

The Rat Arylalkylamine *N*-Acetyltransferase Gene Promoter

cAMP ACTIVATION VIA A cAMP-RESPONSIVE ELEMENT-CCAAT COMPLEX*

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A 10–100-fold rhythm in the activity of arylalkylamine *N*-acetyltransferase (AA-NAT; EC 2.3.1.87) controls the rhythm in melatonin synthesis in the pineal gland. In some mammals, including the rat, the high nocturnal level of AA-NAT activity is preceded by an ~100-fold increase in AA-NAT mRNA. The increase in AA-NAT mRNA is generated by norepinephrine acting through a cAMP mechanism. Indirect evidence has suggested that cAMP enhances AA-NAT gene expression by stimulating phosphorylation of a DNA-binding protein (cAMP-responsive element (CRE)-binding protein) bound to a CRE. The nature of the sites involved in cAMP activation was investigated in this report by analyzing the AA-NAT promoter. An ~3700-base pair fragment of the 5'-flanking region of the rat AA-NAT gene was isolated, and the major transcription start points were mapped. The results of deletion analysis and site-directed mutagenesis indicate that cAMP activation requires a CRE-CCAAT complex consisting of a near-perfect CRE and an inverted CCAAT box located within two helical turns.

The rhythmic nocturnal increase in plasma levels of melatonin (1) in mammals is due to norepinephrine (NE)¹ stimulation of melatonin production in the pineal gland. NE release is regulated by the endogenous circadian oscillator in the suprachiasmatic nucleus (2), which is connected to the pineal gland by a multisynaptic pathway. NE controls melatonin production by regulating the activity of the penultimate enzyme in the melatonin biosynthetic pathway, serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase (AA-NAT), EC 2.3.1.87) (3). The second messengers involved are cAMP and Ca²⁺_i (3–7).

The importance of transcriptional events in the regulation of AA-NAT activity varies remarkably on a species-to-species basis (8). An absolute requirement for *de novo* transcription is

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¹ The abbreviations used are: NE, norepinephrine; AA-NAT, arylalkylamine *N*-acetyltransferase; CRE, cAMP-responsive element; CREB, CRE-binding protein; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; bp, base pair(s); CAT, chloramphenicol acetyltransferase; LUC, luciferase; Pipes, 1,4-piperazinediethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; Bt₂cAMP, dibutyryl cAMP; CATBP, CCAAT-binding protein; (R_p)₈-CPT-cAMP-S, (R_p)₈-(4-chlorophenylthio)adenosine cyclic 3':5'-phosphorothioate; ICER, inducible cAMP early repressor.

most evident in the rat (8, 9), where an ~100-fold increase in AA-NAT mRNA is required for the ~100-fold increase in AA-NAT activity to occur. The mechanism involved in turning on expression of the AA-NAT gene does not require *de novo* protein synthesis (9). Rather, it appears to be initiated by cAMP-dependent phosphorylation of cAMP-responsive element (CRE)-binding protein (CREB) (10) or another member of this growing family (11, 12).

The molecular basis of cAMP stimulation of expression of the rat AA-NAT gene has not yet been investigated at the level of the promoter, and it is not known whether cAMP acts directly or indirectly on the AA-NAT gene. Here we describe the isolation of the rat AA-NAT promoter region and report the results of a functional analysis focused on the question of NE- and cAMP-dependent activation. The results of this study indicate that cAMP can effect gene activation from the AA-NAT promoter. Furthermore, it appears that cAMP acts through a CRE-CCAAT complex, which consists of a near-perfect CRE located within two helical turns of an inverted CCAAT box.

EXPERIMENTAL PROCEDURES

Ligation-mediated PCR—AA-NAT promoter sequences were identified using the Promoter Finder™ DNA Walking kit (CLONTECH, Palo Alto, CA) in conjunction with the nested AA-NAT primers 652, 581, and 587 (see below) derived from the 5'-flanking regions of AA-NAT. The PCR conditions were recommended by the manufacturer. Genomic fragments were subcloned into pCR3 (Invitrogen, San Diego, CA) for sequencing by the dideoxynucleotide chain termination method (13). To generate promoter/reporter hybrid constructs, different PCR-generated fragments of the AA-NAT promoter region were introduced into the *Xba*I site in pCAT-Basic (Promega, Madison, WI) or into the *Nhe*I site in pGL3-BASIC (Promega). These vectors carry the bacterial chloramphenicol acetyltransferase (CAT) and firefly luciferase (LUC) reporter genes, respectively.

