. M., Li, T., Davis, F. C., and Cepko, C. L. (1999) Nat. Genet. 23, 466–470