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Molecular Cloning and Characterization of a cDNA Encoding the Retinal Arylalkylamine *N*-Acetyltransferase of the Rainbow Trout, *Oncorhynchus mykiss*

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ABSTRACT—Melatonin synthesis in the retina as well as in the pineal gland exhibits daily variations with higher levels during the dark phase of light-dark cycles. To analyze the molecular mechanism of melatonin synthesis in the retina, we have cloned, sequenced and characterized a putative cDNA for arylalkylamine *N*-acetyltransferase (AANAT; EC 2.3.1.87), a rate-limiting enzyme in melatonin production, from the retina of the rainbow trout (*Oncorhynchus mykiss*). The trout AANAT cDNA (1,585 bp) contains an open reading frame encoding 240 amino acid protein (predicted molecular weight, 27,420) that is 51-65% identical to avian and mammalian AANAT. The trout retinal AANAT protein contains motifs A and B that are conserved among the *N*-acetyltransferase superfamily and eight potential phosphorylation sites. Southern blot analysis demonstrated that the protein is expressed by a single copy gene. A single AANAT transcript (1.6 kb) was detected in the retina but not in the liver by Northern blot analysis. The levels of AANAT mRNA in the retina exhibited day-night changes with 3.3-fold increase at night. These results indicate that in the rainbow trout retina, the activity of AANAT and thus melatonin synthesis are regulated at least in part at the transcriptional level.

INTRODUCTION

In almost all vertebrates, melatonin production in the pineal gland and retina is controlled by both light and a circadian clock as revealed by *in vivo* and *in vitro* experiments (Yu and Reiter, 1992; Iigo *et al.*, 1994, 1997; Cahill and Besharse, 1995; Tosini and Menaker, 1996). Melatonin production in the pineal gland of mammals is regulated by a circadian clock located in the suprachiasmatic nucleus which is entrained to environmental light-dark (LD) cycles via a multi-synaptic pathway from the retina (Yu and Reiter, 1992), while in a large number of nonmammalian vertebrates, the pineal gland itself contains both a circadian clock and the light input pathway responsible for entrainment of the clock (Iigo *et al.*, 1994, 1997). Melatonin production in the retina, on the other hand, is governed by an ocular circadian clock entrained to LD cycles via its local photoreceptors in some vertebrate species (Cahill and Besharse, 1995; Tosini and Menaker, 1996; Iigo *et al.*, 1997).

In the rainbow trout (*Oncorhynchus mykiss*), melatonin is known to be produced in both the pineal gland and retina (Gern and Ralph, 1979; Morton and Forbes, 1988; Gern *et al.*, 1992; Max and Menaker, 1992; Meissl and Brandstätter, 1992). However, the circadian regulation of melatonin synthesis is lacking, i.e. only light and darkness regulate melatonin production (Gern *et al.*, 1992; Max and Menaker, 1992; Meissl and Brandstätter, 1992). Thus, the rainbow trout is an useful model to investigate the photic regulation of melatonin production. Comparison of the regulatory mechanisms of melatonin production in the rainbow trout with the clock-regulated one might be a possible way to reveal the circadian clock mechanism.

A rate limiting step in melatonin synthesis is *N*-acetylation of serotonin by arylalkylamine *N*-acetyltransferase (AANAT; EC 2.3.1.87) (Yu and Reiter, 1992). Thus, it is important to investigate the regulatory mechanism of AANAT for a better understanding of the cyclic synthesis of melato-

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nin. Recent cloning of cDNAs encoding AANAT in the pineal glands of rat and sheep (Borjigin *et al.*, 1995; Coon *et al.*, 1995) opened a way to study the regulation of AANAT expression at the molecular level. Since then cDNAs encoding AANAT were also cloned in human and chicken (Coon *et al.*, 1996; Bernard *et al.*, 1997). However, to our knowledge, the structure of AANAT in ectothermic vertebrates has not been published.

Toward the molecular analysis of melatonin production in the rainbow trout, in the present study, we have isolated and characterized a putative cDNA encoding AANAT from the retina and examined day-night changes in its mRNA levels by Northern blot analysis.