Total RNA Isolation and S1 Nuclease Analysis—For S1 nuclease protection analysis, a 289-nucleotide end-labeled single-stranded probe was synthesized that was complementary to positions –207 to +82 in the AA-NAT gene (relative to the start of transcription); synthesis was by asymmetric PCR driven by primer 581 (see below). The probe was gel-purified and added (50,000 cpm) to 30 μg of total pineal RNA in 20 μl of S1 hybridization buffer (80% deionized formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl, and 1 mM EDTA, pH 8). The mixture was then denatured (10 min, 65 °C) and hybridized overnight at 44 °C. S1 nuclease digestion was carried out by adding 300 μl of S1 nuclease buffer (0.28 M NaCl, 50 mM sodium acetate, pH 4.5, 4.5 mM ZnSO₄, 10 μg of sheared salmon sperm DNA, and 240 units of S1 nuclease). After a 1-h incubation at 37 °C, 80 μl of stop buffer (4 M ammonium acetate, 20 mM EDTA, pH 8, and 40 μg/ml tRNA) were added. After ethanol precipitation, samples were boiled and electrophoresed on a 6% sequencing gel. A dideoxynucleotide sequencing reaction of –267/CAT primed with primer 581 was run in parallel to locate the transcription start point.

Tissue Dissociation and Transfection—Dissociated rat pinealocytes (5 × 10⁶ cells) were prepared by trypsinization essentially as described (14) with minor modifications. Briefly, 50 pineal glands (Taconic Farms Inc., Germantown, NY) were separated from residual afferent nerve fibers and the surrounding leptomeninges, partially teased apart, and rinsed in 10 ml of BGJb (Life Technologies, Inc.). Glands were incubated (37 °C, 95% O₂ and 5% CO₂) in 5 ml of DMEM containing 0.2%

trypsin and 40 $\mu\text{g/ml}$ DNase I (Boehringer Mannheim) for 50 min. Trypsinization was ended by the addition of 10 ml of DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin (DMEM+). Partially dissociated glands were allowed to sediment and triturated in 3 ml of DMEM+ supplemented with 70 μg of DNase I. Pineal cells were passed through a small funnel-shaped mesh (125- μm opening; Bellco Glass, Inc., Vineland, NJ) to a new tube, spun at $1000 \times g$ for 5 min, and plated in two wells of a six-well plate (Costar Corp., Cambridge, MA) in 2 ml of DMEM+. Twenty-four hours later, cells in suspension were harvested, collected, and resuspended in serum-free Opti-MEM (Life Technologies, Inc.; $\sim 0.5 \times 10^6$ cells/ml); and 0.4-ml samples were transferred to individual wells in a 24-well plate (Costar Corp.). Transfections were performed by overlaying the cells with a precipitate of 3 μl of LipofectAmine™ (Life Technologies, Inc.) and 2 μg of DNA for 30 min before adding $\sim 2 \times 10^8$ adenovirus shuttle particles to enhance transfection efficiency (15). Eighteen hours later, 0.5 ml of DMEM+ were added. Where indicated, dibutyryl cAMP (Bt₂cAMP) was added to the cultures at this point. Cells were harvested 48 h later. CAT assays were performed as described (16). Luciferase activity was measured with the luciferase assay system (Promega) according to the manufacturer's recommendation. Transfection of primary rat pituitary cells or fibroblasts was done using the same procedure as described above. COS-7 and C6 cells (American Type Culture Collection, Rockville, MD) were transfected using LipofectAmine following the manufacturer's recommendations.

Oligonucleotides—The synthetic DNA oligonucleotides and primers referred to in this study were synthesized using an Applied Biosystems 381B DNA synthesizer. Nested primers for promoter cloning were as follows (positions are relative to start of transcription): primer 652 (positions +177 to +158), 5'-CAT GGG TAT CTG GCC ACT GA-3'; primer 581 (positions +82 to +63), 5'-TCC CCA CCA CAG AGC TGG TCA CAC TGG-3'; and primer 587 (positions -14 to -34), 5'-CCT-GAC AGC ATG TGA TGG CTC A-3'. Oligonucleotides used in EMSA and for site-directed mutagenesis were as follows (only upper strand is shown): natCRE, 5'-GAA AAG CTT AGT ACC ACC GAT GAC GCC AGC CCT CAG CAG TCT AGA GC-3'; natCRE_{mut1}, 5'-GAA AAG CTT AGT ACC ACC GAT TAA ACC AGC CCT CAG CAG TCT AGA GC-3'; AP-1, 5'-CGC TTG ATG AGT CAG CCG GAA-3'; CCAAT-binding transcription factor, 5'-CCT TTG GCA TGC TGC CAA TAT-3'; sstCRE (where sst is somatostatin), 5'-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3'; caCRE, 5'-GAG CCC GTG ACG TTT ACA CTC ATT C-3'; AP-2, 5'-GGA TTA TAG TTA GAC CCC AGG CTT AGC CCA TGC TC-3'; CCAAT, 5'-CAG CCC TCA GCA GGA TTG GGT GAG GGC CTG ACT ACC-3'; CCAAT_{mut1}, 5'-CAG CCC TCA GCA GTC TAG AGT-CAG GGC CTG ACT ACC-3'; CCAAT_{mut2}, 5'-CCT CAG CAG GTA-TGG GTC AGG GCC T-3'; and CRECCAAT+5, 5'-GAT GAC-GCC AGC CCT CGA GTA AGC AGG ATTGGG TC-3'.

