# Serotoninergic System in Hamster Skin

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**We have cloned the tryptophan hydroxylase cDNA from hamster pituitary and demonstrated its expression in the skin, melanotic and amelanotic melanomas, spleen, heart, and the eye. We further demonstrated that skin, melanomas, spleen, pituitary, and eye but not heart expressed arylalkylamine *N*-acetyltransferase mRNA. The cutaneous expression of the arylalkylamine *N*-acetyltransferase gene was accompanied by enzymatic activity for the conversion of serotonin and tryptamine to *N*-acetylserotonin and *N*-acetyltryptamine, respectively. There was marked regional variation in the serotonin *N*-acetyltransferase activity, which was higher in ear skin than in corpus skin, and was lower in melanomas than in normal skin. Serotonin *N*-acetyltransferase activity was significantly inhibited by Cole bisubstrate at low concentration ($\leq$ 1 μM); this evidence in conjunction with arylalkylamine *N*-acetyltransferase mRNA expression implies an involvement of arylalkylamine *N*-acetyltransferase in serotonin metabolism in the skin. We also documented both the *in vitro* transformation of serotonin to *N*-acetylserotonin using liquid chromatography/mass spectrometry and the generation/storage of *N*-acetylserotonin in cultured melanoma cells. Thus, we have uncovered a cutaneous pathway displaying capabilities for serotonin biosynthesis and/or its metabolism to *N*-acetylserotonin in rodent skin. As serotonin has powerful vasodilator, immunomodulator, and growth factor actions, this pathway could be involved in skin physiology and/or pathology.** ***Key words: arylalkylamine* N-*acetyltransferase*/N-*acetylserotonin*/*serotonin*/*skin*/*tryptophan hydroxylase*. *J Invest Dermatol 119:934–942, 2002***

Accumulated evidence indicates the expression of neuroendocrine activities in the skin (Slominski and Wortsman, 2000). Thus, the skin has been found to produce locally some of the mediators involved in the systemic response to stress, including the proopiomelanocortin-derived peptides melanocyte-stimulating hormone, adrenocorticotropic hormone, and β-endorphin, corticotropin-releasing hormone, and urocortin (Slominski *et al*, 2000, 2001). In addition to that, the skin also participates in the activation of steroid hormones, e.g., the conversion of testosterone to 5α-dihydrotestosterone or to estradiol (reviewed in Slominski and Wortsman, 2000); and, epidermal keratinocytes specifically produce parathyroid hormone-related protein and vitamin D (Holick, 1994; Philibrick *et al*, 1996). Moreover, epidermal neurotransmitters have also been detected as documented by Schallreuter *et al* (1995) for the catecholaminergic system, and by Grando and Horton (1997) for the cholinergic system.

Serotonin is another neurotransmitter involved in control neuroendocrine pathways, and has been already detected in normal human melanocytes (Johansson *et al*, 1998). Serotonin is the product of a multistep metabolic pathway that starts with the hydroxylation of the aromatic amino acid L-tryptophan by tryptophan hydroxylase (TPH: E.C. 1.14.16.4; Mockus and Vrana, 1998). The resulting metabolite hydroxytryptophan is decarboxylated by an enzyme ubiquitously present in peripheral tissues, aromatic L-amino acid decarboxylase (AAADC: E.C. 4.1.1.28) to generate the active neurohormone serotonin (Yu and Reiter, 1993). Besides its specific actions, serotonin also serves as a precursor to melatonin (Yu and Reiter, 1993). Acetylation of serotonin by arylalkylamine *N*-acetyltransferase (AANAT: E.C. 2.3.1.87), generates *N*-acetylserotonin (NAS), and after methylation, by hydroxyindole-*O*-methyltransferase (E.C. 2.1.1.4) melatonin (Sugden *et al*, 1987; Yu and Reiter, 1993).

We recently uncovered the full expression of novel cutaneous serotoninergic and melatoninergic systems in the human skin (Slominski *et al*, 2002). We found that human skin cells show expression of the genes coding for the biosynthetic enzymes TPH, AANAT, and hydroxyindole-*O*-methyltransferase, as well as expressing the corresponding enzymatic activities (Slominski *et al*, 2002). As we previously showed metabolism of serotonin to NAS in the hamster skin (Gaudet *et al*, 1993; Slominski *et al*, 1996), we investigated whether a serotoninergic system was also expressed in full in this experimental model. As necessary step for this research we had to sequence the reading frame of hamster TPH (the enzyme catalyzing the rate of the limiting step in serotonin synthesis) obtained from pituitary tissue; we then used molecular and biochemical methods, including liquid chromatography mass spectrometry (LC-MS) for the analysis.

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Abbreviations: AANAT, arylalkylamine *N*-acetyltransferase; TPH, tryptophan hydroxylase; LC-MS, liquid chromatography mass spectrometry; NAS, *N*-acetylserotonin.

