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Cloning of an arylalkylamine *N*-acetyltransferase (aaNAT1) from *Drosophila melanogaster* expressed in the nervous system and the gut

EDITH HINTERMANN*, NICOLE C. GRIEDER†, REMO AMHERD*, DANIELA BRODBECK*, AND URS A. MEYER*‡

Departments of *Pharmacology and †Cell Biology, Biozentrum of the University of Basel, CH-4056 Basel, Switzerland

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ABSTRACT **In insects, neurotransmitter catabolism, melatonin precursor formation, and sclerotization involve arylalkylamine *N*-acetyltransferase (aaNAT, EC 2.3.1.87) activity. It is not known if one or multiple aaNAT enzymes are responsible for these activities. We recently have purified an aaNAT from *Drosophila melanogaster*. Here, we report the cloning of the corresponding aaNAT cDNA (aaNAT1) that upon COS cell expression acetylates dopamine, tryptamine, and the immediate melatonin precursor serotonin. aaNAT1 represents a novel gene family unrelated to known acetyltransferases, except in two weakly conserved amino acid motifs. *In situ* hybridization studies of aaNAT1 mRNA in embryos reveal hybridization signals in the brain, the ventral cord, the gut, and probably in oenocytes, indicating a broad tissue distribution of aaNAT1 transcripts. Moreover, in day/night studies we demonstrate a diurnal rhythm of melatonin concentration without a clear-cut change in aaNAT1 mRNA levels. The data suggest that tissue-specific regulation of aaNAT1 may be associated with different enzymatic functions and do not exclude the possibility of additional aaNAT genes.**

Amine acetylation in insects by the enzyme arylalkylamine *N*-acetyltransferase (aaNAT, EC 2.3.1.87) is involved in at least three different physiological functions: (*i*) It appears to be the major route of neurotransmitter catabolism (1–4), inactivating biogenic amines including tryptamine, tyramine, octopamine, serotonin, and norepinephrine. (*ii*) An acetylated amine, acetyldopamine, is subsequently oxidized to the respective quinone, which cross-links different proteins and/or chitin, resulting in stabilization and hardening (sclerotization) of the insect cuticle (5). (*iii*) Acetylation of serotonin plays an important role in the regulation of photoperiodically influenced physiological and behavioral processes (6). The major regulatory hormone is melatonin (7, 8), which is synthesized by the methylation of acetylserotonin. In vertebrates, melatonin is secreted periodically with high concentration at night, caused by a 10- to 100-fold nocturnal increase in aaNAT activity (8). The final steps of aaNAT activation are still unclear and presumably involve new RNA and protein synthesis (9–12). All living organisms are influenced by environmental factors such as light and dark and thus need mechanisms to coordinate their physiological processes in response to seasonal changes. Serotonin, aaNAT activity, and melatonin have indeed been found in several organs (eye, optic lobe, and brain) of various invertebrate species in which melatonin concentration also shows a rhythmic behavior (13). However, it is not known if aaNAT activity and melatonin are subject to diurnal variation in *Drosophila melanogaster*.

aaNAT activity has been described in several insect species (14–18), including *D. melanogaster* (2, 19), but none of these enzymes could be purified to derive antibodies or sufficient internal sequence information for cloning. We have purified an aaNAT from *D. melanogaster* using tryptamine acetylation as a functional enzyme assay (20). In the present study, we document the isolation of the corresponding cDNA (aaNAT1) encoding an enzymatically active aaNAT, and discuss the presence of several aaNAT enzymes in *D. melanogaster*. Moreover, our results indicate a diurnal rhythm of melatonin concentration also in *D. melanogaster*.

MATERIALS AND METHODS

Maintenance of Fly Cultures. The Oregon-R wild-type strain of *D. melanogaster* was maintained at 25°C on standard cornmeal agar medium and live yeast, and entrained to a 12-hr light/12-hr dark photoperiod cycle.

Determination of aaNAT Activity and Melatonin Content. Extracts were prepared by homogenization either of whole flies (1 g per 5 ml) or of heads only (25 heads per 500 μl), and aaNAT activity was measured as described (20). Melatonin concentration was determined by RIA (Nichols Institute, Geneva).

