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# Rodent *Aanat*: Intronic E-box sequences control tissue specificity but not rhythmic expression in the pineal gland

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**Abstract**

Arylalkylamine *N*-acetyltransferase (Aanat) is the penultimate enzyme in the serotonin–*N*-acetylserotonin–melatonin pathway. It is nearly exclusively expressed in the pineal gland and the retina. A marked rhythm of *Aanat* gene expression in the rat pineal is mediated by cyclic AMP response elements located in the promoter and first intron. Intron 1 also contains E-box elements, which mediate circadian gene expression in other cells. Here we examined whether these elements contribute to rhythmic *Aanat* expression in the pineal gland. This was done using transgenic rats carrying *Aanat* transgenes with mutant E-box elements. Circadian expression of *Aanat* transgenes was not altered by these mutations. However, these mutations enhanced ectopic expression establishing that the intronic *Aanat* E-box elements contribute to the gene's pineal specific expression. A similar role of the *Aanat* E-box has been reported in zebrafish, indicating that *Aanat* E-box mediated silencing is a conserved feature of vertebrate biology.


*Keywords:* Arylalkylamine *N*-acetyltransferase; Pineal gland; Transgenic rat; E-box; Circadian rhythm

## 1. Introduction

Daily rhythms of melatonin production in the pineal gland are governed by a rhythm in the synthesis and activity by the penultimate enzyme in the melatonin pathway, arylalkylamine *N*-acetyltransferase, Aanat; (Coon et al., 1995; Klein et al., 1997; Klein, 2006). This enzyme is encoded by *Aanat* which is rhythmically expressed in the rodent pineal gland, resulting in a ∼150-fold increase in the abundance of *Aanat* mRNA at night (Coon et al., 1995; Roseboom et al., 1996; Klein et al., 1997).

The increase in expression of the *Aanat* gene in the rat pineal gland appears to result from *cis*-acting mechanisms that involve a cAMP-responsive element (CRE)-CCAAT complex within the proximal promoter and a CRE located in the first intron (Baler et al., 1997, 1999). Transcription appears to be activated in response to cAMP-dependent phosphorylation of CRE binding protein (CREB) (Roseboom and Klein, 1995; Maronde et al., 1999). The first intron of the rat *Aanat* gene also contains an E-box element (Baler et al., 1999; Fig. 1A), which is of interest because a sub-set of E-boxes, described as circadian E-boxes (Munoz et al., 2002) mediate rhythmic gene expression through interaction with the *trans*-acting clock proteins CLOCK and BMAl1 (Jin et al., 1999; Reppert and Weaver, 2002); *clock* and *Bmal1* are both expressed in the rat pineal gland (Namahira et al., 1999). Furthermore, functional *Aanat* E-box elements have been characterized in other species (Appelbaum et al., 2004; Chong et al., 2000); in the zebrafish these elements appear to form part of an enhancer (PRDM, pineal-restrictive downstream module) that includes photoreceptor conserved elements (PCEs). PCEs are found in genes expressed in the pineal gland and retina and are thought to be essential for this pattern of expression (Appelbaum and Gothilf, 2006). This enhancer contributes not only to the control of rhythmic expression of *Aanat* but also to the determination of tissue-specific zebrafish (zf) *Aanat* expression; the mechanism appears to reflect the interaction of OTX proteins and PCE in the enhancer (Appelbaum et al., 2005).

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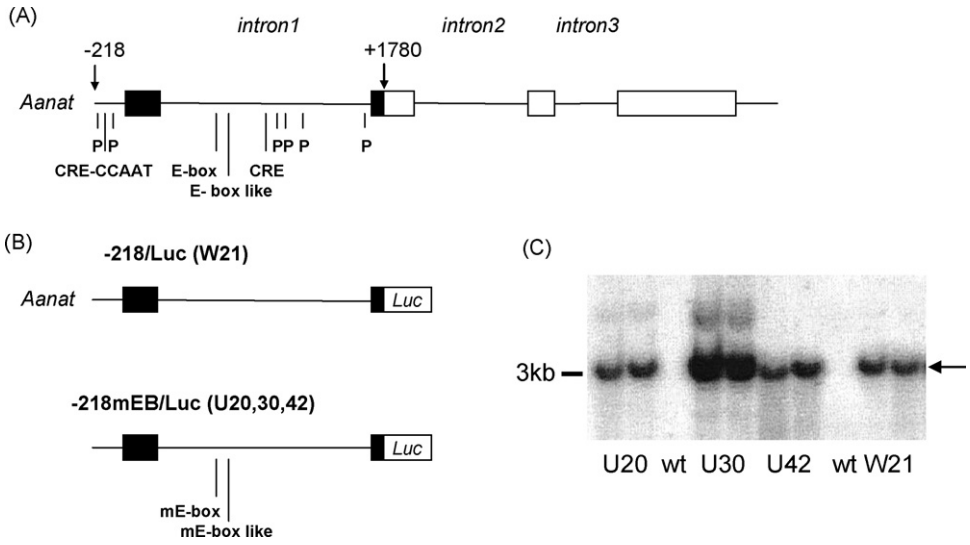


