

# Pineal Serotonin *N*-Acetyltransferase: Expression Cloning and Molecular Analysis

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Pineal serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, or AA-NAT) generates the large circadian rhythm in melatonin, the hormone that coordinates daily and seasonal physiology in some mammals. Complementary DNA encoding ovine AA-NAT was cloned. The abundance of AA-NAT messenger RNA (mRNA) during the day was high in the ovine pineal gland and somewhat lower in retina. AA-NAT mRNA was found unexpectedly in the pituitary gland and in some brain regions. The night-to-day ratio of ovine pineal AA-NAT mRNA is less than 2. In contrast, the ratio exceeds 150 in rats. AA-NAT represents a family within a large superfamily of acetyltransferases.

Amounts of circulating melatonin increase 10-fold at night in all vertebrates. This rhythm is generated by a variation in the activity of AA-NAT (E.C. 2.3.1.87), the penultimate enzyme in melatonin synthesis (serotonin to *N*-acetylserotonin to melatonin) (1). The nocturnal increase in pineal AA-NAT activity also markedly decreases serotonin (1–3). The rhythm in melatonin is essential for seasonal reproduction (4), modulates the function of the circadian clock in the suprachiasmatic nucleus (SCN), and influences activity and sleep (5).

AA-NAT is expressed primarily in the pineal gland and to a variable degree in the retinas of some vertebrates (6). The enzyme is rapidly inactivated (half-time, or  $t_{1/2} \approx 3$  min) when animals are exposed to light at night (1). Arylalkylamines are strongly preferred as substrates over other amines (7). Regulation of AA-NAT activity is controlled by a complex system (1) that includes the circadian oscillator in the SCN. At night, the SCN transmits signals to the pineal gland by a neural circuit passing through central and peripheral structures. Light acts on this system through a retinal-to-SCN projection to reset the SCN clock and to gate transmission to the pineal gland. SCN-generated signals stimulate the release of norepinephrine, which acts through  $\alpha$ - and  $\beta$ -adrenergic receptors to elevate pineal cyclic adenosine monophosphate and calcium. Studies in rats indicate that these second messengers elevate enzyme activity through a mechanism that

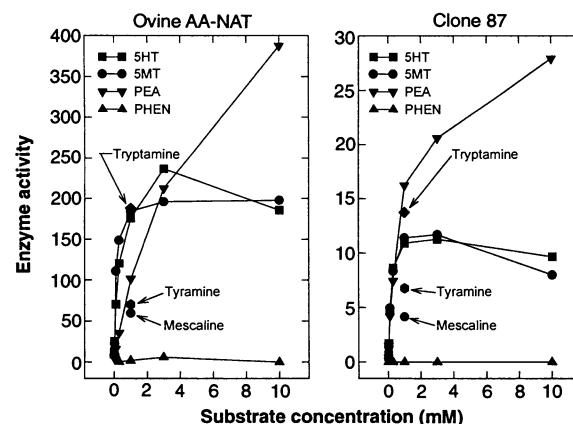
requires new gene expression, new protein synthesis, and stabilization (1). However, the precise molecular details of AA-NAT

regulation are not known, primarily because the complementary DNA (cDNA) encoding AA-NAT was not formerly available.