Reverse Transcriptase–PCR. Total RNA was isolated by the guanidine thiocyanate/CsCl method. First-strand cDNA was produced by Moloney murine leukemia virus reverse transcriptase (GIBCO), using 1 μg of total RNA and 3.4 μM of one of the four antisense primer mixtures: $(dT)_{12}MA=apA$, $(dT)_{12}MC = apC$, $(dT)_{12}MG = apG$, and $(dT)_{12}MT = apT$, where M = A, C, or G. The first-strand cDNAs of the four reverse transcription reactions were amplified by PCR in 30 μl final volume under the following conditions: 2 μl template, 3.4 μM sense primer 5′-GA(C/T)CA(A/G)GA(C/T)TG(C/T)CC(A/C/G/T)TA(C/T)AC-3′ = sp20 (residues 2–8, DQDCPYT, of the 20 amino acid peptide described in ref. 21), 3.4 μM appropriate antisense primer mixture, 50 μM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris·HCl (pH 8.3), and 1 unit Ampli*Taq* polymerase (Perkin–Elmer) at 40 cycles at 94°C for 1 min; 42°C for 1 min; 72°C for 1 min 30 sec. PCR products were purified and then dissolved in 10 μl TE (10 mM Tris·HCl/0.1 mM EDTA, pH 8), and 5 μl aliquots were reamplified in 15 cycles. PCR products were subcloned, and the nucleotide sequence was determined using the Sequenase version 2.0 DNA sequencing kit (Amersham).

Library Screening. The 298-bp PCR fragment was labeled with [α-^{32}P]dATP to a specific activity of 10^8 cpm/μg. This probe was used to screen a ZapII cDNA library from adult *D. melanogaster* Canton-S strain (Stratagene). Filters were hybridized at 55°C in 6× SSC (20× SSC = 3 M NaCl/0.3 M sodium citrate·$2H_2O$, pH 7.0), 20 mM NaH_2PO_4, 0.4% SDS, and 500 μg/ml salmon sperm DNA for 16 hr, and washed at 50°C in 2× SSC and 0.1% SDS twice for 20 min each time.

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Abbreviation: aaNAT, arylalkylamine *N*-acetyltransferase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. YO7964).

‡To whom reprint requests should be addressed.

Expression in COS-7 Cells. The aaNAT1 cDNA (*Eco*RI fragment) was inserted into the pcDNAI vector (Invitrogen) in both directions, which were denoted as paaNAT1s (*s*ense) and paaNAT1a (*a*ntisense). COS-7 cells were cultured as described (21). Plasmid DNA was introduced by DEAE-dextran-mediated transfection (22). Three days after transfection, the monolayers were rinsed with PBS (137 mM NaCl/8 mM $Na_2HPO_4 \cdot 12\ H_2O$/2.7 mM KCl/1.47 mM KH_2PO_4, pH 7.2) and harvested in 400 μl ice-cold homogenization buffer (20). Cells were sonicated on ice and centrifuged at 15,000 *g* for 5 min, and enzyme activity was measured (20) in 50 μl supernatant with tryptamine, serotonin, and dopamine (0.02 mM to 4 mM) at a [1-^{14}C]acetyl CoA concentration of 2 mM. K_m was determined by the data analysis program GRAFIT (23).

Southern and Northern Blot Analyses. Ten micrograms of digested genomic DNA were separated by gel electrophoresis on a 0.6% agarose gel and transferred to nylon membranes. High stringency consisted of hybridization at 60°C in 6× SSC, 20 mM NaH_2PO_4, 0.4% SDS, and 500 μg/ml salmon sperm DNA for 16 hr either with a 260-bp long aaNAT1 5′-probe (bp 36 to 295) or with a 304-bp long 3′-probe (bp 522 to 825); both probes were random labeled with [α-^{32}P]dATP. Blots were washed in 2× SSC/0.1% SDS for 15 min at room temperature, followed by 20 min at 60°C in 2× SSC/0.1% SDS and 20 min at 60°C in 0.5× SSC/0.1% SDS. A last wash at 60°C was performed in 0.2× SSC/0.1% SDS for 15 min. Low stringency consisted of hybridization either with the 3′-probe or with a 757-bp long probe (bp 36 to 792) at 37°C in 5× SSC, 5× Denhardt's solution (50× Denhardt's solution = 1% bovine serum albumin/1% polyvinylpyrrolidone/1% Ficoll), 250 μg/ml salmon sperm DNA, 50 mM sodium phosphate buffer (pH 7.0), 0.1% SDS, 10% dextran sulfate, and 25, 30, or 43% formamide for 42 hr. Washes were performed in 2× SSC/0.1% SDS for 15 min at room temperature, for 30 min at 42°C and for 15 min at 45°C. Further washes were done in 0.5× SSC/0.1% SDS at 45°C for 30 min and at 50°C for 15 min. The final wash was in 0.2× SSC/0.1% SDS at 45°C for 15 min. Northern blot analysis was performed with the radiolabeled 757-bp probe using 20 μg total RNA from whole flies to assess transcript length and with 10 μg total RNA from heads only for the day/night experiment. The same high stringency conditions as described for Southern blot analysis were used.

***In Situ* Hybridizations.** *In situ* hybridizations of whole-mount embryos and polytene chromosomes were carried out with RNA and DNA probes, respectively, as described (24, 25). Both probes were designed to recognize the central cDNA region (bp 36 to 792).