Fig. 1. Transgene design and transgenic line genotype. (A) Schematic representation of the rat *Aanat* gene showing the four exons (boxes) and three introns. The positions of selected regulatory elements are indicated by vertical lines. *P* = photoreceptor conserved element. (B) Schematic representation of the two transgene constructs: a wild-type transgene (−218/LUC) composed of 218 bases of 5′ *Aanat* promoter sequence together with the next 1786 bases of downstream sequence that includes exon 1 and partial exon 2 sequence and the intervening intron 1, linked to the luciferase reporter gene; a mutant transgene (−218mEB/LUC) that includes mutations in the *Aanat* intronic E-box and E-box like sequences. (C) Phosphor screen image of a representative Southern blot of *PstI*-digested genomic DNA extracted from each of the four transgenic lines (U20, U30, U42, W21) and two wild-type rats (wt). Note the presence of a diagnostic ∼3 kb transgene-specifc band (arrow) that is present at different intensities according to the transgene copy number of individual lines.

The ∼2 kb region between −218 and +1786 (relative to transcription start site) in the rat *Aanat* gene is sufficient to mediate both rhythmic and tissue-specific expression of rat *Aanat* (Burke et al., 1999; Smith et al., 2001). Of special interest is the importance of intron 1 to the rhythmic expression of *Aanat*. Little is known about this region, other than gross deletion results in decreased pineal expression and increased ectopic expression (Burke et al., 1999) and that it appears to influence the basal level of expression of the gene in non-pineal cells; in addition the CRE in intron 1 amplifies *in vitro* cAMP responsiveness of reporter constructs containing the CRE/CCAAT complex (Baler et al., 1999). The role of other putative regulatory elements in intron 1 has not been determined.

Here, we examined the role of two related E-box elements in intron 1, a consensus E-box element (CACGTG) and an E-box-like element (CACATG) (Fig. 1A). This was done using a reporter transgene composed of the ∼2 kb sequence that contains the promoter region and intron 1 of *Aanat* described above. The role of the intronic E-box elements was examined *in vivo* in animals carrying a reporter construct in which these elements were distrupted by the insertion of one or two bases. The results of these studies are presented here.

## 2. Methods

### 2.1. Design of constructs and transgenic rat production

The *Aanat*-luciferase transgene construct used in the production of the novel lines of transgenic rats reported here has been described (Burke et al., 1999). This transgene (−218/Luc, which served as the wild-type control for the current study, contains *Aanat* genomic sequence from position −218 to +1786 relative to the transcription start site, and includes the entire 1612 nucleotide intron 1 sequence (Fig. 1A and B). A mutant E-box transgene (−218mEB/Luc was derived from the wild-type transgene by introducing three point mutations: the consensus E-box at position +623 was changed from CACGTG to CACAGTG and the E-box like sequence at position +636 was changed from CACATG to CACTTATG (Fig. 1B; see Chen and Baler, 2000). These sequences were selected for mutation firstly because the +623 E-box is the only consensus E-box within intron 1, and secondly because both this E-box and the +636 E-box-like sequence are both fully conserved between rat and mouse, lying within a 24 bp run of intronic sequence that is 100% conserved between these rodent genomes (Ensembl genes: *ENSRNOG00000011182* and *ENSMUSG00000020804*). In contrast, other E-box-like CACATG sequences within intron 1 of both the rat (+221 and +1486) and mouse (+1336 and +1354) genomes are not located within highly conserved sequence. To confirm that the mutant sequences had been properly incorporated into the genome of transgenic rats, genomic DNA was isolated from tail biopsies and subjected to DNA sequencing using the Prism™ Ready Reaction Dye-Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA, USA) and an ABI Prism™ automated DNA sequencer (377; Perkin-Elmer).