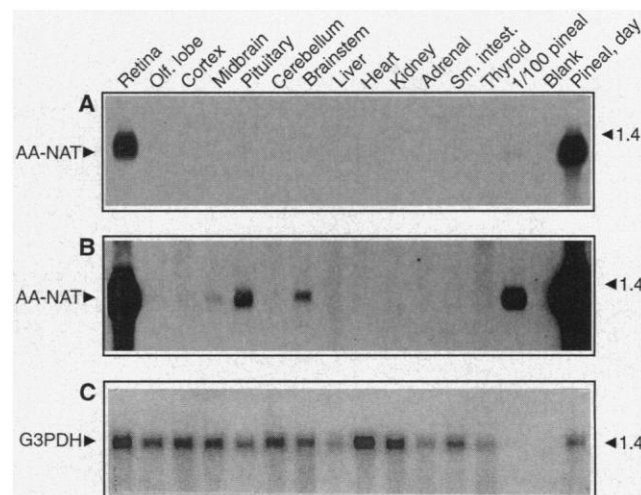
We cloned AA-NAT by using a cDNA expression library (8, 9). Pools of clones were transfected into COS-7 cells that were screened for AA-NAT expression by measuring acetylation of the arylalkylamine 5-methoxytryptamine and of the arylamine phenetidine. This screening identified an AA-NAT clone (clone 87) as indicated by substrate specificity of the encoded protein (Fig. 1) (10–12).

The largest amounts of ovine AA-NAT mRNA were found in the pineal gland (Fig. 2). One-quarter as much was found in the retina, consistent with reports of AA-NAT activity in chicken and frog retinas and human retinoblastoma Y79 cells (6, 13). We also found that the AA-NAT gene was expressed at low levels in ovine brain and pituitary gland (Fig. 2). This led to the finding of AA-NAT activity in the retina,

**Fig. 1.** Substrate specificity of partially purified ovine AA-NAT (11) compared with a homogenate of COS-7 cells expressing clone 87. Acetylation was determined in 20- $\mu$ l assays containing the indicated concentration of amine and 40  $\mu$ M [ $^{14}$ C]acetyl CoA (60 Ci/mol). [ $^{14}$ C]Acetylated 5-methoxytryptamine (5MT), tryptamine, phenethylamine (PEA), and phenetidide (PHEN) were extracted into chloroform (2). [ $^{14}$ C]Acetylated mescaline, serotonin (5HT), and tyramine were resolved by thin-layer chromatography (10) with acetylated standards (27). Radioactivity was determined by scintillation counting. Different  $V_{max}$  values (enzyme activity) reflect different levels of enrichment; enzyme activity is expressed in nanomoles of acetylated product per milligram of protein per hour.



**Fig. 2.** Extrapineal expression of the gene encoding AA-NAT. Ovine tissue was removed, 1-cm cubes were prepared, and cubes were rapidly frozen. Total RNA was extracted, and Northern (RNA) blot analysis was performed with random-primed cDNA (76). Each lane was loaded with 20  $\mu$ g of total RNA, except the lane marked "1/100 pineal," which received 0.2  $\mu$ g. Blots were probed with a full-length cDNA insert (960 bp) from clone 87, stripped, and re-probed with G3PDH cDNA to monitor loading and degradation. Similar results were obtained in two additional studies. Olf. lobe, olfactory lobe; Sm. intest., small intestine. Size markers are shown on the right in kilobases. (A) A 12-hour autoradiographic image of the blot probed with the insert from clone 87. No other bands of radioactivity were detected. (B) A 96-hour autoradiographic image of the same blot. Faint 3- and 5-kb bands were detected inconsistently in pineal glands and retinas (not shown). (C) A 12-hour PhosphorImager exposure of the blot probed with G3PDH.



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pituitary, and olfactory lobe (0.5, 0.2, and 0.1 nmol of acetylated product per milligram of protein per hour, respectively) (14). The discrepancy between amounts of AA-NAT mRNA and enzyme activity in extrapineal sites may reflect different levels of translation or posttranslational activation. Extrapineal AA-NAT is of special interest because it could influence serotonin concentrations and metabolism.

Ovine pineal AA-NAT mRNA increased less than twofold at night (Fig. 3A), which was much smaller than the nocturnal sevenfold increase in AA-NAT activity (Fig. 3B). In rats, pineal AA-NAT mRNA was very low during the day and increased more than 150-fold at night (Fig. 3A) (15, 16), similar to the increase in enzyme activity (1). The rat pineal AA-NAT mRNA rhythm is circadian and is regulated by the

same neural pathway that controls enzyme activity (15).