RESULTS

Molecular Cloning of an aaNAT1 Specific cDNA. With the primer combination sp20/apG, a PCR product was isolated that was 298-bp long and contained the sequences coding for all four internal peptides identified in aaNAT1 (Fig. 1*A*) (20). Surprisingly, this DNA fragment contained no poly(A) tail, presumably due to amplification by only one primer (26). A λZapII cDNA library from adult *D. melanogaster* was screened with this fragment, 32 phagemid clones were isolated, and three clones were further analyzed by restriction digestion and Southern blotting (data not shown). The longest isolated cDNA contained 1546 bp, of which 825 bp encoded a 275 amino acid protein (Fig. 1*A*). The first ATG in 5′ to 3′ direction was flanked by a region matching the consensus sequence for translation initiation sites, ^{C}/AAA^{A}/CATG in *D. melanogaster* (27) (data not shown). The calculated molecular mass of the protein was 30,975 kDa with a calculated isoelectric point of 5.08. The cDNA had a 5′-untranslated region of 488 bp and a 3′-untranslated region of 217 bp [the poly(A) tail was not included in the analysis]. Northern blot analysis of *Drosophila* total RNA revealed a hybridizing transcript of ≈1650 ± 50 nucleotides (*n* = 2; mean range, data not shown).

Sequence Comparison with Other *N*-Acetyltransferases. A GenBank data base search with the cDNA encoding aaNAT1 revealed no homology to cDNAs or genes encoding other *N*-acetyltransferases. A search for homologies in the protein data base SWISSPROT using the BLAST algorithm (28) revealed a sequence of 73 amino acids of aaNAT1 that was 33% identical to a region of the MAK3 *N*-acetyltransferase of *Saccharomyces cerevisiae* (Fig. 1*B*), which is required for double-stranded RNA virus propagation via acetylation of the NH_2 terminus of the viral major coat protein (29). The homology extends over two regions, A and B, that are statistically conserved in NAT enzymes of several bacterial strains and yeast: aminoglycoside 6′-*N*-acetyltransferase from

A

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                               *          *                                              o          *
MEVQKLPDQS LISSMMLDSR CGLNDLYPIA RLTQKMEDAL TVSGKPAACP VDQDCPYTIE LIQPEDGEAV IAMLKTFFFK DEPLNTFLDL GECKELEKYS  100
 *#                               o               o                                         *
LKPLPDNCSY KAVNKKGEII GVFLNGLMRR PSPDDVPEKA ADSCEHPKFK KILSLMDHVE EQFNIFDVYP DEELILDGKI LSVDTNYRGL GIAGRLTERA  200
#          *
YEYMRENGIN VYHVLCSSHY SARVMEKLGF HEVFRMQFAD YKPQGEVVFK PAAPHVGIQV MAKEVGPAKA AQTKL                          275
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B

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aaNAT1     178   GKILSVDTNYRGLGIAGRLTERAYEYM-RENGINV-YHVLCSSHYSARVMEKLGFHEVFRMQFADYKPQGEVVFK  250

MAK3        81   IGMLAVESTYRGHGIAKKLVEIAIDKMQREHCDEIMLETEVENSAALNLYEGMGFIRMKRM-FRYYLNEGD-AFK  153
Consensus        h..h.h...Y..HGIGK.Lh...h                h.hE....N..A...Y...GF.........Y...DG
                             K  AR                         D                            E

                        REGION A                                     REGION B
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FIG. 1. Amino acid sequence and conserved regions of aaNAT1. (*A*) Amino acid sequence numbered from the initiator methionine. Double underligned regions indicate internal peptide sequences derived from purified aaNAT1 (20). Seven amino acids of the first underligned sequence (amino acids 52 to 70) were used for the design of the primer sp20 for PCR amplification. Boldface type represents amino acid sequence derived from the PCR product used for library screening. Underlined regions indicate the two conserved motifs A and B. Putative phosphorylation sites: –, cAMP- and cGMP-dependent protein kinase; ○, casein kinase II; *, protein kinase C; #, tyrosine kinase. (*B*) Region of aaNAT1 amino acid sequence with homology to a stretch of the yeast MAK3 *N*-acetyltransferase. "Consensus" represents the two regions (A and B) of homology among different bacterial and yeast *N*-acetyltransferases. h, Hydrophobic; a period represents any amino acid. Residues underlined in the "Consensus" sequence are those that were changed in MAK3 by site-directed mutagenesis (29).

Citrobacter diversus, streptothricin acetyltransferase from *Streptomyces lavendulae*, rimL and rimJ of *Escherichia coli*, which acetylate ribosomal proteins, and the enzyme system NAT1/ARD1 acetylating the NH_2 termini of yeast proteins.