Transgenic rats were generated as described (Burke et al., 1999). The genotype of individual transgenic founder animals was characterized by Southern blot analysis of *PstI*-digested genomic DNA using a 1.6 kb NcoI/XbaI, luciferase-specfic fragment of the transgene (Fig. 1B). Following the characterization of founders, multiple distinct lines of transgenics were established: three lines harboring the mutant E-box transgene (U20, U30, U42), and one line harbouring the wild-type transgene (W21).

### 2.2. Animals and sampling

Animal studies were conducted in accordance with both UK Home Office regulations, and local ethical review. Adult (3–4 month) Sprague-Dawley (CD) transgenic rats were maintained in standard laboratory conditions in a 14:10 light:dark cycle (lights on: 05.00 h), and then transferred to constant darkness for a period of three days prior to sampling. Animals were killed by cervical dislocation at the indicated times of the daily cycle; tissues were rapidly dissected, and frozen on dry ice and stored.

### 2.3. qRPA analysis of transcript rhythms

Quantitative ribonuclease protection assays (qRPA) of gene rhythms were conducted using the RPA III kit according to the manufacturers instructions (Ambion, Austin, TX). qRPA probes were constructed by annealing and ligat-

Table 1
Oligonucleotides used in construction of qRPA probes

| Gene | Acc. no. | Position | Sequence |
|---|---|---|---|
| GAPDH s | NM_017008 | 585–611 | tgtggatggcccctctggaaagctgtggcgtgatg |
| as | | | ttaacatcacgccacagctttccagaggggccatccacaat |
| AANAT s | NM_012818 | 399–433 | atgagatccggcacttcctcaccctgtgtccagag |
| as | | | ttaactctggacacagggtgaggaagtgccggatctcatat |
| LUC s | U03687 | 1084–1118 | ggagttgcagttgcgcccgcgaacgacatttataa |
| as | | | ttaattataaatgtcgttcgcgggcgcaactgcaactccat |

Oligonucleotide sequences are 5′–3′. LUC: luciferase; s: sense; as: antisense.

ing pairs of complementary oligonucleotides into MultiProbe vectors: GAPDH, vector 2; AANAT, vector 6; luciferase, vector 9 (Ambion). Oligonucleotide sequences are provided in Table 1. The assay was designed according to the Ambion qRPA guidelines. Individual assays were conducted for each transcript. Assays were conducted on triplicate biological replicates (each replicate containing two pooled pineal glands) sampled from each of the four individual transgenic lines (U20, U30, U42, W21). For each time point, 0.5 mg of total pineal RNA was used. Protected fragments were resolved on 10% TBE-Urea polyacrylamide gels and exposed to a storage phosphor screen (Kodak K, Eastman Kodak, Rochester, MN, USA). The abundance of protected fragments was quantified by densitometry (ImageQuant™ 3.0, GE Healthcare, Chalfont St. Giles, Bucks, UK), and *Aanat* and transgene transcript levels were corrected against levels of GAPDH mRNA. Statistical comparison of gene rhythms between wild-type and mutant transgenic lines was conducted by determining area under curve (AUC) of individual time courses (GraphPad Prism, 2.01, GraphPad Software Inc., Calabas, CA, USA), and then comparing groups of AUC values with independent samples Student's *t*-tests (SPSS 13, SPSS Inc., Chicago, IL, USA).

### 2.4. Northern blot analysis of tissue expression

Total RNA was extracted and Northern blot analysis of tissue expression was conducted as described previously (Burke et al., 1999). The hypothalamic and cortical brain samples were obtained from a coronal brain slice as described in the dissection protocol of Glowinski and Iversen (1966). The sample of neocortex was obtained by cutting a 5 mm block through cortical layers I–VI of the right hemisphere at the level of the somatosensory cortex. Whole pituitary glands including both the adenohypophysis and neurointermediate lobe constituted the pituitary sample. The luciferase-specific probe (see above) was used to detect transgene transcripts and a truncated *Aanat* cDNA probe (Smith et al., 2001) used to specifically detect *Aanat* transcripts. Northern blots were stripped (boiling 0.1% SDS, 3–5 × 2 min) and re-probed with a commercially available 18S cDNA (DecaTemplate™, Ambion, Austin, TX, USA). Assays were conducted on duplicate biological samples from three transgenic lines: U30, U42, W21. Densitometric comparison of mRNA levels between tissue samples was performed using ImageQuant™ 3.0 (Amersham Pharmacia Biotech), correcting values against the corresponding level of 18S RNA.