These observations indicate that posttranscriptional mechanisms dominate regulation of AA-NAT activity in sheep, whereas in rats both transcriptional and posttranscriptional mechanisms are involved (1, 17, 18). The involvement of different molecular strategies among species to increase amounts of melatonin at night underscores the biological importance of this signal. These different regulatory mechanisms may explain why in sheep melatonin increases at night immediately after exposure to darkness, whereas in rats there is a delay (19). This delay could be linked to the time required to elevate AA-NAT mRNA.

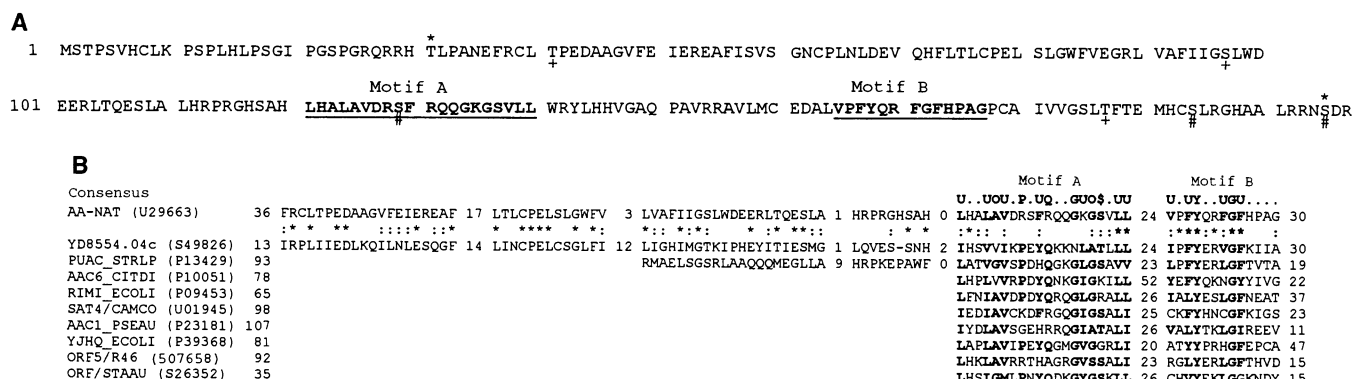
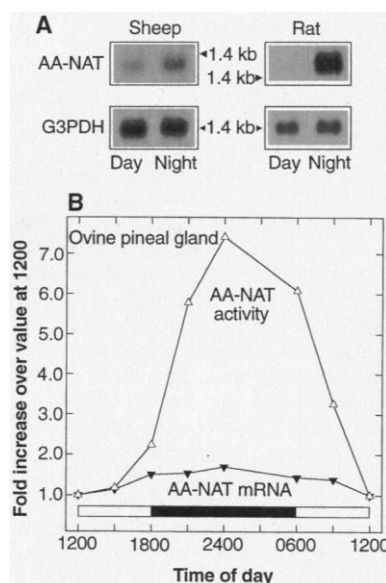
Clone 87 contains a putative 621-base pair (bp) open reading frame (ORF) encoding a 23.1-kD protein (Fig. 4A). The pre-

dicted size was confirmed by expression of clone 87 (20). Putative regulation sites exist (Fig. 4A): the two cyclic nucleotide-dependent protein kinase phosphorylation sites may be involved in rapid inactivation (1, 17); the two protein kinase C phosphorylation sites may mediate the effects of calcium (21), and one or more of the seven cysteines may be involved in activation and inactivation (18).