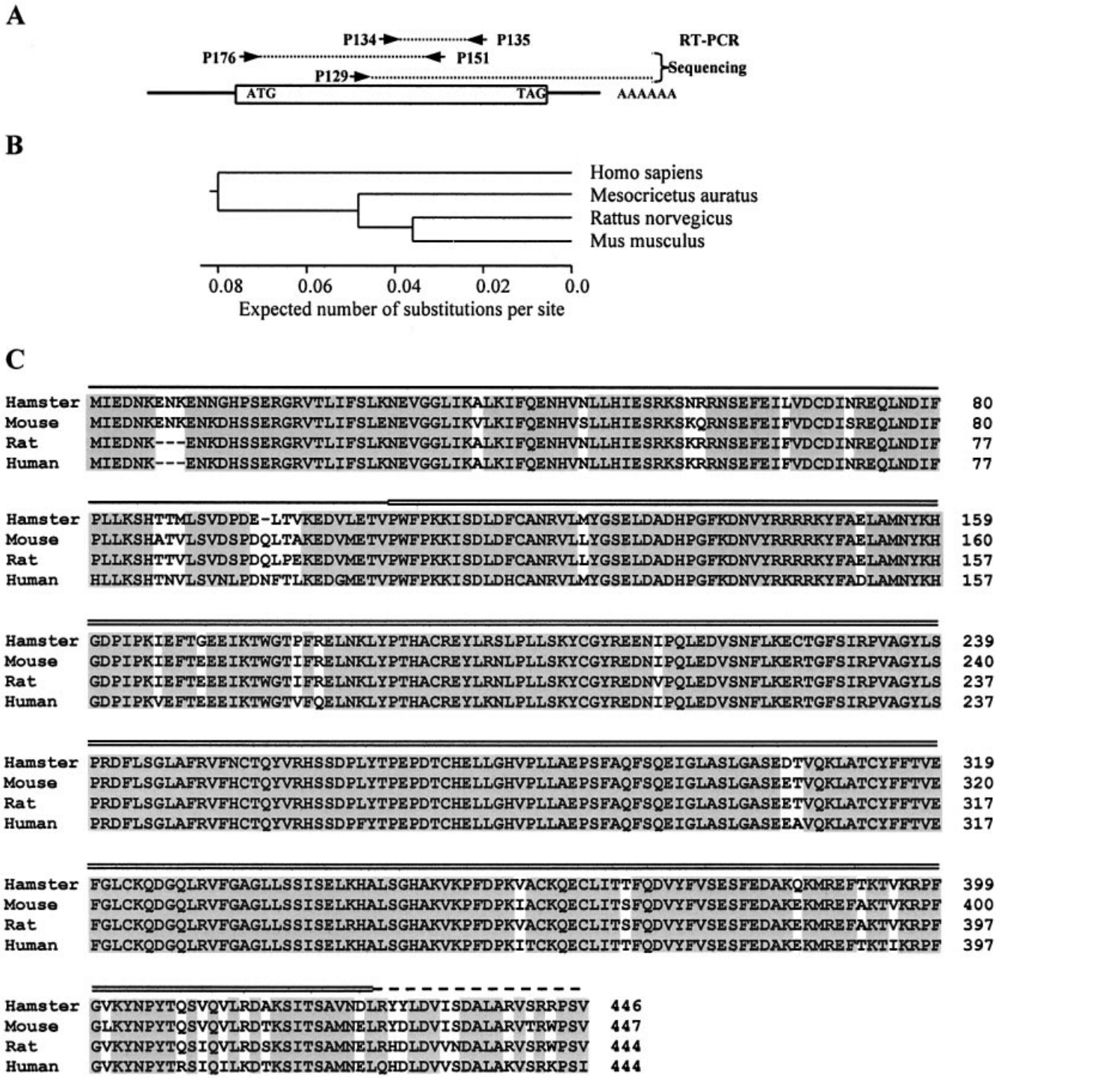


**Figure 1. Cloning and structural analysis of the open reading frame of hamster TPH gene.** (*A*) Sequencing of hamster tryptophan hydroxylase. Open box represents a reading frame of gene. Solid lines: 5′ and 3′ untranslated regions; dashed lines: amplified regions. Primers used for sequencing and amplification are shown above by *arrows*. (*B*) Phylogenic analysis of TPH mRNA sequences from different organisms by DNAMLK program (DNA Maximum Likelihood program with molecular clock, version 3.5c, PHYLIP package). (*C*) Predicted amino acid sequence of hamster TPH (accession no. AY034600), and comparison with mouse (accession no. NM_009414), rat (Accession No. X53501) and human (accession no. XM_006391) sequences. Conserved amino acids are shadowed. The numbers in the right-hand column refer to the amino acid number. The single line marks regulatory domain of the protein over the amino acid sequence. The double line represents the catalytic domain and the dashed line represents the leucine zipper.

## MATERIALS AND METHODS

**Chemicals** Trizol reagent, Superscript preamplification system was purchased from Gibco-BRL (Gaithersburg, MD), pGEM-Teasy cloning vector and Taq polymerase were from Promega (Madison, WI), DNA marker and nucleotides from Fermentas (Hanover, MD), GFX polymerase chain reaction (PCR) DNA and gel band purification kit from Amersham-Pharmacia-Biotech (Piscataway, NJ), plasmid purification kit from Qiagen (Valencia, CA). SMART RACE cDNA amplification kit was from Clontech (Palo Alto, CA). Primers were

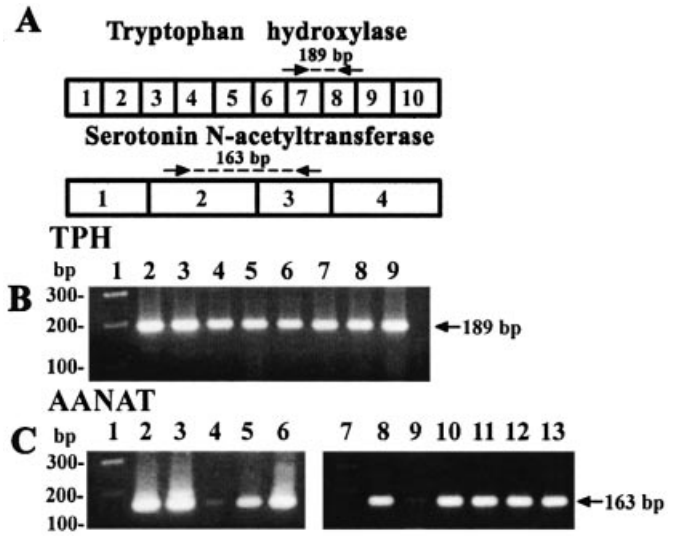


**Figure 2. Reverse transcription–PCR amplification of hamster TPH and AANAT mRNA.** (*A*) Organization of hamster TPH and AANAT genes. Numbers correspond to exons. *Arrows* represent PCR primers. Dashed lines show the PCR fragments. (*B*) Detection of TPH mRNA in hamster tissues: 2, eye; 3, pituitary; 4, heart; 5, spleen; 6, skin; 7, melanoma Ma; 8, melanoma MI; 9, melanoma AbC1. *Lane 1*: DNA ladder. (*C*) Detection of AANAT in hamster tissues: 2, pituitary; 3, eye; 4, skin; 5, spleen; 6, 8, and 10 untreated AbC1 melanoma cell line; 9, melanized AbC1; 11, AbC1 irradiated by 5 mJ per $cm^2$ of UVB; 12, AbC1 irradiated by 20 mJ per $cm^2$ of UVB; 13, AbC1 irradiated by 50 mJ per $cm^2$ of UVB. *Lanes 1 and 7*: DNA markers.

synthesized by Integrated DNA Technology Inc. (Coralville, IA). Cell culture media included Ham's F10 medium, Dulbecco's modified essential medium, fetal bovine serum, anti-mycotic/antibiotic mixture and phosphate-buffered saline (all from Gibco-BRL). All reagents used for enzymatic assays and for high-performance liquid chromatography (HPLC) separations were of the highest purity. L-[5-$^3$H]tryptophan was from Amersham Life Sciences Inc (Arlington Heights, IL). All chemicals for biochemical assays not listed above, including dye reagents for protein assay and chemicals for cell culture or molecular assays were purchased from Sigma (St Louis, MO). Cole bisubstrate inhibitor was obtained from Dr David Klein (National Institute of Health, Bethesda, MD).

**Tissue and cell lines** Syrian hamsters (males 3 months old) were purchased from Charles River Laboratory (Wilmington, MA) and housed in community cages at the animal facilities of the Albany Medical College (AMC), Albany NY or were derived from an in-house colony at the Belarus State University, Minsk, Belarus. The animals were killed under pentobarbital anesthesia and selected organs as well as back skin were collected following protocols routinely used in our laboratory (Slominski *et al*, 1996). Tissue specimens were frozen rapidly in liquid nitrogen. Hamster tissue samples consisted of pituitary, eye, heart, skin, and spleen. Hamster Bomirski Ma melanotic, MI hypomelanotic, and Ab amelanotic melanomas were propagated in male Syrian hamsters by subcutaneous inoculation of tissue suspension as described previously (Bomirski *et al*, 1988). After killing the animals tumor tissue was freed from connective and necrotic tissues and frozen rapidly in liquid nitrogen. Tissue specimens were stored at −80°C until analysis. The original research protocol was approved by the Institutional Animal Care and Use Committee at AMC, and a similar protocol was approved at the University of Tennessee Health Science Center (Memphis, TN).