MATERIALS AND METHODS

RNA preparation

The rainbow trout (130 ± 20 g in body weight) purchased from a local dealer were maintained under LD cycles of 14 hr:10 hr (LD 14: 10; light on 06:00 – 20:00) at 14°C for at least a week. Fish were anesthetized with 0.06% 2-phenoxyethanol and sacrificed by decapitation at mid-day (12:30 – 13:30) or mid-night (00:30 – 01:30). After dissection, the retina was used for total RNA extraction immediately, while the liver was frozen in liquid nitrogen and stored at –80°C until RNA preparation. Total RNA was extracted with an RNA extraction solution, Isogen (Nippongene, Toyama, Japan) according to the manufacture's protocol. Poly(A)$^+$ RNA was prepared from total RNA using Oligotex dT30<Super> (Takara, Tokyo, Japan) according to instructions of the manufacture.

Primers

The following oligonucleotide primers were chemically synthesized: primer 1, 5'-TG(C/T)CCIGA(A/G)(C/T)TI(T/A)(C/G)I(C/T)TIGGITGG-3'; primer 2, 5'-GGIACIA(A/G)IGC(A/G)T(C/T)(C/T)TC(A/G)CACAT-3'; primer 3, 5'-CTCACACATGAGCACTGCAC-3'; primer 4, 5'-CATCAGGATAGGGCCCTTG-3'; primer 5, 5'-TTGAGGAGGGGAGACTAGTAGC-3'; primer 6, 5'-CCAAGAGAGAAGACTTGTGC-3'; primer 7, 5'-GGTGTGCACTTTAGACTACC-3'; anchor primer, 5'-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3'; AUAP primer, 5'-GGCCACGCGTCGACTAGTAC-3'; *Not* I d(T) primer, 5'-AACTGGAAGAATTCGCGGCCGCAGGAAT$_{18}$-3'; RTG primer, 5'-AACTGGAAGAATTCGCGG-3'.

Polymerase chain reaction (PCR)

Each PCR amplification was performed by using GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA) in a total volume of 20 µl with Taq DNA polymerase (Takara) and primers (250 nM each).

Amplification of a trout AANAT cDNA fragment by PCR

PCR was first performed with degenerated primers (the primers 1 and 2) deduced from the conserved amino acid sequences of AANAT of rat (GenBank Accession number U40803), ovine (U29663) and human (U40347). The primers 1 and 2 correspond to the amino acid positions 75–82 (CPELSLGW) and 157–164 (MCENALVP) of the rat AANAT, respectively. First strand cDNA was synthesized from the retinal total RNA of the rainbow trout collected at mid-night with Ready-To-Go™ T primed first strand cDNA synthesis kit (Pharmacia Biotech, Tokyo, Japan) and subjected to 30 cycles of PCR amplification with the primers 1 and 2. Each reaction cycle consisted of incubations at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min. The final elongation step was carried out at 72°C for 7 min.

5'-Rapid amplifications of cDNA ends (RACE)

First strand cDNA was synthesized from the retinal total RNA of the rainbow trout collected at mid-night using 5'-RACE PCR kit (GIBCO/BRL, Gaitherburg, MD, USA) according to the manufacture's instructions. The primer 3 was used to prime cDNA synthesis. Subsequently, a dC tail was added to the 3'-end of the synthesized single-stranded cDNA and PCR amplification for 35 cycles was performed with the primer 4 and 5'-anchor primer (GIBCO/BRL). Each reaction cycle consisted of incubations at 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. The final elongation step was carried out at 72°C for 7 min.

3'-RACE

First strand cDNA was synthesized with Ready-To-Go™ T primed first strand cDNA synthesis kit as described above and used as a template for 30 cycles of PCR amplification with the primer 5 and RTG primer containing the complementary sequence to the *Not* I d(T) primer for 3'-RACE. Each reaction cycle consisted of incubations at 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min. The final elongation step was carried out at 72°C for 7 min.

Confirmation of the obtained sequence by PCR

The obtained nucleotide sequence by 5'- and 3'-RACE over the coding region (nucleotide position 29-915 of the trout AANAT sequence) was reconfirmed by PCR amplification using the primers 6 and 7.

DNA sequencing

The cDNA fragments amplified by the PCR described above were separated by agarose gel electrophoresis and purified using Prep-A-Gene DNA Purification Kit (Bio Rad, Richmond, CA, USA). The purified cDNA fragments were then subcloned into pBluescript II SK(–) vector (Stratagene, La Jolla, CA, USA). Both strands of the plasmid DNA were sequenced with a Model 373A DNA sequencer using dye terminator cycle sequencing kits (Applied Biosystems, Foster City, CA, USA). The nucleotide sequence was determined from two to four clones obtained by independent reverse transcriptions and amplifications to avoid PCR errors.