Expression Studies and Kinetic Analysis of aaNAT1 in COS-7 Cells. Cells transiently transfected with paaNAT1s (sense) (Table 1) had a high enzyme activity as compared with cells transfected with paaNAT1a (antisense) or mock-transfected cells. This result provides conclusive evidence that the isolated cDNA codes for an enzymatically active aaNAT from *D. melanogaster*. Expressed aaNAT1 enzyme had the highest affinity for tryptamine, followed by dopamine and serotonin (Table 2).

Southern Blot Analysis and Chromosomal Localization in *D. melanogaster*. Restriction analysis of *Drosophila* genomic DNA was performed by Southern blotting with a panel of five restriction enzymes that do not cut in the cDNA sequence. With *Hin*dIII, *Pst*I, or *Xba*I, one single DNA band hybridized with the 5′- and the 3′-probe (Fig. 2, lanes C–E and C′–E′), suggesting the presence of one gene coding for aaNAT1. Digestion with *Bam*HI or *Eco*RI led to the identification of two bands with each probe (Fig. 2, lanes A, B, A′, and B′), consistent with the presence of at least two introns in this gene. When low stringency hybridization was performed in a buffer containing 43% formamide at 37°C, the 757-bp probe hybridized with additional bands (Fig. 2, arrowheads in lane B*), suggesting the presence of gene(s) related to aaNAT1. *In situ* hybridization to polytene chromosomes of salivary glands revealed the location of the aaNAT1 gene in band 60B on the second chromosome (data not shown).

***In Situ* Hybridization of aaNAT1 mRNA in Whole-Mount Embryos.** aaNAT1 gene expression was investigated by *in situ* hybridization studies in whole-mount embryos of different stages (Fig. 3). aaNAT1 transcripts were first detected in stage 14 embryos (stages according to ref. 30) in cells of the future proventriculus. From stage 15 onward, aaNAT1 transcripts were strongly expressed in the proventriculus as well as in endodermal cells of the anterior and posterior midgut, whereas the remainder of the midgut showed only very weak expression. aaNAT1 transcripts were excluded from cells of the developing gastric caeca similar to transcripts of the POU-box gene pdm1 (31). Expression of aaNAT1 mRNA in the ventral cord and the brain was complex and seemingly nonsegmental; in addition, older embryos contained more aaNAT1-expressing cells than younger ones. Some tracheal cells and lateral cells, probably oenocytes, as well as cells of the amnioserosa seemed to express aaNAT1 in a dynamic fashion (data not shown). In general, aaNAT1 expression appeared relatively late in embryogenesis but resembled the developmental profile of dopamine acetyltransferase activity determined by Marsh and Wright (32).

Day/Night Studies of aaNAT1 in Adult *D. melanogaster*. Adult animals entrained to a 12-hr light/12-hr dark photoperiod at a constant temperature of 25°C were sampled at 3-hr intervals to determine aaNAT1 mRNA level and aaNAT activity in isolated heads and melatonin content in whole flies. aaNAT1 mRNA concentration showed no significant circadian rhythm during 24 hr, although mRNA contents at night were slightly lower than during the day (Fig. 4 *A* and *B*). When aaNAT activity was determined, lowest levels (2100 ± 10 nmol/mg × hr; mean ± range) were detectable at midnight (Fig. 4*C*). Enzyme activity increased slightly to 2287 ± 109 nmol/mg × hr at 9:00 a.m. We observed rhythmic secretion of melatonin with a peak value at noon (Fig. 4*D*). The lowest melatonin concentration of 5.62 ± 0.65 pg/mg (mean range) was measured at 9:00 a.m. This value increased within 3 hr to the value of 11.45 ± 0.15 pg/mg at 12:00 p.m. A comparable melatonin concentration of 9.2 pg/mg was determined in adult flies collected between 9:00 and 11:00 a.m. by Finocchiaro *et al.* (33).

Table 1. Expression of aaNAT1 cDNA in COS-7 cells

	Activity (nmoles × mg^{-1}·hr^{-1})
Mock (without DNA)	2.4 ± 1.1
paaNAT1a	11.5 ± 3.3
paaNAT1s	636 ± 170

COS-7 cells transfected with paaNAT1s (sense) or paaNAT1a (antisense). Specific aaNAT1 activity with tryptamine was measured in the supernatant fraction of cell homogenate (n = 2; mean ± range).

Table 2. Kinetics of expressed aaNAT1 cDNA in COS-7 cells

	K_m, mM		
	Tryptamine	Dopamine	Serotonin
paaNAT1s	0.19 ± 0.01	1.15 ± 0.9	1.62 ± 0.16

K_m values determined in COS-7 cells transfected with paaNAT1s in a concentration range of 0.02–4 mM for all three substrates (n = 4; mean ± SD).