### 2.5. In situ hybridization analysis of retinal transgene expression

*In situ* hybridization (ISH) analysis of retinal transgene expression was conducted on fresh frozen sections using standard non-radioactive protocols as described: (http://wwwetb.info.nih.gov/lcmr/snge/Protocols/ISHH/ISHH.html) using sense and antisense orientation RNA probes corresponding to bases 1737–2141 of the pGL3 luciferase vector (Promega, Madison, WI). ISH analysis was conducted on multiple 12 μm sections from each of the U30, U42, and W21 transgenic lines. Retinal sections were viewed under bright field optics (Leica DM-LB microscope) and images were captured using a Leica DFC-300FX digital camera, and Leica QWin software (V3; Leica Microsystems Imaging Solutions Ltd., Cambridge, UK).

## 3. Results

### 3.1. Circadian expression

A marked circadian rhythm was detected in the expression of the wild-type transgene with transcripts being minimal during subjective day (08.00 h–18.00 h) and intensely expressed during the middle period of subjective night (22.00 h–03.00 h). For the purposes of the present study we therefore selected a sampling period of 18.00 h–08.00 h. In confirmation of previous findings (Burke et al., 1999) the pattern of expression of the wild-type transgene (−218/Luc) is characterized by a rhythm with peak values at either the 24.00 h or 02.00 h time-points (Fig. 2). A similar rhythm of expression was observed for the endogenous *Aanat* transcript (Fig. 2). Comparison of the time-courses generated for these two transcripts obtained by plotting values relative to the daily peak revealed that they were nearly identical, except for a minor difference in the shape of the two curves (Fig. 2B). The similar overall pattern and amplitude of the rhythm is consistent with similar rates of transcription of both transcripts and confirms previous studies using a similarly designed construct with a chloramphenicol acetyltransferase (CAT) reporter (Burke et al., 1999).

Analysis of the expression of the mutated transgene (−218mEB/Luc) revealed that it exhibited a marked daily rhythm of expression (Fig. 2) that was generally similar, albeit not identical to the rhythm of native *Aanat* mRNA in the same samples. Statistical comparison of the mutant (−218mEB/Luc) and wild-type (−218/Luc) transgene rhythms failed to reveal significant differences in any of the three mutant transgenic lines (Fig. 2). The absence of differences in rhythmic gene expression between the mutant and wild-type lines could not be attributed to differences in transgene copy number because the three mutant lines had variable copy numbers and the wild-type line has a copy number that is very similar to the U20 and U42 mutant lines (Fig. 1C). These observations indicate that the E-box sequences in intron 1 are not required for rhythmic expression of the gene in the rat pineal gland.

### 3.2. Tissue specific expression

The issue of issue specific expression was examined using Northern blot analysis (Fig. 3). In the wild-type transgenic line (−218/Luc) transgene expression was restricted to the pineal