Sequence analysis

Sequence analysis was performed with the GeneWorks 2.45 (IntelliGenetics, Mountain View, CA, USA). A phylogenetic tree of vertebrate AANAT was constructed by the neighbor-joining method (Saitou and Nei, 1987) based on the amino acid identities by the PHYLIP program (Felsenstein, 1989) using *Drosophila* AANAT (Hintermann *et al.*, 1996) as an outgroup.

Isolation of genomic DNA and Southern blot analysis

Genomic DNA (10 µg) isolated from the trout liver was digested with the restriction enzymes (*Eco*R I, *Pst* I, or *Xba* I) and used for Southern blot analysis according to the method as described (Kikuchi *et al.*, 1995). A partial AANAT cDNA (nucleotide position 9-570 of the trout sequence) labeled with [α-^{32}P]dCTP (Amersham Japan, Tokyo, Japan) by Random primer DNA labeling kit ver. 2 (Takara) was used as a probe (5×10^9 cpm/ml).

Northern blot analysis

Poly(A)$^+$ RNA (5 µg) prepared from the pooled retina of 10 individuals and the liver of a individual was separated on a formaldehyde-agarose gel and blotted onto a nylon membrane (Hybond-N$^+$, Amersham Japan). The membrane was UV crosslinked using Spectrolinker™ XL-1500 (Spectronics Corporation, Westburg, NY, USA) for 30 sec. The membrane was prehybridized in 6 × SSC containing 50% formamide, 1 × Denhardt's solution, 0.5% sodium dodecylsulphate (SDS) and calf thymus DNA (100 ng/ml) at 42°C for 12 hr and then hybridized with the randomly [α-^{32}P]dCTP-labeled probe (the same cDNA probe as in the Southern blot analysis, 5×10^9 cpm/ml) in the hybridization buffer (6 × SSC containing 50% formamide, 1 × Denhardt's solution and 0.5% SDS) at 42°C overnight. The mem-

brane was washed in 2 × SSC containing 0.5% SDS at room temperature for 10 min, and then washed at 65°C for 10 min in 1 × SSC containing 0.5% SDS and exposed to Fuji X-ray film (Fuji Film, Tokyo, Japan) at –80°C for 2 weeks. The hybridized membrane was also scanned by FUJIX BAS 1000 Mac Bio-Imaging Analyzer (Fuji Film) to count the hybridization signal. The hybridized probe on the membrane was washed out in the washing buffer (5 mM Tris-HCl containing 2 mM EDTA and 0.1 × Denhardt's solution) at 65°C for 2 hr. Then the membrane was rehybridized with the [α-^{32}P]dCTP-labeled β-actin probe of masu salmon, *O. masou*, (4,000 cpm/ml; 444 bp; Gen *et al.*, in preparation) corresponding to the nucleotide position 499-942 of mouse β-actin (GenBank accession number X03672) to confirm the integrity of the mRNA loading and transfer efficiency. The membrane was washed, exposed and scanned as described above.

RESULTS

Molecular cloning of trout AANAT

A trout retinal AANAT cDNA fragment (222 bp) was first obtained by PCR using degenerate primers (the primers 1 and 2) designed from the conserved amino acid sequences of mammalian AANAT. This cDNA fragment is 73 and 72% identical to the corresponding region of the rat sequence at the nucleotide and amino acid levels, respectively. Based on this sequence, we designed trout AANAT specific primers (the primers 3-5) and isolated several clones including 5'- and 3'-franking regions by RACE methods. A pair of primers (the primers 6 and 7) was also synthesized and used in reverse transcription-PCR to amplify a cDNA (888 bp) covering the entire coding region of the trout AANAT. The overlapping cDNAs for the trout AANAT spanned 1,585 bp (Fig. 1). The