EMSA—Pineal glands were quick-frozen on dry ice. To prepare extracts, a 30- μl sample of ice-cold buffer C (20 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, 1 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ leupeptin, 1 mM sodium fluoride, 5 μM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 25% glycerol) was added to two glands. The glands were homogenized on ice with 20 strokes of a tight-fitting 1.5-ml microtube pestle. Lysates were centrifuged at 12,000 rpm for 10 s, vortexed, and refrozen on dry ice for 5 min. They were next incubated in ice water for 15 min and centrifuged at 12,000 rpm for 15 min at 4 °C. This procedure extracted 40–50 μg of soluble protein/pineal gland. EMSA was performed using a ³²P-radio-labeled double-stranded oligonucleotide probe containing the natCRE or natCCAAT sequence (see Fig. 1) and 3 μl of whole pineal extract as described previously (17). Competition EMSA was performed as described above with a 200-fold molar excess of unlabeled double-stranded oligonucleotides added before the probe or 1 μl of one of the following antisera (added 20 min after the probe): anti-c-Fos(3–16) (SC-52X), anti-Fra-1(3–22) (SC-183X), anti-Fra-2(285–299) (SC-57X), anti-FosB(102–117) (SC-48X), anti-JunD(329–341) (SC-74X), and anti-CREM-1 (SC-440X) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) or rabbit anti-rat CREB-(1–205) polyclonal antiserum (Upstate Biotechnology, Inc., Lake Placid, NY).

Site-directed Mutagenesis—*In vitro* mutagenesis of the CRE and CCAAT *cis*-acting elements within the AA-NAT promoter was done with the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Initial screening of putative mutant clones was performed by *Bsa*HI and *Xba*I restriction endonuclease digests, respectively. Mutations were then confirmed by dideoxynucleotide sequencing. Promoter fragments were

subsequently excised with *Xba*I and inserted into an *Nhe*I-linearized pGL3-BASIC reporter vector.

RESULTS

Isolation of the Rat AA-NAT Promoter—Putative regulatory regions in the sequence immediately upstream of the AA-NAT coding sequence were isolated using a ligation-mediated PCR-based technique as described under "Experimental Procedures." This generated an ~ 3700 -bp genomic fragment that contains the first intron (intron 1; Fig. 1A) located in the 5'-untranslated region after position +114; the intron is 1628 bp long.²

The 2160-bp putative promoter sequence was analyzed to determine if it contains known transcription factor-binding sites (Fig. 1A). This failed to identify a canonical TATA box; however, an A/T-rich sequence resembling a TATA box (ATTA-GAAT) is located 38 bp upstream of the start of transcription, as defined below. This putative TATA box is preceded by a GC-rich region (positions -47 to -65), which could function as an SP1-binding (18) and/or AP-2-binding (19) site. An inverted CCAAT box (natCCAAT) exists at position -120. In addition, we detected an alternate purine-pyrimidine repeat (20) between positions -1165 and -1104, an AP-1-like element (21) at position -32, and a CRE-related sequence at position -139 (natCRE). The natCRE sequence (TGACGCCA) closely resembles (seven out of eight) the perfect CRE consensus sequence (TGACGTCA) (22, 23).

Determination of the Transcription Start Point—The transcription initiation site was established using S1 nuclease protection analysis of total RNA isolated from Bt₂cAMP-treated pineal glands. Three transcription start points were detected (Fig. 1B), the strongest of which generates the longest leader sequence (173 nucleotides). The leader sequences generated by the weaker start points are 148 and 136 nucleotides long. The upstream initiation site is located within an apparent initiator consensus sequence, characterized by an initiating A with a 3'-pyrimidine-rich flanking sequence (Fig. 1C) (24, 25).

Functional Characterization of the AA-NAT Promoter—Regions necessary and sufficient for AA-NAT promoter activity were identified using partial promoter constructs. These contained decreasing portions of the longest 5'-flanking genomic fragment placed in front of the CAT reporter gene.