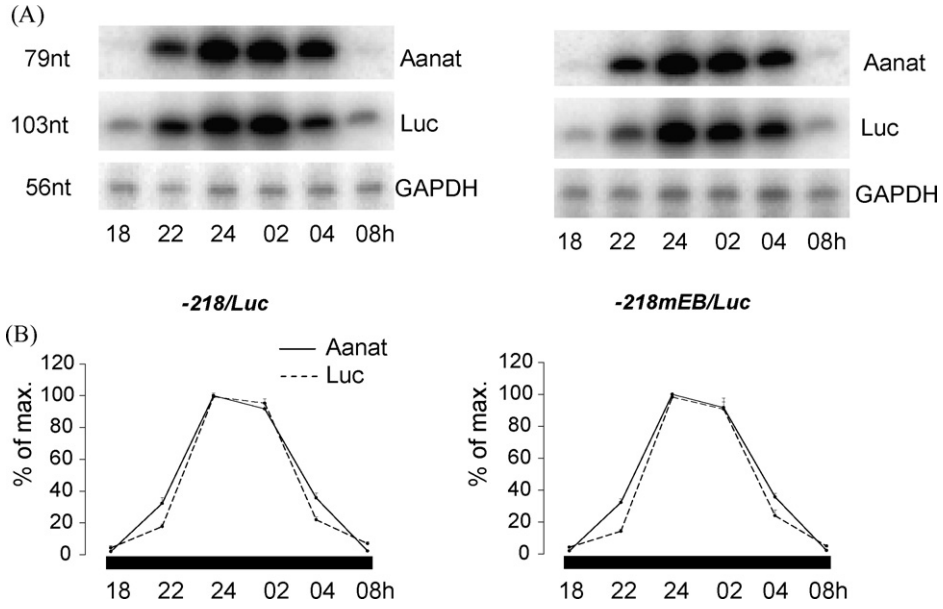


Fig. 2. Rhythms of transgene expression in rats transgenic for either a wild-type (−218/Luc) or mutant (−218mEB/LUC) *Aanat* promoter–reporter transgene. Total RNA was extracted from pineal glands of rats killed at different times of the circadian cycle. Levels of transgene (luciferase reporter gene, Luc) and endogenous *Aanat* transcripts were quantified with a ribonuclease protection assay (RPA, see text), and compared with levels of a control transcript (GAPDH). (A) Representative phosphor screen images of protected RPA products (sizes in nucleotides, nt) in samples taken across the circadian cycle. (B) Graphical representation of summated RPA data showing the levels of Luc and *Aanat* transcripts plotted as a percentage of peak values. Each data point is mean ± S.E.M. of three independent pools of pineal glands. Statistical analysis of wild-type transgene transcript (Luc) abundance across the cycle revealed a highly significant between group difference ($F = 775.119$; $p < 0.0001$, 1-way ANOVA). Comparison of the mutant (−218mEB/Luc) and wild-type (−218/Luc) transgene rhythms by Area under curve measurement and Student's *t*-test did not reveal any significant differences ($p = 0.132$ for the U30 line shown in this Figure).

gland and retina; this has been observed previously for the CAT transgenic lines (Burke et al., 1999) and for native *Aanat* mRNA. However, a different tissue expression pattern was observed in the −218mEB/Luc lines (Fig. 3), characterized by significant ectopic expression in the cortex, hypothalamus and spleen. In the cerebral cortex of the U30 line animals, transgene transcript levels were elevated above retinal levels (Fig. 3B). Similar results were observed in a duplicate biological replicate of the U30 line and in duplicate replicates of the U42 line.

Northern analysis of retinal extracts (Fig. 3) indicated that the levels of expression of the −218/Luc and the −218mEB/Luc transgenes were not markedly different. We also performed *in situ* hybridization analysis to determine whether there were marked differences in the pattern of transgene expression in the retina. This overall intensity of transgene expression in the −218mEB/Luc and −218/Luc lines was similar, however, there was a marked change in the pattern of cellular expression characterized by a greater spread across the ONL and increased expression in the INL of the −218mEB/Luc lines. The change in the expression pattern within cell layers of the retina is consistent with the view that the intronic *Aanat* E-Boxes function to mediate ectopic silencing of the gene (Fig. 4).

Comparison of light and dark phase retinal samples indicated that the transgene was rhythmically expressed in the ONL in the −218/Luc line but not in that of the −218mEB/Luc lines; however, the extended distribution of the transcript across the ONL precludes clear visualization and objective analysis of rhythmic expression within a subpopulation of ONL cells.

## 4. Discussion

The present study has provided a number of insights into the regulation of rat *Aanat* gene expression. First, we have discovered that E-box sequences within intron 1 are not required to drive or modulate the physiological circadian expression of the gene in the pineal gland (Baler et al., 1999; Chen and Baler, 2000; Burke et al., 1999). Second, we have determined that E-box sequences within intron 1 are required for the maintenance of tissue-specific expression because mutation of the +623 E-box and +636 E-box-like sequence in a transgenic context resulted in ectopic expression of the transgene within the brain and retina. These results are entirely consistent with previous studies that indicated a role, firstly, for intron 1 (Burke et al., 1999), and secondly, specifically for the intron 1 E-box in specifying rat *Aanat* gene expression (Baler et al., 1999; Chen and Baler, 2000). The findings of the current study provide convincing evidence because they were generated *in vivo*, in a physiological context.

Our finding that intron 1 E-box mutations do not affect rhythmic pineal expression of *Aanat* is also consistent with previous studies that found no evidence that Bmal1 and Clock proteins act through the intron 1 E-box to alter expression in cultured pinealocytes (Chen and Baler, 2000). The latter result is intriguing because it indicates that this element is functionally refractory to these clock proteins in a pinealocyte context where these proteins appear to be expressed (Namahira et al., 1999; Karolczak et al., 2004). In contrast, similar experiments using retinal photoreceptor cells indicate that Baml1and Clock can up-regulate transcription from this element (Chen and Baler,