The AA-NAT amino acid sequence was not strongly similar to any functionally characterized protein in the databases; however, it was statistically similar to an uncharacterized, hypothetical yeast protein (YD8554.04c) (22). Marginal similarity was also found to *Streptomyces lipmanii* putomycin acetyltransferase (PUAC\_STRLP) and a hypothetical protein (ORF5/R46) (Fig. 4B). The similar regions included the portion of AA-NAT that aligned with YD8554.04c, which suggests that these regions may be functionally relevant. This interpretation is supported by the conservation in PUAC\_STRLP and AA-NAT of two essential motifs (designated A and B in Fig. 4B) found in several acetyltransferases (23). These motifs may function in acetyl coenzyme A (CoA) binding, acetyl group transfer, or both (24).

We searched for other AA-NAT-related proteins by using a block that encompasses motif A from the alignments of AA-NAT with the three proteins described above (25). Of the 79 related proteins identified in this search, 30 are acetyltransferases and the remainder are uncharacterized, hypothetical proteins. Therefore, it appears that motif A is a reliable identifier of an acetyltransferase superfamily. Subsequent multiple-alignment analysis confirmed the statistical significance of the similarity between AA-NAT and other acetyltransferases and identified additional re-

**Fig. 3.** Day and night studies of pineal AA-NAT mRNA. **(A)** Northern blot analysis. Day pineal glands were removed at ~1200 hours and night pineal glands at ~2400 hours. Sheep were maintained outdoors (8). Rats were housed in an automatically regulated 14:10 light-dark cycle (lights on at 0500 hours). Night samples were obtained from animals killed in dim red light. Northern blot analysis was as in Fig. 2. A rat cDNA probe was used to probe the rat blot (28). **(B)** A 24-hour study of ovine AA-NAT activity and mRNA. Enzyme activity was measured as described (2). The value at 1200 hours was 7.4 nmol of acetylated product per milligram of protein per hour. AA-NAT mRNA was analyzed by Northern blot; values were normalized to G3PDH mRNA. Enzyme activity and mRNA values are expressed relative to values at 1200 hours. Each point represents the average of enzyme activity or mRNA in two or three pineal glands; individual values were within 20% of the value given.



**Fig. 4.** Amino acid sequence and conserved motifs of ovine AA-NAT. **(A)** Deduced amino acid sequence (GenBank accession number U29663) (29). Putative phosphorylation sites: \*, cyclic nucleotide-dependent; #, protein kinase C; +, casein kinase II. The two conserved motifs are bold and underlined. **(B)** Alignment of the AA-NAT amino acid sequence with sequences of known and putative acetyltransferases (25). The number of amino acid residues from the protein termini and between the aligned blocks are indicated.

Identities between AA-NAT and YD8554.04c are indicated by asterisks; similarities, by colons. Consensus residues are conserved in most aligned sequences (bold residues conform to the consensus): U, bulky hydrophobic residue (I, L, V, M, F, Y, W); O, small residue (G, A, S); \$, S or T; dot, any residue. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

gions of limited similarity between AA-NAT, YD8554.04c, and PUAC\_STRLP (Fig. 4B) (25).

Although motifs A and B define a large acetyltransferase superfamily, this superfamily excludes arylamine *N*-acetyltransferase and many other acetyltransferases (23, 26). AA-NAT appears to represent a new family within this superfamily because there are no closely related proteins in the databases. The AA-NAT family may have emerged relatively late in evolution, perhaps in association with the evolution of melatonin as a photochemical signal. The cloning of AA-NAT opens the door to research on the function of extrapineal AA-NAT and should lead to the resolution of many long-standing issues in research on circadian rhythms and photoperiodism.