Southern Blot Analysis of Different Vertebrate Species. With genomic DNA from *Xenopus*, mouse, and human, the 757-bp probe of aaNAT1 hybridized to several restriction fragments (Fig. 5, arrowheads) when blots were incubated in a buffer containing 30% formamide. At higher stringency (43% formamide), binding to the mammalian DNA remained, whereas signals with *Xenopus* disappeared. Hybridization with fish DNA was only detectable when the formamide concentration was lowered to 25%. The 757-bp probe showed no signal with the chicken DNA, whereas the 3′-probe hybridized to several DNA fragments when incubated in a buffer containing 30% formamide.

DISCUSSION

In this report, we describe the sequence of an aaNAT from *D. melanogaster* and provide evidence for its capacity to catalyze different enzymatic pathways. The isolated cDNA encodes an enzyme that may represent the first member of a new subfamily of acetyltransferases since it showed no obvious homology at

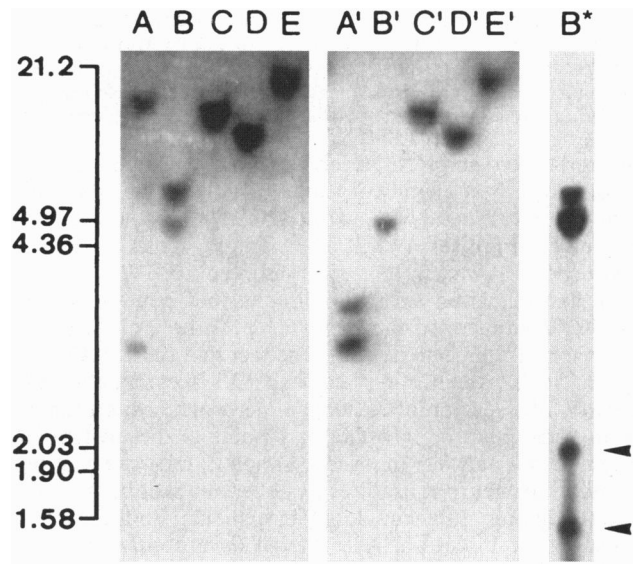

FIG. 2. Southern blot analysis of genomic *Drosophila* DNA with aaNAT1 probes. Ten micrograms of genomic *Drosophila* DNA were digested by the indicated restriction enzymes: A and A′, *Bam*HI; B, B′, and B*, *Eco*RI; C and C′, *Hin*dIII; D and D′, *Pst*I; E and E′, *Xba*I. Lanes A–E: 5′-probe, high stringency conditions; lanes A′–E′: 3′-probe, high stringency conditions; lane B*: 757bp probe, low stringency conditions. *Eco*RI/*Hin*dIII digested λ DNA marker is indicated on the left (numbers represent kb).