Bomirski hamster AbC-1 melanoma cells were grown in Ham's F10 medium as described previously; the media was supplemented with 10% fetal bovine serum and antibiotics (Slominski *et al*, 1988). To induce melanin pigmentation the cells were cultured for 3 d in Dulbecco's modified essential medium (Slominski *et al*, 1988). After washing with PBS, melanoma cells were detached using Ca and Mg free Tyrode's solution containing 1 mM ethylenediamine tetraacetic acid. The cells were centrifuged and pellets were used for analyses.

**Reverse transcription–PCR amplifications and cloning of the hamster tryptophan hydroxylase gene** Total RNA was extracted using Trizol isolation kit. The synthesis of first-strand cDNA was performed using the Superscript pre-amplification system. An aliquot (5 μg) of total RNA per reaction were reverse transcribed according to the manufacturer's protocol using oligo(dT) as the primer. For this analysis, all samples were standardized by amplification of the housekeeping gene GAPDH as described previously by Robbins and McKinney (1992).

**Table I. Expression of TPH and AANAT in hamster tissue samples and cell lines**

| Tissue sample | TPH | AANAT |
|---|---|---|
| Pituitary | + | + |
| Eye | + | + |
| Heart | + | - |
| Spleen | + | + |
| Skin | + | + |
| Melanoma Ma | + | + |
| Melanoma MI | + | + |
| Melanoma Ab | + | + |
| Melanoma AbC1 | + | + |
| Melanoma AbC1 melanized | + | + |
| AbC1 irradiated by UV | + | + |

PCR amplifications were performed under standard conditions with modifications where indicated. Thus, the reaction mixture (25 μl) contained 2.5 mM $MgCl_2$, 2.5 mM of each deoxyribonucleoside triphosphate, 0.4 μM of each primer, 75 mM Tris–HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, and 1.25 units of Taq polymerase. The mixture was heated to 94°C for 2.5 min and then amplified for 35 or 30 cycles as specified: 94°C for 30 s (denaturation), 55°C for 45 s (annealing) for hamster tryptophan hydroxylase or 60°C for serotonin *N*-acetyltransferase and 72°C for 1 min (extension).

A full-length sequence of hamster tryptophan hydroxylase mRNA was obtained from alignment of two separate fragments. The first fragment was amplified by RACE method (random amplification of cDNA ends). Amplification of pituitary cDNA was performed according to the manufacturer's protocol (SMART RACE cDNA amplification kit, Clontech). For the 3′RACE we used primer P129 (5′-TCCGTCCTG-TGGCTGGTTACCTCTC-3′). Amplified fragment was sequenced. A second anti-sense primer P151 (5′-GAACAGTGTCCTCTGACGCTC-CAAG-3′) was designed. This primer did not produce a single band in 5′RACE. Therefore, using mouse gene sequence we designed primer P176 (5′-GATTGAAGACAACAAGGAGAACAAAG-3′) with a sequence homologous to the region starting from the initiation codon of the mouse tryptophan hydroxylase (Stoll *et al*, 1990). Amplification of hamster pituitary cDNA by primers P151 and P176 produced a single band. This band corresponded to the 5′ fragment of the hamster gene. Amplification products were separated by agarose electrophoresis and visualized by ethidium bromide staining, according to a standard protocol used in our laboratory (Pisarchik and Slominski, 2001), and the corresponding fragments sequenced.

The identified PCR products were excised from the agarose gel and purified by GFX PCR DNA and gel band purification kit (Amersham-Pharmacia-Biotech). PCR fragments were cloned in pGEM-T easy vector system and purified by a plasmid purification kit. Sequencing was performed in the Molecular Resource Center at the University of Tennessee Health Science Center (Memphis, TN) using Applied Biosystems 3100 Genetic Analyzer and BigDye™ Terminator Kit (Foster City, CA).

Amplification of hamster tryptophan hydroxylase was conducted by a single PCR using primers P134 (5′-CAGACACCTGCCATGAACTC-3′) and P135 (5′CAAAGACTCTCAGCTGTCCATC-3′). These primers were designed specifically for the hamster gene. PCR conditions were the same as described above, except that the annealing temperature was 55°C.

Hamster serotonin *N*-acetyltransferase was amplified by nested PCR. Primers were designed according to the already published sequence (GenBank accession no. AF092100). The first round of amplification was performed with primers P242(5′-CCAGCGAGTTCCGTTGCCTTAC-3′) and P243(5′-GCCTGTGCAGTGTCAGTGACTC-3′). An aliquot of the mixture from the first PCR round was transferred into a new tube and amplified again with primers P244(5′-CGTGTTTGAGA-