Primary pinealocytes were transiently transfected with this series of constructs and tested for basal and stimulated CAT activity (Fig. 2). CAT activity was low in untreated cells in all constructs. Bt₂cAMP treatment produced comparable levels of CAT expression from a family of intronless promoter deletion constructs with 5'-boundaries ranging from positions -2160 to -267 and encompassing sequences up to position +82. Similar results were obtained following treatment with NE or a selective β -adrenergic agonist (isoproterenol), but not following selective α -adrenergic treatment (phenylephrine + propranolol; data not shown). Maximum Bt₂cAMP induction was achieved using a promoter construct that begins at position -207, spans the entire length of intron 1, and ends just prior to the first codon (-207/iCAT). However, a similar construct containing >90% of intron 1 but no 5'-untranslated region (+371/CAT) failed to drive detectable CAT enzyme activity, indicating that the key elements promoting Bt₂cAMP responsiveness are not likely to reside in intron 1. Taken together, these results suggest that the region between positions -267 and +82 is sufficient for conferring cAMP responsiveness. This region contains the natCRE and natCCAAT sequences described above.

The -267/CAT construct fragment was also able to drive cAMP-dependent expression of CAT when tested in non-pineal

² R. Baler, S. Covington, and D. C. Klein, unpublished results.

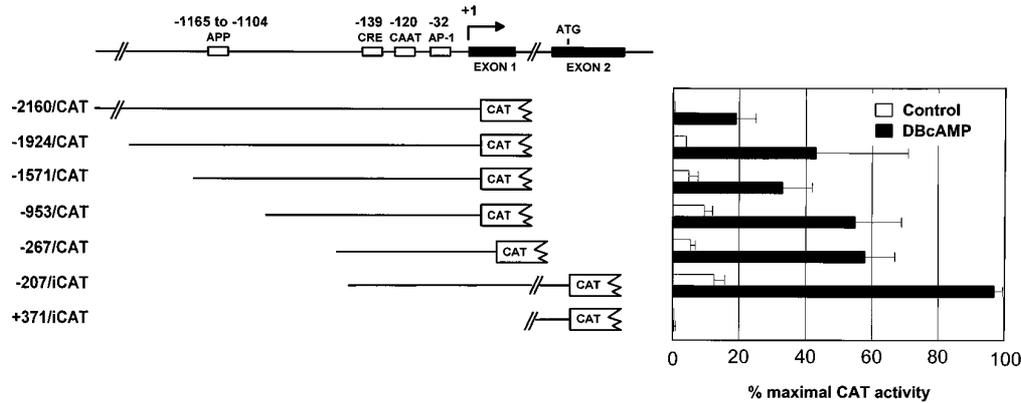


FIG. 2. **Functional analysis of the AA-NAT promoter.** Nested fragments of the AA-NAT promoter were generated by PCR, placed in front of the bacterial CAT gene, and transfected into primary pinealocytes as described (see "Experimental Procedures"). Eighteen hours later, individual cultures were stimulated with Bt_2cAMP (DBcAMP; 1 mM) or left untreated. Whole cell extracts were prepared 48 h later, and CAT enzyme activity was measured as described (16). Transfection efficiency was assessed by cotransfection with Rous sarcoma virus- β -galactosidase. Results represent data collected from five independent experiments. APP, alternate purine-pyrimidine repeat.

TABLE I

The minimal AA-NAT promoter responds to increased cAMP in non-pineal cell types.

Primary rat pinealocytes and pituitary cells and established C6 glioma and COS-7 immortalized cell lines were transfected with the -267/CAT AA-NAT promoter construct (see "Experimental Procedures"). Twenty-four hours later, cells were stimulated with 1 mM Bt_2cAMP or left untreated. Basal and stimulated levels of CAT enzyme activity were assessed 48 h later. With the exception of C6 cells, for which transfection efficiencies varied dramatically between different transfections, values represent the result of independent duplicate experiments.

Cell type	CAT activity		Stimulation index
	Basal	Stimulated	
	* % conversion \pm S.E.		
Pinealocytes	0.8 \pm 0.7	20 \pm 2	25
Pituitary cells	1.3 \pm 0.4	5 \pm 1.3	3.8
COS-7 cells	2.5 \pm 1.2	37 \pm 12	14.7
C6 glioma cells	6.3 (NA) ^a	15.4 (NA)	2.4

^a NA, not applicable.

changes (TGACGCCA \rightarrow TTA AACCA), including a G/T transversion at position -141, which disrupts CRE function (26). The triple mutation abolished binding to specific nuclear proteins (data not shown). In transfection studies using primary pinealocytes, the CRE_{mut}/CAT reporter construct partially reduced the response to Bt_2cAMP (60–80% inhibition) (Fig. 3B) as compared with the response exhibited by the unmutated control. The possibility that this apparent inhibition was due to uncontrolled reporter gene-dependent artifacts was ruled out using equivalent firefly luciferase constructs, indicating that the triple mutation partially reduced effects of cAMP (Fig. 4B, wt/LUC versus CRE_{mut}/LUC).