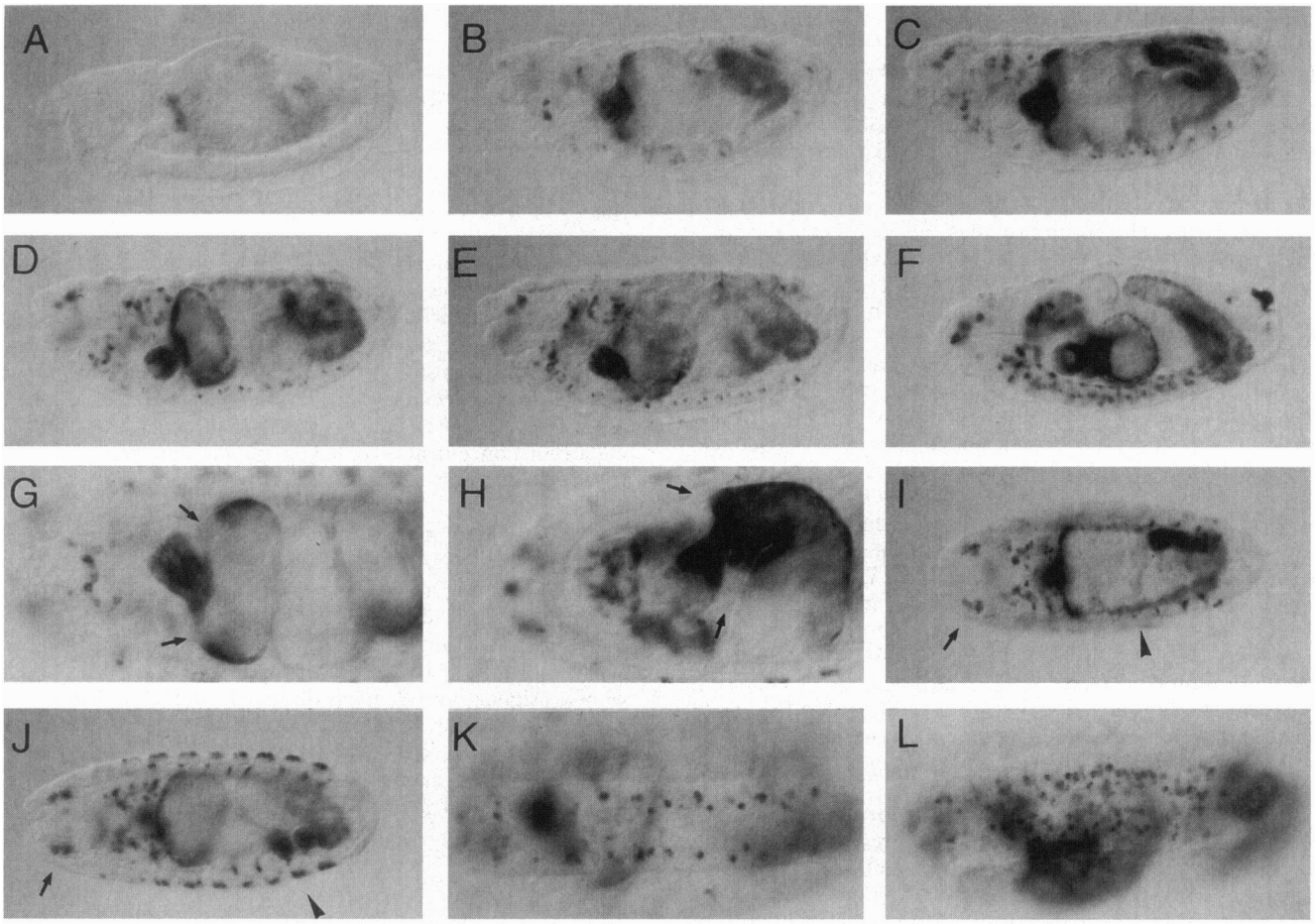

FIG. 3. Expression pattern of aaNAT during embryonic development. (*A–L*) Whole-mount embryos hybridized with an antisense aaNAT1 RNA probe. (*A*) Lateral view of a stage 14 embryo: aaNAT1 transcripts start to be expressed in the future proventriculus. (*B*) Lateral view of a stage 15 embryo: strong staining of the proventriculus and the anterior as well as the posterior midgut; however, only faint staining throughout the central midgut, single cells in the ventral cord and the brain, and a cluster of cells in the head stain positive for aaNAT1 mRNA. (*C*) Lateral view of a stage 16 embryo. (*D* and *E*) Lateral views of progressively older stage 17 embryos: the spatial distribution of the staining pattern remains unchanged from stage 15 onwards. Staining of the median tooth and the Filzkörper in *F* is unspecific as it is also seen with the sense probe. (*G* and *H*) Dorsal view of a stage 16 and a stage 17 embryo, respectively: strong staining is detectable in the proventriculus and in the first midgut compartment, however not in cells of the developing gastric caeca (arrow). (*I* and *J*) Dorsal view of a stage 16 and a stage 17 embryo, respectively, showing staining in a cluster of cells in the head (arrows). Some cells of the tracheal system (arrowhead) and cells possibly representing the oenocytes are also staining. (*K* and *L*) Ventral view of a stage 17 embryo: only single cells of the ventral cord are staining; their spatial distribution does not appear to be segmental; in addition, more cells seem to stain in older embryos.

the amino acid and DNA levels to already known acetyltransferases. The best alignment (homology of 33% at the amino acid level) was found with a stretch of 73 amino acids of yeast *N*-acetyltransferase MAK3. The homology was confined to two regions, A and B, that are conserved in NATs of several bacterial strains and yeast. Site-directed mutagenesis studies in MAK3 demonstrated the importance of these consensus regions for enzyme activity (29). The fact that these NATs have amino acids as substrates and aaNAT1 acetylates arylalkylamines like serotonin and dopamine, which are synthesized from tryptophan or tyrosine, may point to the evolutionary origin of this aaNAT. In insects, arylalkylamine acetylation is required for neurotransmitter turnover, melatonin formation, and hardening of the cuticle. In agreement with these diverse functions of insect aaNAT, the product of the transiently expressed aaNAT1 cDNA catalyzed the acetylation of serotonin, dopamine, and tryptamine in COS-7 cell homogenates, albeit with different affinities (Tables 1 and 2). The question remains if only one or different genes or gene products are responsible for the acetylation of these different arylalkylamines *in vivo*.

High stringency Southern blot analysis with genomic *Drosophila* DNA indicated that aaNAT1 is encoded by one gene and that the aaNAT1 cDNA contains no homologies to other *Drosophila* genes. However, using low stringency conditions, additional hybridization signals became visible (Fig. 2, lane B*), consistent with the presence of related genes. During our purification of aaNAT1 based on tryptamine acetylation activity (20), fractions with biphasic enzyme kinetics, separation of two activity peaks on hydroxylapatite columns, and evidence for two additional protein spots after two-dimensional SDS/PAGE also suggested the presence of additional aaNAT enzymes (data not shown).