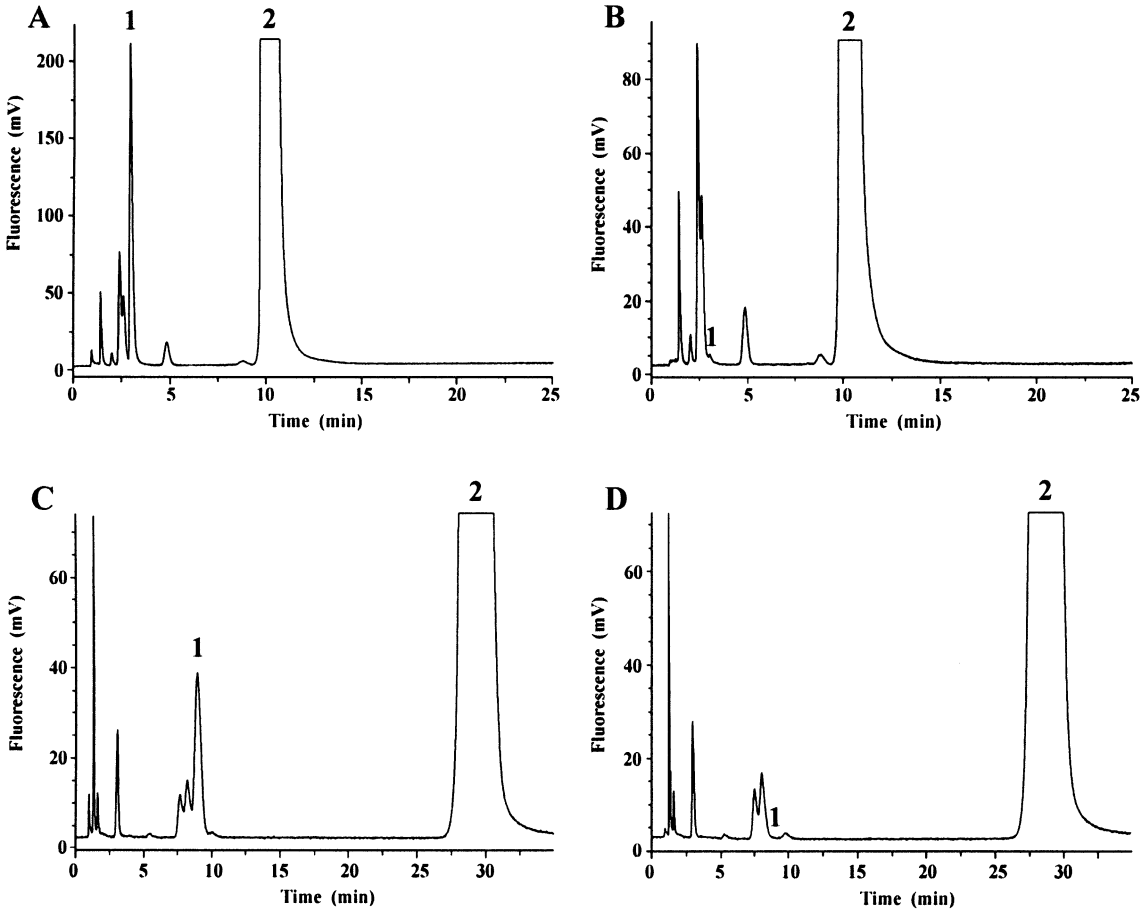


**Figure 3. Representative HPLC chromatograms obtained from reaction mixture in which hamster skin from ears was the enzyme source.** Experimental incubation with acetyl-CoA and serotonin (*A*) or tryptamine (*C*), and (*B,D*) corresponding controls represented by incubation without acetyl-CoA. The numbers in the figure indicate the elution position of standards. (1) NAS (*A,B*) or *N*-acetyltryptamine (*C,D*); (2) serotonin (*A,B*) or tryptamine (*C,D*).

TTGAGCGTGAAG-3′) and P245(5′-CTTGTCCCAAAGTGAGCCG-ATG-3′). The resulting 163 bp long PCR band represented exons 2 and 3 of the hamster gene.

Ultraviolet (UV) irradiation of hamster melanoma cells was performed as described previously (Pisarchik and Slominski, 2001). After irradiation PBS was substituted by standard culture medium, cells were incubated for 24 h, and then detached, collected, and processed for RNA isolation.

**Assays for tryptophan hydroxylase and serotonin *N*-acetyltransferase activities** The radiometric assay for TPH (kindly performed by Dr Wilfred Pinto) followed the methodology described by Beevers *et al* (1983) with minor modifications. Cells pelleted by low speed centrifugation (≈ 1000 × *g*) were homogenized in 3–5 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.6 at room temperature) with a Tekmar tissuemizer (Tekmar Co., Cincinnati, OH) at high setting (13,500 r.p.m., 10 s). The homogenizing buffer as well as the assay buffer contained 2 mM dithiothreitol in order to maintain the enzyme in the active conformation. The crude cell homogenate was utilized as the source material for the tryptophan hydroxylase assays. The assay mixture contained, in a final volume of 200 μl, 50 mM Tris buffer containing 2 mM dithiothreitol, 100 μM L-tryptophan, ≈ 10 nM L-[5-$^3$H]tryptophan as radioactive tracer (≈ 100 000 cpm, 31 Ci per mmol, Amersham Life Sciences Inc.), 0.5 mM 6-methyl-5,6,7,8-tetrahydropterine, and crude cell homogenate containing 30–50 μg protein. The pterin cofactor dissolved in 0.1 M HCl to a concentration of 5 mM was stored frozen in multiple aliquots at −80°C until the assay. Adding the crude cell homogenate to the prepared assay mixture, and mixing the contents by vortexing initiated the reaction. Background "blank" tubes were prepared substituting the cell extract for an equivalent volume of assay buffer. Following incubation for 120 min at 37°C, 40 μl of 60% perchloric acid ($HClO_4$) were added to the assay mixture to the final $HClO_4$ concentration of 12%; the contents were then mixed and incubated for an additional 30 min at 37°C. The assay contents were allowed to reach room temperature before the addition of 250 μl of a Norit-Dextran T70 slurry (50 mg each per ml $H_2O$). After vortexing the samples remained at room temperature for 5 min and then centrifuged for 5 min at 10,000 × *g*. Subsequently, 200 μl aliquots of the supernatant were added to minivials containing scintillation cocktail (Scintiverse, Fisher Scientific Co., Fair Lawn, NJ) and counted in a Beckman LS7000 liquid scintillation spectrometer (Beckman Coulter Co., Fullerton, CA).

Serotonin *N*-acetyltransferase activity was measured in samples of hamster skin and melanoma cell lines by a modification of the method described by Thomas *et al* (1990) and further detailed by Slominski *et al* (2002). All preparatory procedures were performed at 0–4°C. The samples of melanoma tumors and melanoma cell line were homogenized with a glass homogenizer in an ice-cold 0.25 M potassium phosphate buffer (pH 6.8) containing 1 mM dithiothreitol, 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, protease inhibitor cocktail (2 μl per ml homogenization mixture) and 0.625 mM acetyl-coenzyme A (acetyl-CoA). The homogenization medium for the samples of hamster skin contained the same compounds with the exception of absence of acetyl-CoA. Homogenates were centrifuged at 15,000 r.p.m. (5,000 g) in microcentrifuge (Hermle Z 231M, Labnet, Woodbridge, NJ) for 10 min at 4°C.

When melanoma tumors and melanoma cells were the enzyme source, the final concentrations of acetyl-CoA and amine substrate were 0.5 mM and 1 mM, respectively. When assaying AANAT activity in hamster skin, the final concentration of serotonin, tryptamine, and acetyl coenzyme were 0.55 mM, 1 mM, and 0.1 mM, respectively. The enzymatic reactions were run at 37°C and after various time stopped by the addition of $HClO_4$ (Slominski *et al*, 2002).After centrifugation the supernatant was subjected to HPLC in a system equipped with a Novapak $C_{18}$ reverse-