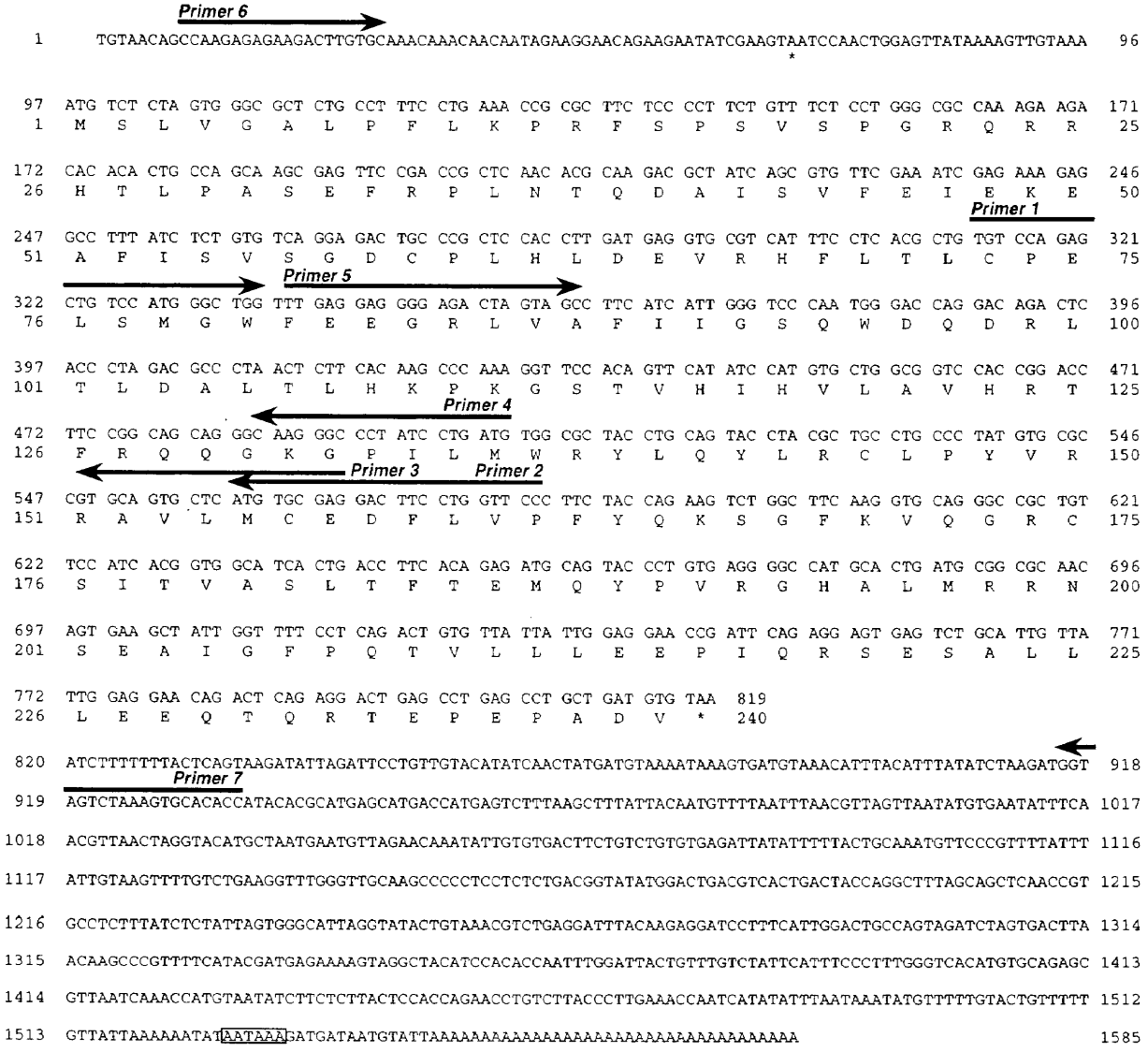

Fig. 1. The nucleotide and deduced amino acid sequences of the trout retinal AANAT. Nucleotide and amino acid sequences are numbered from the first and indicated in the left and right of each line. Arrows indicate the regions used as the primers in PCR. The stop codon is indicated by the asterisk and a consensus sequence for polyadenylation signal is boxed. The sequence has been deposited in DDBJ, EMBL and GenBank nucleotide databases under accesion number AB007294.

trout retinal AANAT cDNA contains a 723 bp open reading frame encoding 240 amino acids with a predicted molecular weight of 27,420 and an isoelectric point of 7.78. The 5'- and 3'-untranslated regions were 96 and 766 bp long, respectively.

Analysis of deduced protein

The deduced amino acid sequence of the trout AANAT is aligned with those of the chicken, rat, bovine, ovine and human (Borjigin *et al.*, 1995; Coon *et al.*, 1995, 1996; Bernard *et al.*, 1997) (Fig. 2). The sequence of trout retinal AANAT is very similar in amino acid sequences to those of other vertebrates (65, 54, 54, 53 and 51% identical to chicken, rat, ovine, bovine and human AANAT, respectively) but shows lower identity (14%) to the *Drosophila* AANAT (Hintermann *et al.*, 1996). The carboxy tail of the trout AANAT is 37 amino acid longer than the avian and mammalian AANAT.