In situ hybridization studies further substantiated these observations: aaNAT1 was expressed in the central nervous system, consistent with functions in neurotransmitter catabolism and melatonin production. Further hybridization signals were found in oenocytes, the predicted location of a dopamine acetyltransferase involved in sclerotization, studied by Maranda and Hodgetts (19) and mapped to position 60B1-10 on chromosome 2 (32). Interestingly, the aaNAT1 gene was also localized to band 60B. Additional aaNAT1 transcripts were found in certain areas of the gut. This result is consistent with the finding that in higher vertebrates the gut might be an extrapineal site of melatonin synthesis (34). Taken together, these observations strongly support the concept that more than

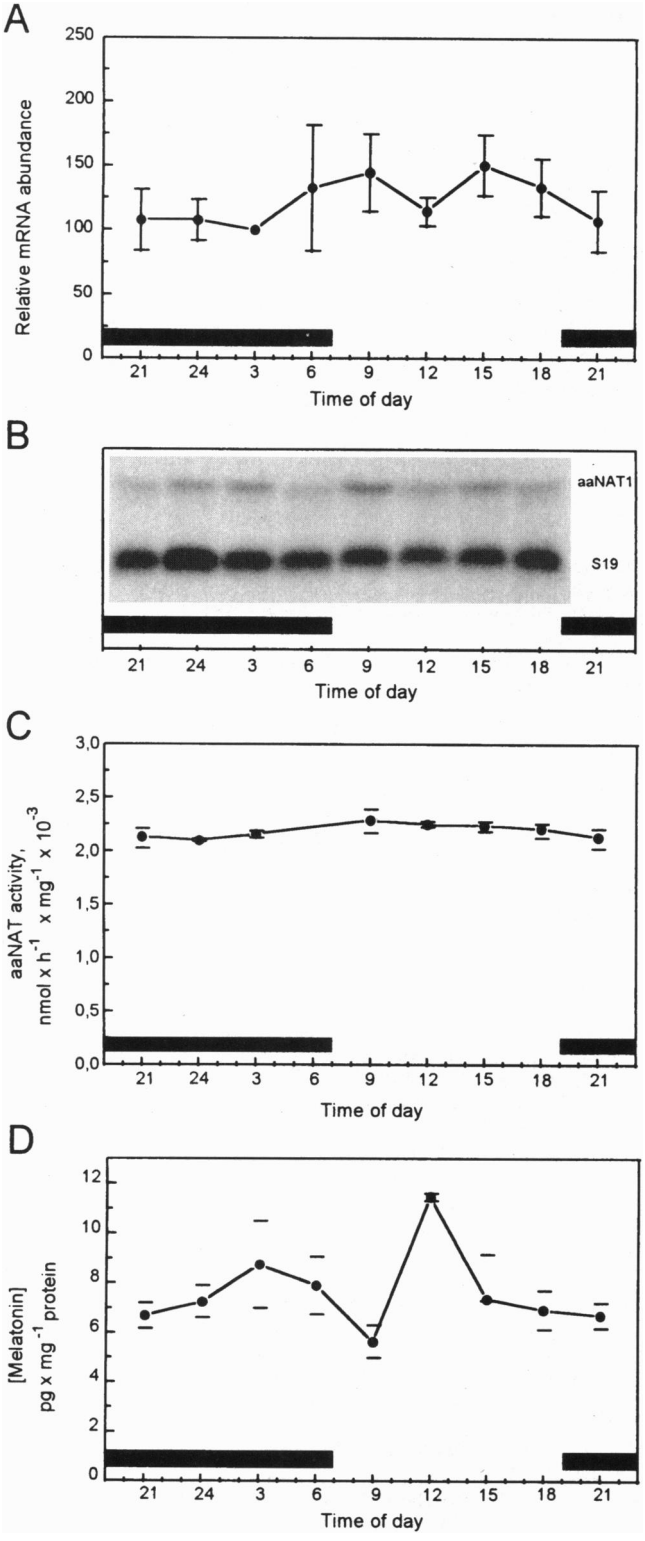

FIG. 4. aaNAT1 mRNA levels, aaNAT activity, and melatonin production during 24 hr. (*A*) Northern blot analysis of total head RNA with the 757-bp aaNAT1 probe under high stringency conditions. RNA extractions from three different day/night experiments were analyzed (n = 3, mean ± SD). Values were normalized to ribosomal S19 RNA and represent % of mRNA level relative to the value at 3:00 a.m. (*B*) One typical Northern blot. (*C*) aaNAT activity (tryptamine acetylation) measured in 25 heads per time point. Values represent mean range of triplicates of one fly collection. (*D*) Melatonin content for each time point was determined from one fly collection in two independent homogenate extractions and each sample analyzed in duplicates (values = mean ± range).

one aaNAT enzyme may be expressed in insects as previously suggested (14, 16). The fact that the gene encoding a dopamine acetyltransferase activity (5) and the aaNAT1 gene described here both were localized to the 60B locus on chromosome 2 suggests that either aaNAT1 exclusively encodes a dopamine acetyltransferase described earlier (19) or that additional aaNAT genes are located side by side at the 60B locus. The cDNA described here provides the first tool to answer this interesting question. We are currently isolating the aaNAT1 gene to study its potential to produce different products in different tissues.