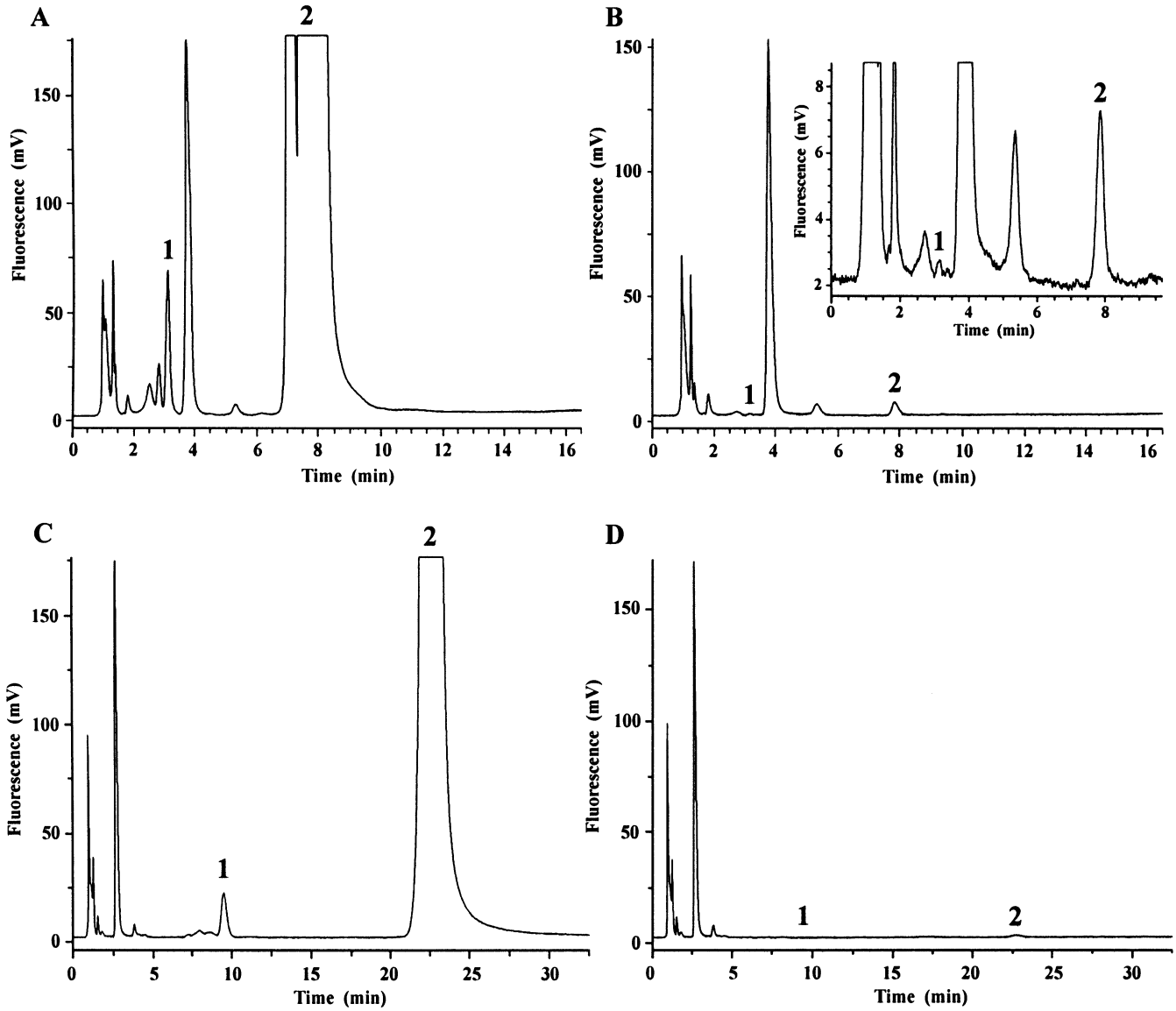


**Figure 4. Representative HPLC chromatograms obtained from reaction mixture in which Ab amelanotic melanoma transplantable in hamsters was the enzyme source.** Experimental incubation with acetyl-CoA and serotonin (*A*) or tryptamine (*C*), and (*B,D*) corresponding control extracts without amine substrate. The numbers in the figure indicate the elution position of standards. (1) NAS (*A,B*) or *N*-acetyltryptamine (*C,D*); (2) serotonin (*A,B*) or tryptamine (*C,D*). *Right insert*: expanded scale showing detectable peaks with retention time corresponding to NAS and serotonin.

phase column (100 × 5 mm, i.d.) and fluorimetric detector (Waters, Milford, MA). The detector was calibrated with excitation and emission wavelengths set at 285 and 360 nm, respectively. The elution was carried out isocratically at ambient temperature with a flow rate 1.5 ml per min for the different mobile phases depending upon the amine substrate used. The mobile phase contained 4 mM sodium 1-octane sulfonate as an ion-pairing agent, 50 mM ammonium formate (pH 4.0) *vs* methanol (80 : 20) for serotonin and (75 : 25) for tryptamine. Elution peaks of NAS and *N*-acetyltryptamine were identified by retention time. Their identity was verified by their coelution with authentic standards. Comparing the peak areas with known concentrations of standards produced the quantitative determination of NAS and *N*-acetyltryptamine. For the background controls, the reaction mixture was incubated without substrates or without an enzyme source. To identify unknown peaks, standards of assorted indole compounds were added to the samples.

To test the effect of Cole bisubstrate inhibitor CoA-S-N-acetyltryptamine (BSI) the samples of hamster skin were homogenized with a glass homogenizer in an ice-cold 0.25 M potassium phosphate buffer (pH 6.8) containing 1 mM dithiothreitol, 1 mM ethyleneglycol-bis-(β-amino-ethylether)-*N,N,N′,N′*-tetraacetic acid and 0.125 mM acetyl-CoA. Homogenates were centrifuged at 13,000 r.p.m. (11,500 g) in centrifuge (Biofuga Fresco, Heraeus, Kendro Laboratory Products, Germany) for 10 min at 4°C. Aliquots of supernatant were mixed with serotonin in 0.25 M potassium phosphate buffer (pH 6.8) and incubated for 1 h at 37°C. The final concentrations of acetyl-CoA and serotonin were 0.1 mM and 10 mM, respectively. Inhibition of AANAT activity was determined using the specific AANAT bisubstrate inhibitor CoA-S-N-acetyltryptamine (BSI) at five concentrations ranging from 0.1 to 10 μM. The enzymatic reaction was stopped by the addition of 20 μl of 6 M $HClO_4$. After centrifugation at 13,000 r.p.m. (11,500 g) in a centrifuge (Biofuga Fresco, Heraeus, Kendro Laboratory Products) for 5 min at 4°C, 20 μl of the supernatants were separated on a LC-MS-QP8000α (Shimadzu, Japan) through Restec Allure C18 reverse-phase column (150 × 4.6 mm; 5 μm particle size; 60 Å pore size). The elution was carried out isocratically at a flow rate of 0.3 ml per min at 30°C by mobile phases consisted of 20% acetonitrile and 0.1% trifluoroacetic acid. The effluent from the HPLC system was routed to the MS electrospray interface used in positive mode. Nitrogen was used as nebulizing gas. The MS parameters were as follows: the nebulizer gas flow rate was 4.5 l per min; the electrospray voltage was set to 4.5 kV; the CDL (curved desolvation line) heater temperature was 250°C. SIM (selected ion monitoring) mode was applied and ions with m/z = 219 was detected.

The LCMS workstation Class-8000 software was used for system control and data acquisition (Shimadzu, Japan). For the controls, a reaction mixture was incubated without substrate or without an enzyme source. Quantitative determination of NAS was made by comparing its peak areas with those given by known concentrations of NAS standard.

Protein concentration was determined by a dye-binding method with BSA as the standard (Bradford, 1976).

**LC-MS for NAS determination in cultured melanoma cells** The experimented samples were analyzed by LC-MS using a model M-8000 LC/3DQ-MS quadropole ion trap mass spectrometer (Hitachi Instruments Inc., San Jose, CA) in the tandem mode. Briefly, AbC-1 hamster melanoma cell pellets were suspended in 0.03 M perchloric acid containing 40 μM pargyline at a density of 17–20 $\times$ $10^7$ cells per ml. The extract was sonicated, centrifuged for 10 min at 15 000 $\times$ *g* and supernatants were filtered through a Millex-LH filter (0.45 μm pore size). Fifty microliter aliquots were separated by reverse phase-HPLC on a Hitachi 7000 System through a C18 Wydac column (250 $\times$ 2 mm; 5 μm particle size; 300 Å pore size; cat. no. 218TP52) (Hesperia, CA) with a mobile phase A (water with 0.2% acetic acid), and separating phase B (methanol with 0.2% acetic acid). Separation was performed with methanol at the following gradients: 5% (0–5 min), 90% (5–40 min), and 90% (40–45 min), while maintaining the flow rate at 0.3 ml per min. The effluent from the HPLC system was routed to the MS through atmospheric pressure chemical ionization. The atmospheric pressure chemical ionization conditions were as follow: nebulizer temperature 220°C; desolvator temperature 250°C; aperture 1 temperature 170°C; aperture 2 temperature 120°C; needle voltage 4 kV; drift voltage 30 V; focus voltage 30 V. The NAS standard was analyzed by LC-MS under the same conditions.