The above results indicate that natCRE plays a key role in translating the adrenergic stimulus into an increase in AA-NAT gene transcription, consistent with predictions regarding the role of CREB phosphorylation in activation of the rat AA-NAT gene (10). However, a discrepancy is apparent: the triple natCRE mutation completely abolished binding, but only partially abolished the effects of Bt_2cAMP on promoter activity. This raised the possibility that cAMP-mediated gene activation requires another element(s) in the -267 construct. The basis of this residual responsiveness was examined below.

The Inverted CCAAT Box at Position -120 Is Required for Full cAMP Responsiveness—As indicated above, an inverted CCAAT box is located downstream and two helical turns away from natCRE. The CCAAT box is of special interest because of two observations: a CRE-CCAAT complex in the fibronectin gene promoter is necessary for full cAMP activation of gene

expression (27–29), and an inverted CCAAT box mediates cAMP activation of expression of the apparently CRE-less tryptophan hydroxylase promoter in primary pinealocytes (30).

To test whether natCCAAT binds pineal proteins, we analyzed the region containing the inverted natCCAAT sequence by EMSA using whole pineal protein extracts. The double-stranded oligonucleotide used did not contain natCRE. A distinct nucleoprotein complex was generated using either day or night pineal extracts. Proteins in this complex are referred to here as CCAAT-binding proteins (CATBPs) (Fig. 4A). Binding specificity was demonstrated by successful competition with a 200-fold molar excess of the wild-type natCCAAT sequence (Fig. 4A, compare lanes 7 and 8). In contrast, binding was not inhibited by double-stranded oligonucleotides containing either of two mutated natCCAAT oligonucleotides (Fig. 4A, lanes 9 and 10) or by AP-2 or AP-1 sequences (lanes 11 and 12). These studies indicate that natCCAAT specifically binds to pineal CATBPs.

The contribution of the natCCAAT site to gene expression was determined by site-directed mutagenesis of position -120 within the minimal promoter (construct -267) using the firefly luciferase reporter system. Promoter activity of the resulting construct (CCAAT_{mut}/LUC) was reduced by 60–80% when compared with that obtained using wt/LUC and CRE_{mut}/LUC (Fig. 4B).

These observations raise the possibility that both natCRE and natCCAAT are important for inducible promoter activity. To determine whether both sites are required to achieve full transactivation, we generated a double mutant without functional natCRE and natCCAAT sites ((CRECCAAT)_{mut}/LUC). Whereas the single mutant constructs CRE_{mut}/LUC and CCAAT_{mut}/LUC were partially active, the double mutant (CRECCAAT)_{mut}/LUC was essentially refractory to cAMP stimulation (Fig. 4B). This supports the interpretation that both sites participate in cAMP regulation of the AA-NAT gene.

When the natCRE and natCCAAT sites are considered within the context of coiled DNA, their centers are separated by approximately two turns of a helix and are therefore likely to be in phase. To address the question of whether this precise alignment is crucial for cAMP-inducible promoter activity, phasing was disrupted by inserting a 5-bp sequence between the two elements (Fig. 4B, CRECCAAT+5/LUC). This did not affect promoter activity, indicating that precise phasing between bound factors does not appear to be important when the distance between these sites is not significantly affected.