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8. Timed ovine pineal glands were purchased from the University of Auckland Medical School, Auckland, New Zealand. Animals were maintained outdoors and housed indoors (12 hours light:12 hours dark) for 1 day. Other tissues and pineal glands were obtained from the University of Michigan and were from adult sheep killed at ~1200 hours.
9. Size-selected (>1 kb) oligo(dT)-primed cDNA, prepared from polyadenylated RNA from the pineal glands of night-killed sheep, was used to construct a unidirectional cDNA library ( $1.7 \times 10^6$  independent recombinants) in the eukaryotic expression vector Zap Express (Stratagene, La Jolla, CA). pBK-CMV phagemids were excised from pools of 1000 phage from the unamplified library and transfected into COS-7 cells with Lipofectamine (Gibco/BRL, Gaithersburg, MD). After 48 hours, the cells were harvested and assayed for arylamine *N*-acetyltransferase and AA-NAT activities (10). Three positive pools were detected out of the first 50,000 clones; one was purified to a single colony (clone 87).
10. Samples were incubated with amine substrate and  $40 \mu\text{M}$  [ $^3\text{H}$ ]acetyl CoA (60 Ci/mol) in 0.1 M sodium phosphate buffer, pH 6.8, for 1 hour. One volume of ethanol containing 1 mM HCl, 1 mM authentic acetylated product, and 0.001% ascorbic acid was added, and the acetylated product was resolved by thin-layer chromatography (PE SIL G/UV sheets, Whatman, Maidstone, England; 30 min in chloroform-methanol- $\text{NH}_4\text{OH}$ , 80:20:1). The acetylated products were located, and radioactivity was determined (PhosphorImager, Molecular Dynamics, Sunnyvale, CA).
11. Glands were homogenized (1:10 w/v) at  $4^\circ\text{C}$  by use of a Polytron (Brinkmann Instruments, Westbury, NY) in 5 mM citrate, pH 5.5, containing 1 mM dithiothreitol (DTT) and 5% glycerol (buffer A); the homogenate was centrifuged (1 hour, 100,000g), and the supernatant was applied (1 ml/min,  $4^\circ\text{C}$ ) to a WAX column (Zorbax Bioseries, Dupont, Wilmington, DE) equilibrated with buffer A. The adsorbed material was eluted with NaCl (0 to 1 M; 55 min, 1 ml/min) and the eluent assayed for AA-NAT activity (2). Chromatography enriched AA-NAT activity 10-fold and removed arylamine *N*-acetyltransferase.
12. The apparent  $K_m$  (Michaelis constant; acetyl CoA =  $40 \mu\text{M}$ ) values for 5-methoxytryptamine with partially purified pineal AA-NAT and with expressed AA-NAT were 90 and 120  $\mu\text{M}$ , respectively; those for 5-hydroxytryptamine were 180 and 140  $\mu\text{M}$ , respectively. Neither enzyme preparation acetylated 1 mM aniline, sulfamethazine, 2-aminofluorene, isoniazid, *p*-aminobenzoic acid, octopamine, dopamine, norepinephrine, epinephrine, amphetamine, histamine, tryptophan, puromycin, glucosamine, ethanolamine, or chloramphenicol.
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14. Tissues (Fig. 2) from two animals were homogenized in 10 volumes of 0.1 M sodium phosphate buffer, pH 6.8, containing 1 mM DTT, 2  $\mu\text{M}$  leupeptin, 0.5 mM *p*-aminoethylbenzenesulfonyl fluoride, and 1 mM sodium fluoride. The homogenates were assayed in duplicate (2, 10); mean values are given. Pineal AA-NAT activity was 22 nmol of acetylated product per milligram of protein per hour.
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20. Expression in a reticulocyte lysate translation system (R. Baler and D. C. Klein, *J. Biol. Chem.*, in press) generated an ~23-kD protein with AA-NAT substrate specificity. Expression in bacteria of a fragment encoding the full predicted ORF of AA-NAT fused to hexahistidine (pET-15b vector, Novagen) produced an ~24-kD protein identified as AA-NAT by microsequencing and substrate specificity (R. Baler and D. C. Klein, unpublished data).
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22. The comparison was done with the BLASTP program [S. F. Altschul *et al.*, *J. Mol. Biol.* **215**, 403 (1990)]. The probability of AA-NAT and YD8554.04c matching by chance is  $\sim 10^{-4}$ ; there is 31.7% identity and 60.3% similarity in two aligned segments of a total length of 63 amino acid residues.
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