## RESULTS AND DISCUSSION

**Cloning of the hamster tryptophan hydroxylase gene** The cloning strategy for the hamster pituitary is shown in **Fig 1**(***A***) A full-length sequence of hamster TPH mRNA was obtained by alignment of two separate fragments. First, we amplified a fragment of 933 bp by spanning one-half of the coding sequence and the 3′-untranslated sequence by the 3′-RACE (**Fig 1*A***). The amplified fragment was then sequenced and used to design an anti-sense primer (P151). As this initial primer did not produce a clear band in 5′RACE, we then designed another primer, P176, homologous to mouse TPH. Amplification of hamster pituitary cDNA with primers P151 and P176 did produce a single band of 906 bp. The complete coding sequence of composite hamster TPH cDNA was retrieved by alignment of the two fragments. The open reading frame contained 1338 bp and encoded a protein composed of 446 amino acids with a predicted molecular weight of 48.5 kDa (accession no. AY034600).

We then compared the sequence of the hamster gene with that of TPH genes available from the GenBank database using the DNAMLK program from the PHYLIP package (**Fig 1*B***). Mouse

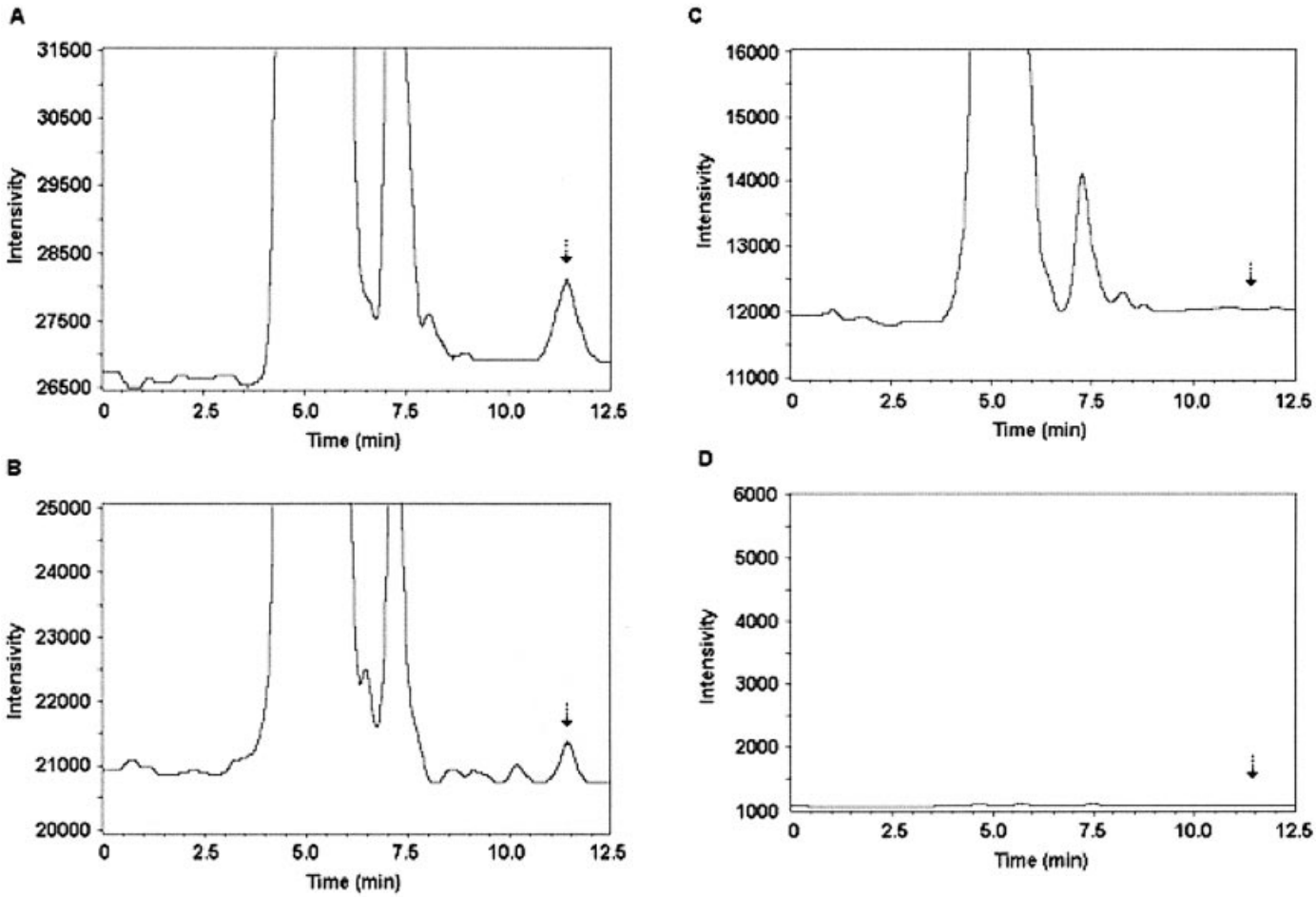


**Figure 5. LC-MS analysis of the products of serotonin acetylation by skin extracts.** SIM (selected ion monitoring) of adduct ion $[M + H]^+$ with m/z 219 was used to detect the NAS. (*A*) Experimental incubation with acetyl-CoA and serotonin. (*B*) Inhibition of enzymatic activity by specific AANAT bisubstrate inhibitor CoA-S-N-acetyltryptamine (5 μM). (*C*) Sample incubated without serotonin (enzymatic reaction control). (*D*) Acetyl CoA and serotonin incubated without enzyme source (chemical acetylation control). *Arrow* identifies the compound with retention time (retention time) 11.4 min and with m/z 219 (calculated mass was 218) corresponding to NAS standard.

and rat genes appeared to be the closest to hamster. The hamster cDNA sequence had 91%, 91%, and 86% identity with mouse, rat, and human sources, respectively. Comparison of deduced TPH amino acid sequences between hamster and mouse, rat, and human proteins showed an even higher degree of identity (92%, 92%, and 87%, respectively) (**Fig 1*C***). TPH is organized into N-terminal regulatory and C-terminal catalytic domains; the latter contains a leucine zipper involved in the formation of the tetrameric holoenzyme (Mockus and Vrana, 1998). Additional inspection of the amino acid sequences showed that the structure most conserved was the catalytic domain (94–97% homology) with intermediate amino acid homology for the N-terminal regulatory domain (84–86%), and lowest identity for the C-terminal fragment containing the leucine zipper (67%) (**Fig 1*C***). That the catalytic domain would be the most conserved portion is not surprising as that is critical for enzyme activity.