A phylogenetic tree of vertebrate AANAT is shown in Fig. 3. The branching patterns are highly reliable and the tree topology agreed well with phylogeny.

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                                           cnpk          ck2   ck2
Trout      MSLVGALPFL KP-----RFS PSVSPGRQRR HTLPASEFRP LNTQDAISVF    45
Chicken    MPVLGAVPFL KPTPL---QG PRNSPGRQRR HTLPASEFRC LSPEDAVSVF    47
Rat        ---MLSIHPL KPEALHLPLG TSEFLGCQRR HTLPASEFRC LTPEDATSAF    47
Bovine     -MSTPSIHCL KPSPLHLPSG IPGSPGRQRR HTLPANEFRC LTPKDAAGVF    49
Ovine      -MSTPSVHCL KPSPLHLPSG IPGSPGRQRR HTLPANEFRC LTPEDAAGVF    49
Human      -MSTQSTHPL KPEAPRLPPG IPESPSCQRR HTLPASEFRC LTPEDAVSAF    49
Consensus  -........L KP........ .......QRR HTLPA.EFR. L...DA...F

                                                             ck2
Trout      EIEKEAFISV SGDCPLHLDE VRHFTTLCPE LSMGWFEEGR LVAFIIGSQW    95
Chicken    EIEREAFISV SGDCPLHLDE IRHFLTLCPE LSLGWFEEGR LVAFIIGSLW    97
Rat        EIEREAFISV SGTCPLHLDE IRHFLTLCPE LSLGWFEEGC LVAFIIGSLW    97
Bovine     EIEREAFISV SGNCPLNLDE VRHFLTLCPE LSLGWFVEGR LVAFIIGSLW    99
Ovine      EIEREAFISV SGNCPLNLDE VQHFLTLCPE LSLGWFVEGR LVAFIIGSLW    99
Human      EIEREAFISV LGVCPLYLDE IRHFLTLCPE LSLGWFEEGC LVAFIIGSLW    99
Consensus  EIE.EAFISV .G.CPL.LDE ..HFLTLCPE LS.GWF.EG. LVAFIIGS.W

                                          pkc
Trout      DQDRLTLDAL TLHKPKGSTV HIHVLAVHRT FRQQGKGPIL MWRYLQYLRC   145
Chicken    DQDRLSQAAL TLHNPRGTAV HIHVLAVHRT FRQQGKGSIL MWRYLQYLRC   147
Rat        DKERLTQESL TLHRPGGRTA HLHVLAVHRT FRQQGKGSVL LWRYLHHLGS   147
Bovine     DEERLTQESL TLHRPGGRTA HLHALAVHHS FRQQGKGSVL LWRYLQHAGG   149
Ovine      DEERLTQESL ALHRPRGHSA HLHALAVHRS FRQQGKGSVL LWRYLHHVGA   149
Human      DKERLMQESL TLHRSGGHIA HLHVLAVHRA FRQQGRGPIL LWRYLHHLGS   149
Consensus  D..RL....L .LH...G... H.H.LAVH.. FRQQG.G..L .WRYL.....
                                      Motif A
                                                   ck2     pkc
Trout      LPYVRRAVLM CEDFLVPFYQ KSGFKVQGRC SITVASLTFT EMQYPVRGHA   195
Chicken    LPCARRAVLM CEDFLVPFYE KCGFVAVGPC QVTVGTLAFT EMQHEVRGHA   197
Rat        QPAVRRAVLM CENALVPFYE KFGFQAMGPC AITMGSLTFT ELQCSLRCHT   197
Bovine     QPAVRRAVLM CEDALVPFYQ RFGFHPAGPC AVVVGSLTFT EMHCSLRGHA   199
Ovine      QPAVRRAVLM CEDALVPFYQ RFGFHPAGPC AIVVGSLTFT EMHCSLRGHA   199
Human      QPAVRRAALM CEDALVPFYE RFSFHAVGPC AITVGSLTFM ELHCSLRGHP   199
Consensus  .P..RRA.LM CE..LVPFY. ...F...G.C ......L.F. E.....R.H.
                        Motif B
            cnpk pkc                         pkc  ck2
Trout      LMRRNSEAIG FPQTVLLLEE PIQRSESALL LEEQTQRTEP EPADV 240
Chicken    FMRRNSGC-- ---------- ---------- ---------- ----- 205
Rat        FLRRNSGC-- ---------- ---------- ---------- ----- 205
Bovine     ALRRNSDR-- ---------- ---------- ---------- ----- 207
Ovine      ALRRNSDR-- ---------- ---------- ---------- ----- 207
Human      FLRRNSGC-- ---------- ---------- ---------- ----- 207
Consensus  ..RRNS..-- ---------- ---------- ---------- -----
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Fig. 2. Alignment of amino acid sequences of AANAT of rainbow trout, chicken, rat, bovine, ovine and human. The sequences in chicken, rat, bovine, ovine, and human were obtained from Gen Bank (accession numbers are U46502, U40803, AD000742 , U29663, and U40347, respectively). To maximize homologies, gaps represented by hyphns have been introduced in the sequences. Potential phosphorylation sites are shaded: cnpk, cyclic nucleotide-dependent protein kinase; ck2, casein kinase II; and pkc, protein kinase C. The consensus sequence contains moieties conserved in all six species. The two conserved motifs (A and B) for *N*-acetyltransferase superfamily (Tercero *et al.*, 1992; Coleman *et al.*, 1996; Lu *et al.*, 1996) are underlined.