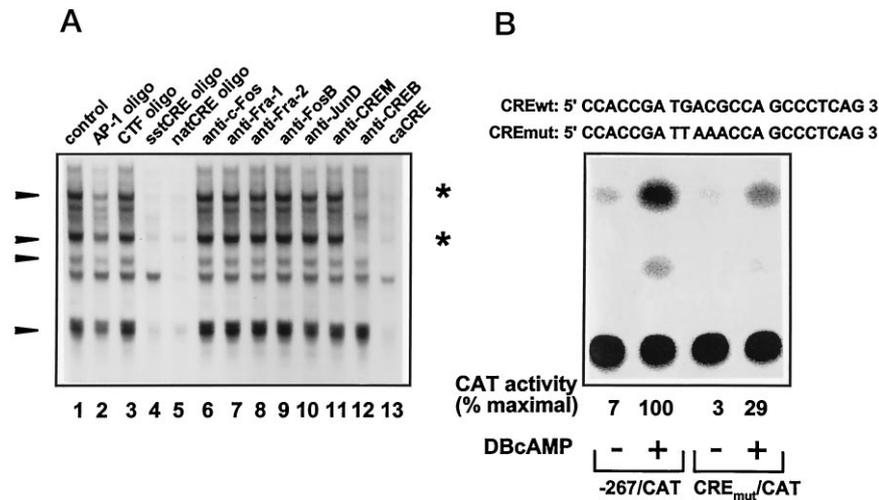


FIG. 3. The AA-NAT promoter contains a functional cyclic AMP response-like element. *A*, detection of natCRE-specific DNA-binding proteins in pineal extracts. EMSA was carried out using a radiolabeled natCRE oligonucleotide probe and a whole pineal protein extract. Unlabeled oligonucleotide competitors (natCRE (lane 5; this study), sstCRE (lane 4; Ref. 22), *c-fos* CRE (*caCRE*, lane 13; see "Experimental Procedures"), CCAAT-binding transcription factor (lane 3; Ref. 34), and AP-1 (lane 2; Ref. 44)) were preincubated with pineal extracts on ice for 20 min before the addition of the probe at ~200-fold molar excess. At the end of the incubations, several binding reactions were supplemented with 1 μ l of a specific antibody (as indicated) and incubated on ice for another 30 min prior to native electrophoresis (lanes 6–12). Specific nucleoprotein complexes are identified by arrowheads. Asterisks denote complexes affected by anti-CREB antibodies. Free probe is not shown. *B*, effect of natCRE mutagenesis on AA-NAT promoter cAMP responsiveness. The natCRE element was mutagenized in the -267/CAT background and transfected into primary pinealocytes. Parallel transfections were carried out with the original wild-type -267/CAT promoter construct. Cultures were stimulated with Bt₂cAMP (DBcAMP; 1 mM) or left untreated for 48 h before measuring CAT activity. Wild-type and mutant sequences are shown at the top of the panel. Similar results were obtained in four independent experiments.

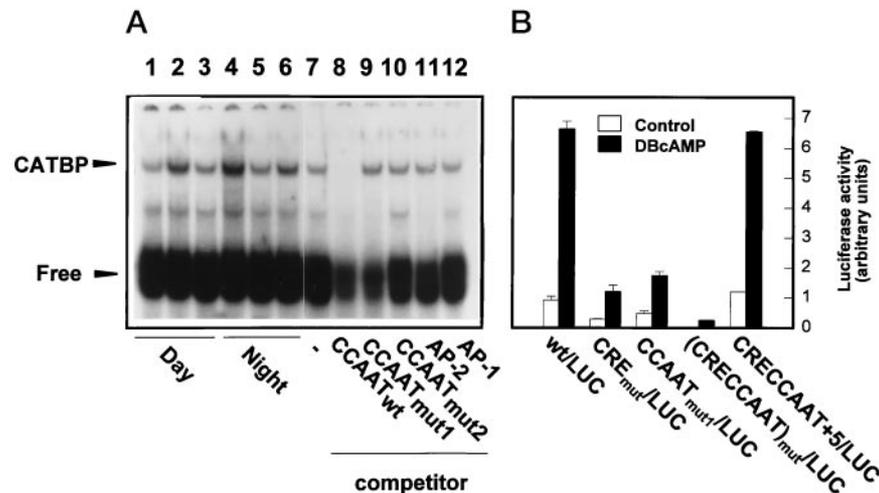


FIG. 4. The inverted CCAAT box in the AA-NAT promoter coparticipates with natCRE to achieve full cAMP inducibility. *A*, detection of natCCAAT-specific DNA-binding proteins in pineal extracts. Electrophoretic mobility shift assay was carried out using a radiolabeled natCCAAT oligonucleotide probe and whole pineal protein extracts prepared from pineal glands (pools of two per lane) harvested at 3:00 p.m. (day) or 02:00 a.m. (night; lights on from 07:00 a.m. to 21:00 p.m.). Unlabeled oligonucleotide competitors (as indicated) were used on a pool of day and night pineal protein extracts as described for Fig. 3. The migration of the specific CATBP-containing complexes is indicated. *B*, effect of single versus double natCRE:natCCAAT mutagenesis on AA-NAT promoter cAMP responsiveness. The natCCAAT element was mutagenized in the wild-type (CCAAT_{mut1}/CAT) and natCRE mutant (CRECCAAT_{mut1}/CAT) vectors. An additional construct (CRECCAAT+5/CAT) contained a 5-bp insertion between natCRE and natCCAAT. The mutated promoters were fully sequenced, spliced in front of the luciferase reporter gene (to yield CCAAT_{mut1}/LUC, (CRECCAAT_{mut1})/LUC, and CRECCAAT+5/LUC), and transfected into primary pinealocytes. Parallel transfections were carried out with wt/LUC and CRE_{mut1}/LUC. In selected experiments, cultures were cotransfected with a CAT reporter vector driven by the dynorphin promoter construct to provide an internal standard for transfection efficiency. Cultures were stimulated with Bt₂cAMP (DBcAMP; 1 mM) or left untreated for 48 h before assessing luciferase and CAT activities. Similar results were obtained in three different experiments utilizing two independent isolates of mutant clones.