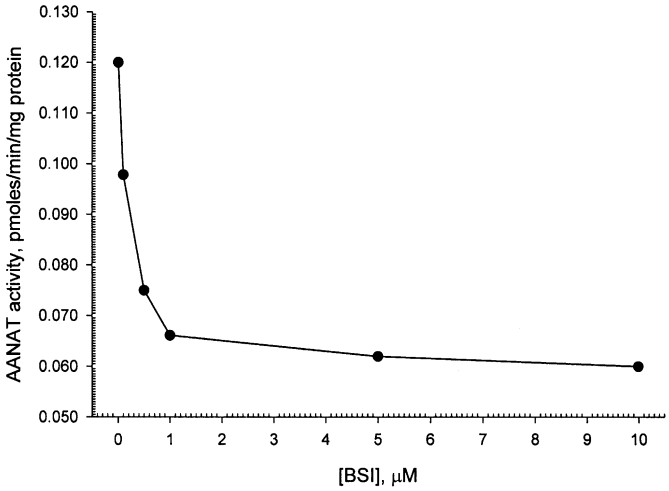


**Figure 6. Effect of the specific AANAT bisubstrate inhibitor CoA-S-N-acetyltryptamine (BSI) on the enzyme activity in hamster skin.**

**Tissue expression of TPH and of AANAT genes** We used hamster-specific primers located at exons 7 and 8 for TPH and exons 2 and 3 for AANAT for the reverse transcription–PCR assays performed on RNA from hamster skin, melanomas, eye, pituitary, heart, and spleen (**Fig 2**). Representative amplifications of a 189 bp TPH fragment and a 163 bp AANAT fragment are shown in **Fig 2(*B,C*)**. The amplified fragments show 100% nucleotide sequence homology with the corresponding genes. Experiments demonstrating expression of the TPH and AANAT genes in normal hamster skin, Bomirski hamster AbC-1 cell line, Ab amelanotic, MI hypomelanotic, and Ma melanotic variants of Bomirski melanoma transplantable in hamsters as well as in hamster pituitary, eye, and spleen are summarized in **Table I**. From these results it can be concluded that rodent skin, including skin-derived melanoma cells, must be added to the list of peripheral tissues expressing genes coding for enzymes involved in the synthesis and transformation of serotonin. We could not investigate the expression of hydroxyindole-*O*-methyltransferase, because neither the hamster nor murine gene sequences are yet available.

We also tested the effect of UV radiation (UVR), and of melanogenesis induction to test for the main differentiated function of the melanocyte on the expression of TPH and AANAT genes in hamster amelanotic melanoma cells. Neither UVR nor induction of melanogenesis had significant effects on TPH gene expression in

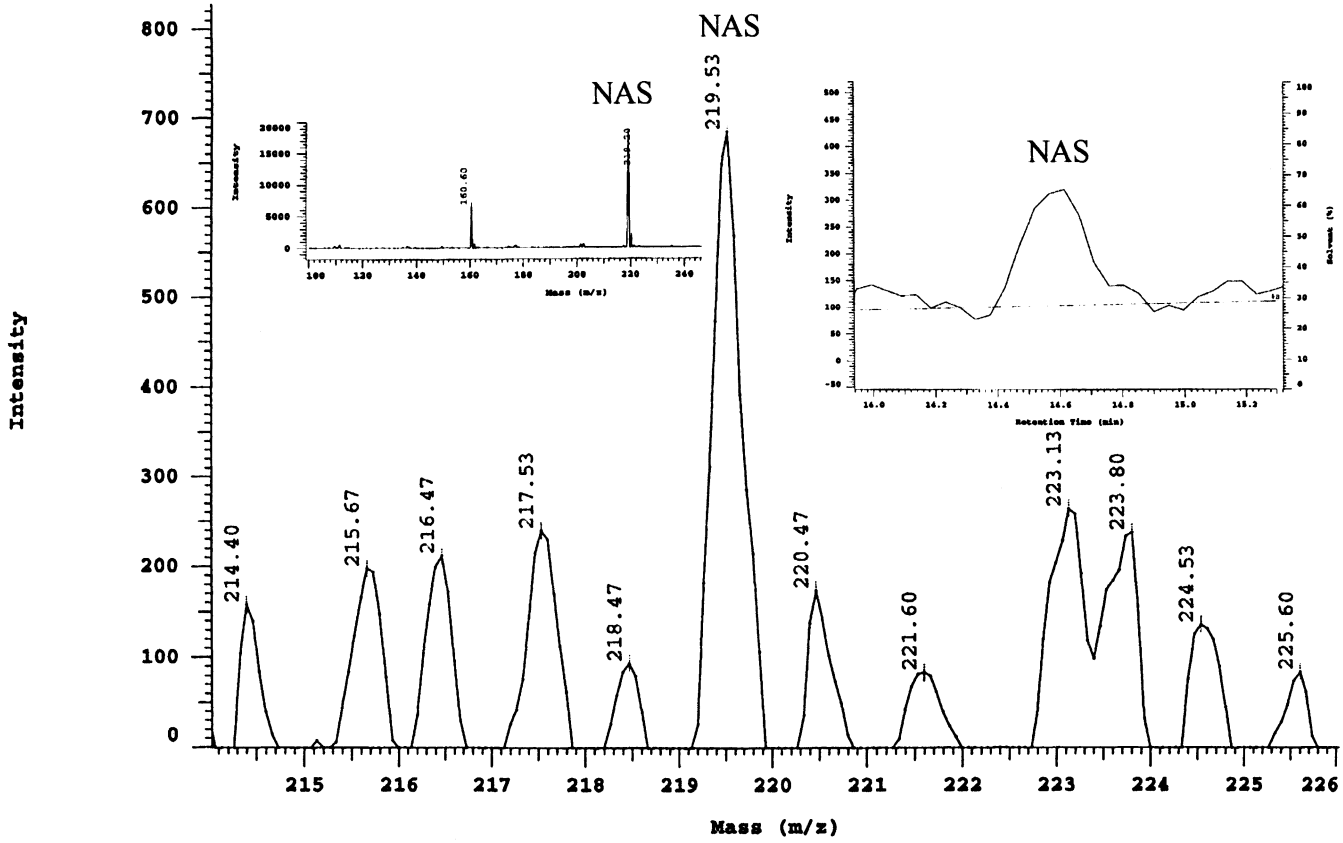


**Figure 7. LC-MS identification of NAS in hamster AbC-1 melanoma cells.** Shown in the center is an adduction $(M + H)^+$ at m/z 219.5, and the corresponding retention time (14.5 min) is in the *right insert*. *Left insert* shows $(M + H)^+$ ion at m/z of 219 of NAS standard.