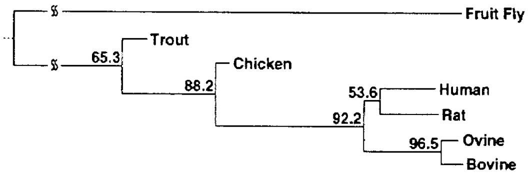

Fig. 3. A phylogenetic tree of AANAT in vertebrates based on amino acid identities. The tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using PHYLIP software (Felsenstein, 1989). The values at the nodes are bootstrap probabilities (%) estimated by 1,000 times replications, and the length of the horizontal lines are evolutionary distances.

The trout AANAT sequence contains putative motifs for acetyl coenzyme A binding, motifs A and B (Tercero *et al.*, 1992; Coleman *et al.*, 1996; Lu *et al.*, 1996), at the amino acid positions of 117–136 and 161–173, respectively. The trout AANAT also contains eight putative phosphorylation sites; two consensus sites for cyclic nucleotide-dependent protein kinase at Thr^{27} and Ser^{201}, four sites for casein kinase II at Ser^{43}, Ser^{93}, Thr^{185} and Thr^{233}, and two sites for protein kinase C at Thr^{125} and Thr^{230}.

Southern blot analysis

Southern blot analysis was carried out to analyze the genomic organization of the trout retinal AANAT. Probing the liver genomic DNA with labeled cDNA probe produced one band each of 19, 2.9, and 14 kbp after digestion with *Eco*R I, *Pst* I, and *Xba* I, respectively (Fig. 4). These results suggest the trout retinal AANAT is encoded by a single copy gene.

Northern blot analysis

Expression of the AANAT gene in the retina and liver was surveyed by Northern blot analysis (Fig. 5A). A single transcript (1.6 kb) was detected in the retina at both mid-day and mid-night, but not in the liver. The mRNA level of the retinal AANAT exhibited 3.3-fold increase at night under LD cycles (Fig. 5B). A similar result was obtained in another experiment.

DISCUSSION

In the present study, we have determined the primary structure of the retinal AANAT of the rainbow trout. The obtained clone contains an open reading frame that codes a 240 amino acid protein. The overlapping cDNA spanned 1,585 bp (Fig. 1), being consistent with the size of mRNA (1.6 kb) estimated by Northern blot analysis (Fig. 5A). The AANAT seems to be coded by a single copy gene in the trout genome.