Low stringency Southern blot analysis with genomic DNA from different vertebrate species (Fig. 5) revealed several restriction fragments hybridizing with defined regions of the aaNAT1 cDNA, indicating that the *Drosophila* aaNAT1 cDNA may provide information on the evolutionary precursor of mammalian aaNATs. Although aaNAT1 mRNA levels in *Drosophila* heads showed no prominent circadian rhythm, an involvement of aaNAT1 in the melatonin synthesis pathway similar to mammals cannot yet be excluded, since little is known about the circadian regulation of aaNAT activity in insects. In contrast to mammals, the melatonin peak value in *D. melanogaster* occurred during the day. Similar findings were made in the giant freshwater prawn, where a melatonin peak was measurable at 3:00 p.m. (35). Surprisingly, aaNAT activity in *Drosophila* homogenates showed no significant circadian rhythm. This may be explained by the presence of several aaNAT enzymes in *D. melanogaster* with overlapping enzyme activities and possibly different circadian rhythms, as discussed above. However, our observation that melatonin content is subject to diurnal variation also in *D. melanogaster* is of particular interest in view of the recent discovery of circadian "clock genes" and clock mutants in *D. melanogaster* such as *per* (36, 37) and *tim* (38). There is evidence for autoregulation of these genes by interaction between the PER and TIM proteins (39, 40) involving timed transfer of a PER–TIM dimer into the nucleus. However, how dimers enter the nucleus and how they shut down the expression of their own genes and affect other genes remains incompletely understood.

In vertebrates, regulation and fine-tuning of circadian melatonin production by photoperiodic signals requires complex interactions of transsynaptic signaling. Indirect evidence suggests that aaNAT activity is regulated via a cAMP-inducible promoter element, the cAMP responsive element (CRE) interacting with cAMP responsive element binding protein (CREB) and inducible cAMP early repressor (ICER) (41). Opposing functions of cAMP responsive element binding protein activators and repressors have recently also been described in *Drosophila* (42). The cloning of the first aaNAT gene from insects will now permit to study its tissue-specific dependence on the cAMP signal transduction pathway and its interaction with clock genes in the control of daily cycles of observable rhythmic behaviors.

Note. After completion of the present study, two independent reports appeared, describing amino acid sequences of two mammalian aaNAT enzymes [in sheep (43) and in rat (44)]. The two consensus motifs A and B of the ovine aaNAT amino acid sequence with bacterial and yeast acetyltransferases also showed homologies to conserved motifs in aaNAT1 (Fig. 1*B*).

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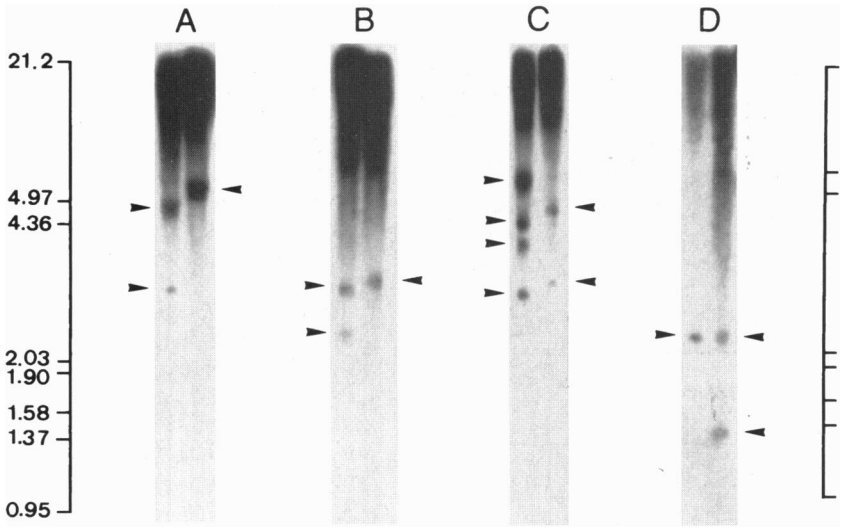

FIG. 5. Genomic Southern blot analysis in different vertebrate species. Genomic DNA from different species was digested with *Bam*HI (*Left*) or *Xba*I (*Right*), except for *D*, in which both lanes reflect *Xba*I digestion. (*A*) *Xenopus*, 757-bp aaNAT1 probe, 30% formamide. (*B*) Carp, 757-bp probe, 25% formamide. (*C*) Chicken, 3′-probe, 30% formamide. (*D*) Mouse (*Left*) and human (*Right*), 757-bp probe, 43% formamide.

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