DISCUSSION

Transcription plays a pivotal role in the nocturnal stimulation of AA-NAT in the rat. This is evident from the findings that NE and cAMP antagonists, including Bt₂cAMP, increase AA-NAT mRNA levels ~100-fold (9) and that the transcription blocker actinomycin D blocks the NE → cAMP stimulation of AA-NAT mRNA and activity (3, 9). We have suspected that cAMP acts to increase AA-NAT mRNA through cAMP-dependent phosphorylation of CREB resident at a CRE site. This was

based on the evidence that NE acts through a cAMP mechanism to phosphorylate CREB in the pineal gland and that the protein kinase A antagonist (*R_p*)-8-CPT-cAMP-S inhibits cAMP-dependent induction of AA-NAT activity (10).

Turning on Expression of the AA-NAT Gene—The studies presented here appear to support this notion because a near-perfect CRE (natCRE) is found in a 2160-bp fragment of the rat AA-NAT promoter. Functional dissection by deletion analysis revealed that a natCRE-containing region from positions

–267 to +82 confers cAMP-dependent responsiveness to either CAT or luciferase reporter genes in primary pinealocytes. Furthermore, the natCRE core sequence binds a CREB-like moiety present in whole pineal protein extracts, and mutagenesis of the natCRE site reduces promoter activity and binding, indicating that it functions as a *bona fide* CRE. Accordingly, it appears very likely that natCRE mediates cAMP stimulation of expression of the rat AA-NAT gene, presumably in response to cAMP-dependent phosphorylation of resident CREB molecules.

In addition to this site, a neighboring sequence appears to be involved in cAMP responsiveness because disruption of natCRE in the –267/+82 region of the promoter does not completely abolish the response to NE or Bt₂cAMP. The most likely element involved in this residual cAMP responsiveness is the inverted CCAAT box (natCCAAT) at position –120. natCCAAT represents a perfect match in reverse orientation to a known regulatory site referred to as the CCAAT box. Such sites are found in many promoters and appear to control gene expression through interaction with members of a growing family of different CATBPs (31–35). The natCCAAT element appears to exert a positive effect on transcription because site-directed mutagenesis abolished binding activity and decreased the level of reporter gene activity under both basal and stimulated conditions (Fig. 4B). In addition, a double mutant with a disrupted CRE-CCAAT complex displayed only basal luciferase activity after Bt₂cAMP stimulation. This finding, together with the partial decrease obtained after mutagenesis of either site alone, is consistent with the hypothesis that both elements are necessary to achieve full activation of the AA-NAT promoter by cAMP.

It should be noted that there are reports in the literature of CRE-CCAAT cooperation and of cAMP activation being mediated by a CCAAT box. As indicated above, a CRE-CCAAT complex is found in the fibronectin gene promoter (27–29), in which occupancy of the CCAAT box is facilitated by the CRE. In addition to this example of a CRE-CCAAT interaction, there also is evidence that the CCAAT box can mediate activation of gene expression in the absence of a CRE; this comes from the tryptophan hydroxylase gene (30). This points to the possibility that cAMP may act through CATBPs, perhaps via phosphorylation, as it does through CREB to control gene expression. It is of further interest to note that tryptophan hydroxylase is very strongly expressed in the pineal gland, suggesting that CATBPs may play a common role in this tissue in gene expression.

Turning Off Expression of the AA-NAT Gene—This investigation focused on the mechanisms through which cAMP activates expression of the rat AA-NAT gene and thereby generates the rhythmic ~100-fold increase in AA-NAT mRNA. Mention should be made here of some of the possible mechanisms that turn off expression of this gene, based on the structure of the promoter revealed in this study and current thinking about this issue. One mechanism that will significantly reduce expression of this gene involves a decrease in cAMP, which would terminate positive downstream effects of this second messenger. An additional theoretical mechanism involves cAMP-dependent coinduction of negative transcription factors such as Fra-2 (Fos-related antigen-2) (17) and the inducible cAMP early repressor (ICER) (36), which could repress transcription through binding to AP-1 and CRE sites, respectively.