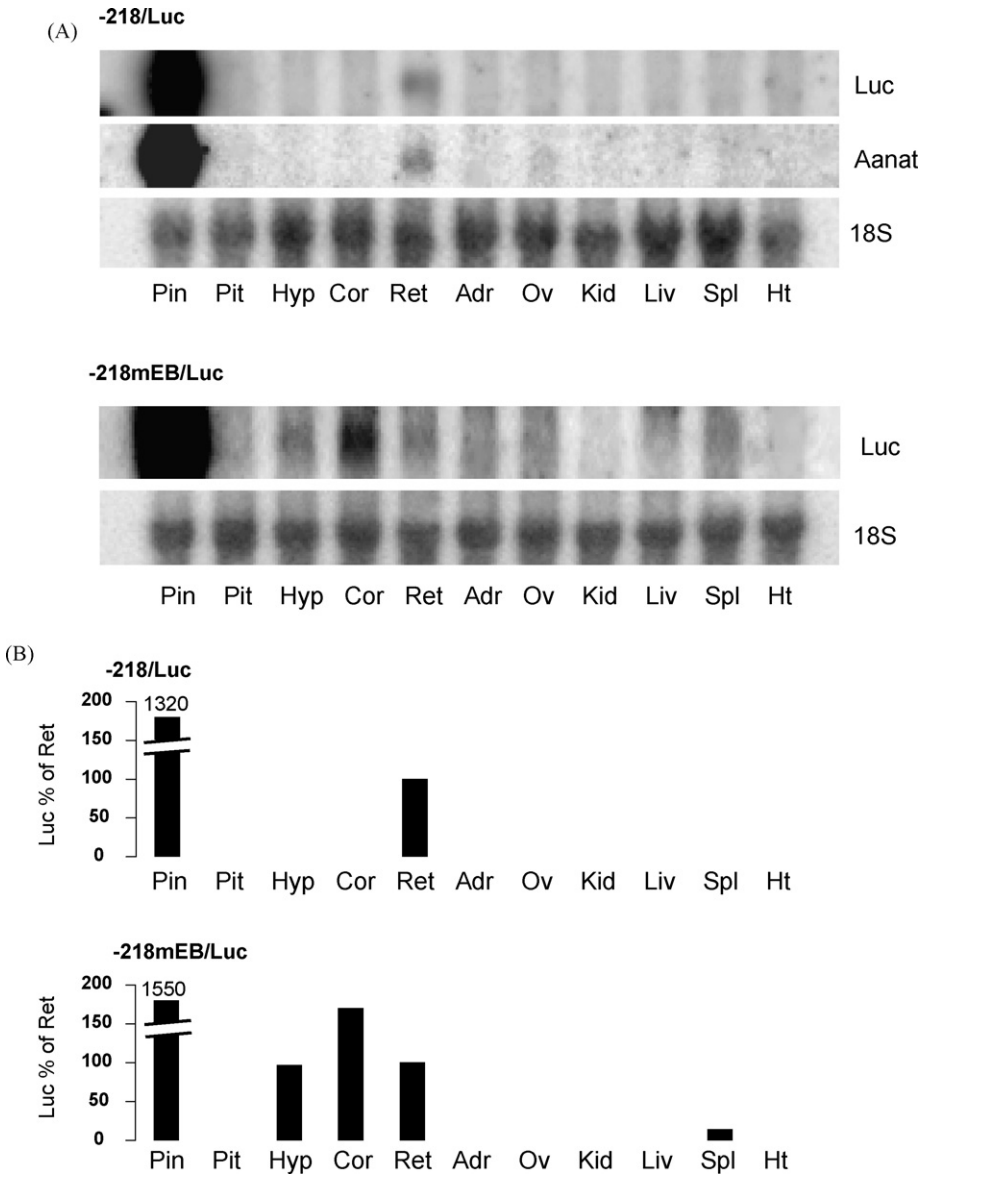


Fig. 3. Tissue distribution of transgene expression. Northern blot of transgene and endogenous *Aanat* transcripts in rats transgenic for either a wild-type (−218/Luc) or mutant (−218mEB/LUC) *Aanat* promoter–reporter transgene. Total RNA (8 mg/lane) was extracted from pineal glands (pool of two) and other tissues (Pit, pituitary gland; Hyp, hypothalamus; Cor, cerebral cortex; Ret, retina; Adr, adrenal gland; Ov, ovary; Kid, kidney; Liv, liver; Spl, spleen; Ht, heart) of rats killed at 24.00 h and subjected to Northern analysis as described in the text. Blots were probed with cDNA probes specific for the transgene transcript (Luc) or *Aanat* transcript, and re-probed with an 18S ribosomal RNA probe. (A) Representative phosphor screen images of Northern blots conducted on the W21 (−218/Luc) and U30 (−218mEB/LUC) lines are shown, and similar results were obtained in a second, independent line of MUT-Tg rats. Note that transgene expression is restricted to the pineal gland and retina in the −218/Luc line, but that expression is relaxed in the −218mEB/LUC line with transgene expression being clearly detected in both the hypothalamic and cortical regions of the brain. (B) Graphical representation of the data in (A). Levels of the transgene transcript (Luc) were quantified by densitometry, corrected against the corresponding level of 18S RNA and expressed as a percentage of the level in retina. Note that the pineal gland values are presented above the corresponding histograms.

2000). This emphasizes both the fundamental differences that exist between the intracellular milieus of these closely related tissues and the importance of cellular context in the analysis of regulatory elements.

The molecular basis of the pineal/retinal difference is unknown but may involve cell-specific factors that possibly interact with elements that flank the E-box sequences (Munoz et al., 2002). The absence of any effect of E-box mutations on rhythmic *Aanat* expression in the pineal gland is not surprising in light of the large body of evidence that supports the view that the dominant mechanism controlling expression of *Aanat* in the rodent involves cAMP acting through a CRE-CCAAT complex element in the proximal 5′ region of the *Aanat* gene (Baler et al., 1997; Klein, 2006). The dominant role