Comparison of the deduced amino acid sequence of trout AANAT with those from other vertebrate species shows a high degree of identity (51–65%). There are two motifs termed A and B (also called regions A and B or domains I and II) that are conserved among the *N*-acetyltransferase superfamily (Tercero *et al.*, 1992; Borjigin *et al.*, 1995; Coon *et al.*, 1995,

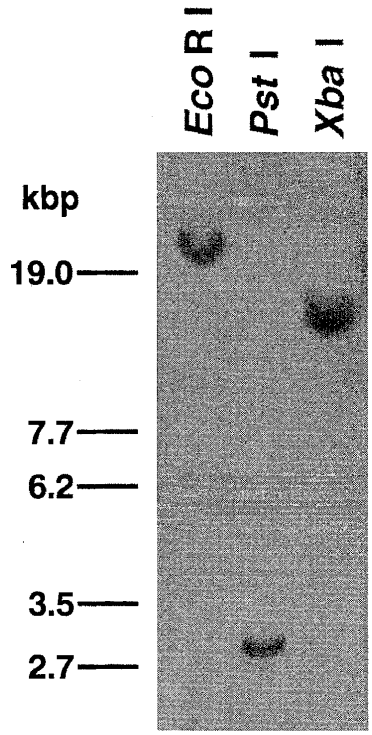

Fig. 4. Southern blot analysis of the trout retinal AANAT gene. When the trout liver genomic DNA (10 μg) was digested with *Eco*RI, *Pst*I, or *Xba*I, hybridization with the labeled cDNA probe produced a single band in each lane (*Eco*R I, 19 kbp; *Pst* I, 2.9 kbp; *Xba* I, 14 kbp), indicating that the trout retinal AANAT is encoded by a single copy gene. Molecular weight markers are *Eco*T14I digests of lambda phage DNA.

1996; Hintermann *et al.*, 1996; Coleman *et al.*, 1996; Lu *et al.*, 1996; Bernard *et al.*, 1997). Site directed mutagenesis in yeast MAK3 and human spermidine/spermine *N*-acetyltransferases revealed that these motifs are important to maintain enzyme activities as acetyl coenzyme A binding sites (Tercero *et al.*, 1992; Coleman *et al.*, 1996; Lu *et al.*, 1996). The amino acid stretch of the trout AANAT at the amino acid position 45–96 might contain the arylalkylamine (substrate) binding domain, which is highly conserved among AANAT sequences of vertebrates (Coon *et al.*, 1995, 1996; Klein *et al.*, 1996). However, further site directed mutagenesis studies will be required to test whether these regions are the binding domains for acetyl coenzyme A and arylalkylamine substrates in AANAT proteins.

In all known vertebrate AANAT sequences, two potential phosphorylation sites for cyclic nucleotide-dependent protein kinase (Thr^{27} and Ser^{201} of the trout sequence) and one for casein kinase II (Ser^{93}) are conserved, suggesting that phosphorylation at these sites is functionally important in the regulation of AANAT activity. This is consistent with the important roles of cAMP in the induction and maintenance of AANAT activity (Yu and Reiter, 1992; Thibault *et al.*, 1993; Klein *et al.*, 1996). The other putative phosphorylation sites, especially two sites in the carboxyl tail of the trout sequence (Thr^{230} and Thr^{233}), might be involved in species-specific regulation of AANAT. Isolation of the trout AANAT cDNA now allows us to study the functional significance of these phosphorylation sites.

It is widely accepted that cysteine residues mediate the formation of intramolecular disulphide bridge. In all known AANAT in vertebrates, four cysteine residues are conserved