Levels of mRNA encoding both factors increase in the pineal gland during the night period of a typical lighting cycle providing 10–12 h of darkness (17, 36). The increase in *fra-2* mRNA drives a >100-fold rhythm in Fra-2 protein that is generally similar to the increase in AA-NAT activity. We suspect that Fra-2 protein binds to AP-1 sites in the AA-NAT promoter,

negatively affecting transcription. One such site, identified above at position –32, is located in close proximity to the major transcription start point. Fra-2 strongly binds to AP-1 sites, but does not possess a strong transactivation domain (37). Binding of Fra-2-containing AP-1 complexes at this location could conceivably disrupt the assembly of the basic transcription machinery, inhibiting cAMP activation of AA-NAT expression. Inhibition of Fra-2 protein synthesis may explain why the cAMP-induced increase in AA-NAT mRNA is greater if protein synthesis is blocked (9).

Accordingly, it is possible to hypothesize that Fra-2 could be part of a complex AA-NAT gene regulatory mechanism controlled by cAMP, in which cAMP rapidly turns on expression of the AA-NAT gene through phosphorylation of CREB and activation of the natCRE-CCAAT complex and coincidentally induces expression of the *fra-2* gene and accumulation of Fra-2 protein, which then progressively turns off expression of the AA-NAT gene.

In contrast to the close association of the rhythm in *fra-2* mRNA and Fra-2 protein, the rhythm in ICER mRNA is not associated with a remarkable increase in ICER protein; rather, under typical lighting schedules, ICER protein appears to be relatively constant and stable (38). Accordingly, it seems reasonable to suspect that ICER and CREB proteins are present at all times of the day and that they compete for CRE occupancy. As a result, the relative abundance of ICER and CREB pools at these sites becomes an important factor because it could influence the magnitude of the AA-NAT response. Although the relative amount of ICER and CREB might not change under typical fixed laboratory lighting schedules, their relative abundance appears to change in response to very long and very short nights; for example, ICER levels are highest if animals are maintained in lighting cycles with 20-h dark periods (38). This could gradually increase the ICER/CREB ratio and might influence the magnitude of the AA-NAT rhythm. Such a mechanism could underlie seasonal changes in melatonin production.

Potentially Important Features of the AA-NAT Promoter—Our analysis revealed two additional features of the AA-NAT promoter that deserve comment. First, in addition to the data obtained by S1 nuclease protection analysis, two observations are consistent with the identification of the major transcription start point. One is the location, 38 bp upstream, of a TATA-like element. The other is the location of the transcription start point within a putative initiator sequence. This sequence belongs to a family of RNA polymerase II start sites (24, 25) and is present in other dynamically regulated genes, including homeotic genes and genes expressed during immunodifferentiation (for review, see Ref. 39). It seems possible that the dynamic regulation of AA-NAT and these genes may involve interactions of this element with the same or similar nuclear factors (40).

A second point relates to the issue of translational control and the finding that large changes in sheep AA-NAT activity and protein³ can occur with little or no change in levels of AA-NAT mRNA. It is reasonable to suspect that this translational control might be a conserved feature of AA-NAT regulation. In this regard, some features of the rat AA-NAT 5'-flanking region are relevant. Translation is known to be influenced by long leaders with extensive secondary structure (41, 42). Such extensive secondary structure is likely to form in the 173-bp leader sequence according to computer analysis of this region (43); this predicts the formation of an elongated stem-loop structure with a free energy of –54.1 kcal/mol (data not shown). It will be of interest to investigate the possible role

³ J. L. Weller and D. C. Klein, unpublished results.

of the leader sequence upon translational regulation of AA-NAT.

Summary—The promoter of the AA-NAT gene has been isolated and partially characterized. It appears that a CRE and an adjacent inverted CCAAT box function as a combined target site for the adrenergic signaling cascade that activates expression of this gene. The pattern of expression of the AA-NAT gene is similar to that of an immediate-early gene in that it is rapidly activated in the absence of *de novo* protein synthesis (9). Thus, rapid induction of AA-NAT gene transcription appears to rely on adrenergically induced post-translational modification and/or recruitment of pre-existing *trans*-acting factors belonging to the CREB and CATBP families.

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Addendum—Following submission of this manuscript, Folkes *et al.* reported the identification of natCRE (Folkes, N. S., Borjigin, J., Snyder, S., and Sassone-Corsi, P. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 14140–14145). In addition, they found a rhythm in AA-NAT mRNA in the normal mouse and in mice lacking ICER and found that the amplitude of the AA-NAT mRNA rhythm in mice lacking ICER was larger. This confirms the proposal here that ICER does not play a dynamic role in rhythmically turning off AA-NAT gene expression, but does play a role in determining the amplitude of the increase in AA-NAT mRNA.

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