of a cAMP/CRE pathway in the control of rat pineal *Aanat* expression appears to contrast, however, with modes of pineal *Aanat* regulation in other species, for example, the zebrafish *Danio rerio* where E-box elements do appear to contribute to circadian regulation of the *zfaanat2* gene (Appelbaum et al., 2005). Similarly, in the avian pineal gland and retina, the primary mechanism controlling *Aanat* expres-

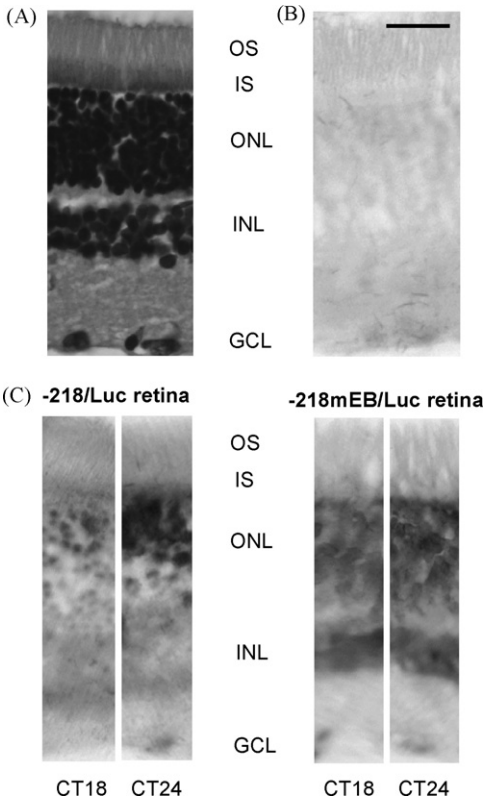


Fig. 4. *In situ* hybridization analysis of transgene expression in the retina of rats transgenic for either a wild-type (−218/Luc) or mutant (−218mEB/Luc) *Aanat* promoter–reporter transgene. Whole eyes were obtained from rats killed at either 18.00 h (CT18) or 24.00 h (CT24) and frozen (12 mm) transverse sections were processed for non-radioactive *in situ* hybridization analysis using digoxigenin-labelled, sense and antisense RNA probes as described in the text. (A) Haematoxylin and eosin-stained section of the retina showing the main cellular layers: outer segments (OS), inner segments (IS) and outer nuclear layer (ONL) of the photoreceptor cells; inner nuclear layer (INL), and ganglion cell layer (GCL). (B) Representative image of a retinal section from a −218/Luc rat probed with a sense orientation transgene probe. Note the detection of a minor level of signal that is apparent in the GCL. The scale bar, which is representative for all images, is 20 μm. C. Representative images of retinal sections from −218/Luc and −218mEB/Luc rats probed with an antisense transgene probe. Note that transgene-positive cells are primarily located in the outer (upper) zone of the ONL in the −218/Luc line, and that the signal intensity is greater at CT24 compared with CT18. In contrast, in the −218mEB/Luc line transgene-positive cells are found in a wider distribution across the ONL, and in addition, there is significant expression within cells of the INL. A consistent temporal difference in expression was not detected in the 218mEB/Luc retina.

sion is the Bmal1/Clock interaction with E-box elements; the role that cAMP plays appears to be secondary (Chong et al., 2000, 2003; Iuvone et al., 2005; Klein, 2006). These differences are in accordance with known diversity in the organization of the systems that control Aanat among vertebrates (Klein et al., 1997; Klein, 2006).