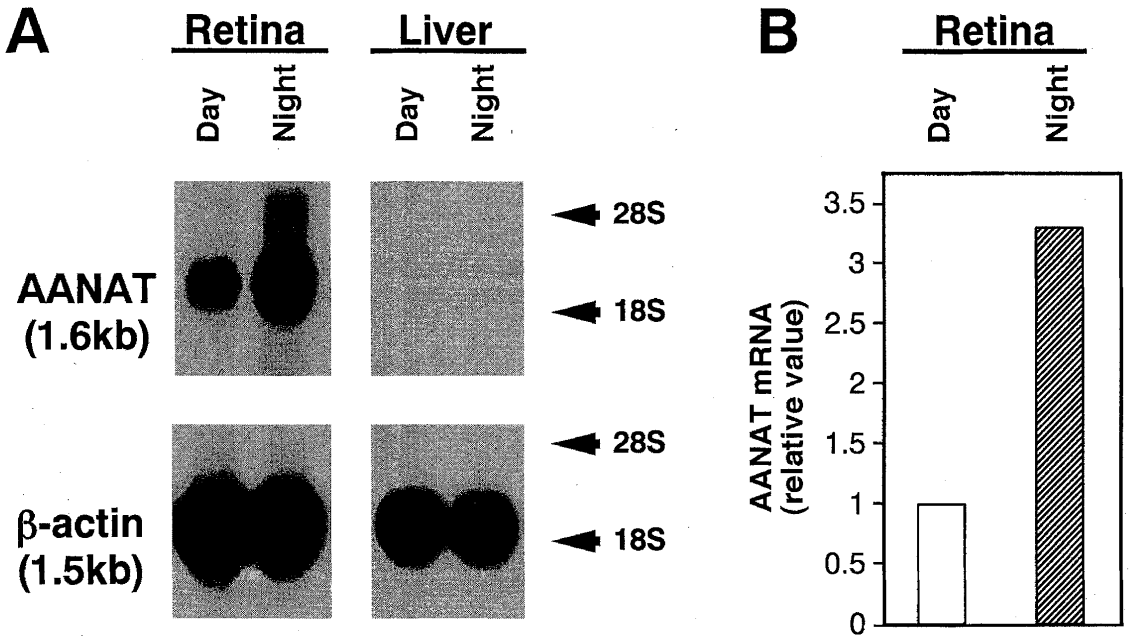

Fig. 5. Northern blot analysis for AANAT mRNA in the retina and liver. (**A**) The rainbow trout reared under LD 14:10 (light on 06:00–20:00) were sacrificed at mid-day (Day, 13:00) or at mid-night (Night, 01:00). Northern blot analysis was performed on Poly(A)$^+$ RNA (5 μg) obtained from the retina and liver. Arrowheads on the right mark the positions of 28S (top) and 18S (bottom) rRNA bands. Blots were stripped and reprobed with β-actin of masu salmon to allow normalization of lanes. (**B**) Day-night changes in the AANAT mRNA in the rainbow trout retina. The abundance of the AANAT transcript has been normalized to β-actin mRNA, to correct for variations in the mRNA loading and transfer efficiency. Data are expressed relative to the mid-day value. The trout retinal AANAT mRNA increased 3.3-fold at mid-night as compared with the mid-day value.

at the amino acid positions of 59, 73, 156 and 175 of the trout sequence. These residues might be involved in the proper folding of the AANAT protein.

Northern blot analysis revealed the presence of AANAT mRNA in the retina but not in the liver. This is consistent with findings in chicken, rat, ovine and human (Borjigin *et al.*, 1995; Coon *et al.*, 1995, 1996; Bernard *et al.*, 1997). A recent study in a teleost (the pike *Esox lucius*) has demonstrated that the retinal and pineal AANAT enzymes have the significant differences in the effects of temperature, substrate concentration, pH and molarity (Falcón *et al.*, 1996). This suggests the presence of at least two types of AANAT molecules in a teleost species. Thus, it is of great interest to see whether the retinal and pineal AANAT molecules are products of two distinct genes or variants of the same gene. The retinal AANAT cDNA isolated in the present study is available as a hopeful probe to clone the pineal homologues or variants.

In the present study, we investigated day-night changes in AANAT mRNA levels in the trout retina. The results demonstrated daily variations in steady state levels of AANAT mRNA in the trout retina with higher values at night under LD cycle. Similar daily variations have been reported in the pineal glands of chicken, rat and ovine and in the retina of chicken (Borjigin *et al.*, 1995; Coon *et al.*, 1995, 1996; Roseboom *et al.*, 1996; Bernard *et al.*, 1997). This is also consistent with the ocular melatonin data (high levels at night) in the rainbow trout (Gern and Karn, 1983; Yamamoto *et al.*, 1992). These results indicate that melatonin production in the trout retina is regulated at least in part by transcription of AANAT mRNA. Since melatonin production in the rainbow trout is regulated by light and darkness, it is interesting to determine how light and darkness regulate AANAT transcription. Further analysis of the promotor region of the AANAT gene and the transcription factors involved would help us to identify "light-responsive" element(s), a possible ubiquitous input for light-dependent events such as melatonin syntheis and photic entrainment of the circadian clock.

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Following acceptance of this manuscript, a partial cDNA sequence encoding the trout pineal AANAT was published (GenBank accession number AF033500; Begay V, Falcón J, Cahill GM, Klein DC, Coon SL (1998) Transcripts encoding two melatonin synthesis enzymes in the teleost pineal organ: circadian regulation in pike and zebrafish but not in trout, Endocrinology 139: 905-912). The amino acid sequence is only 66% identical with the relevant region of the trout retinal AANAT. In addition, they found that the pineal-type AANAT transcript was not detected in the retina. Thus, there are two different AANAT genes in the rainbow trout.