The transgenic demonstration that E-box sequences act in the specification of rat *Aanat* gene expression presented here firmly establish a role for these intronic sequences in the control of tissue/celluar specificity in mammals. Previous *in vitro* analysis of regulatory *Aanat* intronic sequence addressed the gross contribution of blocks of intron 1 sequence to tissue specificity by measuring relative transcriptional activity in a pituitary cell line (AtT20 cells, Baler et al., 1999). The deletion mutant that approximates most closely to the point mutants examined here (Δ 622–1010) was found not to have a significant effect on the relative level of expression in these cells. This finding is in accordance with our current *in vivo* data that does not indicate any up-regulation of pituitary expression in the E-box mutants. In contrast, other deletion mutants in the Baler et al. (1999) study, for example Δ 523–1563, exhibited a marked enhancement of pituitary cell expression. This, together with the finding ectopic expression in the E-box mutant lines, suggests that sequences within the *Aanat* intron contribute differentially to tissue specific repression of ectopic expression. Our results are therefore consistent with the notion that intron 1 E-box sequences contribute to tissue specificity but are not solely responsible for this facet of *Aanat* gene expression. It is apparent that silencing mechanisms exist in non-pineal tissues to suppress ectopic expression and that these may involve tissue/cell specific factors that interact with different elements within *Aanat* intron 1. Previous studies by Chen and Baler (2000) have shown that tissue extracts from a number of brain regions including cerebral cortex contain factors that bind the intron 1 E-box *in vitro*. However, the identity and cellular-specificity of these factors is currently unknown.

A role for E-box elements in the maintenance of appropriate pineal-specific expression has also been addressed in the zebrafish model. In this fish species, it has been shown that a downstream E-box element is necessary but not sufficient for the maintenance of pineal-specific expression of the *zfaanat2* gene in zebrafish (Appelbaum et al., 2004; Appelbaum and Gothilf, 2006). The results of these transgenic fish experiments suggest, in fact, that the E-box has a dual role in both the suppression of ectopic expression and in the enhancement of pineal expression. The present data from the rat is inconsistent with the latter function because the E-box mutant (−218mEB/Luc) transgenic lines did not exhibit a reduction in pineal expression (Fig. 3B). One explanation of this is that the levels of clock genes expressed in the zebrafish pineal are sufficiently high to control gene expression.

Another explanation involves the context within which the E-box exists in the zebrafish *Aanat* gene, where it is functionally interactive with PCEs within the 3′ enhancer (Appelbaum et al., 2004; Appelbaum and Gothilf, 2006). Our current demonstration of a (partially) conserved role, but different gene location (intronic v 3′ flanking sequence) for the *Aanat* E-box sequences is consistent with current comparative genomic studies that have identified location shuffling of *cis*-elements in gene orthologues (Sanges et al., 2006). A number of PCEs are located within rat *Aanat* intron 1 (Fig. 1A) but the functional role of these putative regulatory elements either individually, or in association with the intronic E-box elements has not been investigated. It is apparent, however, that a possible collective (modular) role of the rat intronic E-box and PCE elements may be cell-type specific, being dependent upon *trans*-acting and/or epigenetic components. This supposition is based upon the published evidence of differential activities of the intronic E-box in pineal and retinal photoreceptor cells (Chen and Baler, 2000), and the supportive findings of the present study which show the putative circadian E-box elements in intron 1 of the *Aanat* gene

do not contribute to rhythmic expression in the pineal gland of transgenic rats. Further studies of the functional role of *Aanat* E-boxes are warranted, particularly given the presence of these sequences within the *Aanat* gene in representative species across vertebrate evolution (Ensembl: http://www.ensembl.org).

In conclusion, we have found that E-box elements play a role in the regulation of tissue-specificity of the rat *Aanat* gene but not pineal rhythmicity. This finding further advances our understanding of E-boxes, important DNA elements that mediate a broad range of regulatory mechanisms following rules that remain poorly defined (Kewley et al., 2004; Munoz et al., 2002).

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