**Table II. AANAT activity in hamster skin, melanoma tumors and melanoma cell line**

| Sample | AANAT activity (pmol per min per mg protein) | |
|---|---|---|
| | Serotonin substrate | Tryptamine substrate |
| Hamster skin from body (2) | 26.45 ± 2.3 | 35.07 ± 2.89 |
| Hamster skin from ears (2) | 100.95 ± 4.32 | 113.93 ± 5.73 |
| Ab amelanotic melanoma tumor (2) | 2.88 ± 0.2 | 2.52 ± 0.04 |
| Ma melanotic melanoma tumor (2) | 5.20 ± 0.49 | 4.45 ± 0.02 |
| MI hypomelanotic melanoma tumor (2) | 6.49 ± 0.08 | 6.45 ± 0.04 |
| AbC-1 amelanotic melanoma cell line (2) | 25.86 ± 1.72 | 27.58 ± 3.44 |

The data represent mean ± SEM from two assays.

that cell line. AANAT gene expression also remained unaffected by UVR; although, it did decrease after induction of melanin synthesis (**Fig 2*C***). Thus, in contrast to the effect of UVR on induction of melanin synthesis in normal and malignant melanocytes, and expression of melanocyte-stimulating hormone, adrenocorticotropic hormone, and corticotropin-releasing hormone neuropeptides together with their corresponding receptors (corticotropin-releasing hormone-R1 and MC1-R) (Chakraborty *et al*, 1999; Slominski *et al*, 2001), the rodent cutaneous serotoninergic pathway appears to be resistant to UVR regulation at least in malignant melanocytes. Induction of melanogenesis in hamster amelanotic melanoma cells are further associated with increased expression and activity of melanogenesis related proteins, and with the formation of specialized organelles—melanosomes (Slominski *et al*, 1988). As the level of expression of the AANAT gene in pigmented cells was generally low, it is possible that the induction of melanogenesis may be paradoxically connected with downregulation of the conversion of serotonin to NAS. This finding obviously requires further study.

**Enzymatic activities and LC-MS detection of NAS** Direct biochemical assays showed high activity for the enzymatic conversion of tryptophan to hydroxytryptophan in hamster AbC-1 melanoma cells, at levels comparable with rat brain. Thus, the respective TPH activities for rat brain and for hamster melanoma homogenates were similar, at 10.92 ± 0.97 and 9.42 ± 1.9 nmol of OH-tryptophan formed per mg protein per hour (n = 3).

We established the definitive presence of serotonin and tryptamine *N*-acetyltransferase activity in hamster skin and hamster melanomas using reverse phase-HPLC with fluorimetric detection (**Figs 3 and 4**). Extracts of both skin and tumor samples readily transformed serotonin to NAS and tryptamine to *N*-acetyltryptamine after addition of acetyl-CoA. These results have not only confirmed the presence of the enzyme in the skin, but are also consistent with our detection of expression of the corresponding gene. In addition, hamster melanoma extracts subjected to reverse phase-HPLC showed fluorescence at the retention times of serotonin and NAS standards (**Fig 4*B***, insert), implying intracellular production/storage of both serotonin substrate and NAS product.

Final confirmation of local serotonin acetylation was provided by LC-MS analysis of the products of enzymatic reaction showing the compound at m/z 219 with retention time 11.4 min and calculated mass 218, which matched the retention time and m/z of the NAS standard (**Fig 5**). Importantly, the specific AANAT bisubstrate inhibitor CoA-S-N-acetyltryptamine (BSI) inhibited the observed enzymatic activity (**Fig 5*B***). The effect of the inhibitor on the serotonin *N*-acetyltransferase activity is shown in **Fig 6**. There was a steep decrease in activity to approximately 50% at low concentrations of BSI ($\leqslant 1$ μM) and a further increase of BSI concentrations (5–10 μM) produced minimal or no effect. The inhibitory effect of low concentrations of BSI ($\leqslant 1$ μM) is characteristic for AANAT (Coon *et al*, 2001), whereas inhibition of arylamine acetyltransferase activity requires high concentrations (> 10 μM) of BSI (Khalil and Cole, 1998). Therefore, we suggest that serotonin acetylation is both mediated by AANAT (sensitive to low concentrations of BSI) and by arylamine acetyltransferase (resistant to BSI) (**Fig 6**). This is in agreement with our previous detection of two isozymic forms of arylamine *N*-acetyltransferase in Syrian hamster skin (Gaudet *et al*, 1993). One had the characteristics of pineal AANAT and the second of arylamine acetyltransferase (Gaudet *et al*, 1993).

*In vivo*, the local production of NAS was ultimately confirmed by LC-MS analysis of extracts from hamster AbC-1 melanoma cells, which showed an adduct ion $(M + H)^+$ at m/z 219 (**Fig 7**), correspondent with the $(M + H)^+$ at m/z of 219 of the NAS standard (calculated mass = 218 Da) (**Fig 7**, left insert). The adduct ion from cell extracts had a retention time of 14.5 min (**Fig 7**, right insert), identical to that of the NAS standard. As regards the serotonin standard, this eluted at retention time 3 min and presented as $(M + H)^+$ ion at m/z 177; a detected fragment ion $(M + H\text{-}17)^+$ at m/z 161 represents a product of serotonin deamination (not shown). A specific search for serotonin (retention time of 3 min) disclosed buried within the background (noise level) two species with mass spectrum ion at m/z 177 and 160 and retention time 3 min, similar to the $(M + H)^+$ ion at m/z 177 and a fragment ion $(M + H\text{-}17)^+$ at m/z 160 of the serotonin standard (not shown). Thus, we conclude that hamster skin cells contain NAS, express the TPH and AANAT genes together with their enzymatic activities, hence delineating a novel serotoninergic system.

Whereas both gene expression and activity of the enzyme catalyzing serotonin to NAS conversion (AANAT) were detected in skin and in all melanoma lines, the measured enzymatic activities were variable (**Table II**). For example, hamster skin showed generally high activity, and dependence on anatomic site, e.g., 4-fold higher in the skin from ears than in back corporal skin. In contrast, the same activity was one to two orders of magnitude lower in transplantable melanomas. Thus, besides anatomic location, expression of enzymatic activity is also dependent on existing pathology (at least of melanoma). It is also interesting that AANAT activity was higher in the slow-growing MA and MI melanomas than in the fast-growing Ab melanoma cell line (Bomirski *et al*, 1988). That pattern shown in **Table II**, suggests an inverse relation between severity of skin pathology (melanoma) and acetylation of serotonin. Nevertheless, the possibility that this may represent an incidental finding related to this particular tumor type, e.g., family of Bomirski hamster melanomas, remains open.

## CONCLUSIONS

Mammalian TPH shares approximately 50% amino acid homology with tyrosine hydroxylase and/or phenylalanine hydroxylase, enzymes that may have evolved from a common ancestor and require tetrahydrobiopterin (BH4) as a cosubstrate (Mockus and Vrana, 1998). Epidermal expression of the phenylalanine hydroxylase, tyrosine hydroxylase, and the BH4 generating system (described by Schallreuter *et al*, 1997) suggest local involvement, probably in melanogenesis and catecholamine synthesis. Our

current findings of a serotoninergic system in the hamster skin complements the existing data supporting the cutaneous synthesis of biogenic amines. Also in agreement with our previous functional characterization of cutaneous arylamine *N*-acetyltransferase as having two isozymic forms of which one (NAT-2) could acetylate serotonin to NAS, is the current detection of AANAT gene expression and enzyme activity (Gaudet *et al*, 1993). Taken together, these data provide definitive evidence for the capability of rodent (hamster) skin cells to metabolize tryptophan to serotonin and further to NAS, similar to our previous finding of serotoninergic and melatoninergic systems in human skin (Slominski *et al*, 2002).

Given the multiple systemic actions of serotonin, as neurotransmitter, regulator of vascular tone, immunomodulator, and growth factor (reviewed in Seuwen and Pouyssegur 1990, and Slominski and Wortsman, 2000), the current findings could potentially underscore significant physiologic and/or pathologic implications. In fact, it has already been shown that in a mouse skin organ culture model, serotonin stimulates epidermal keratinocyte proliferation (Maurer *et al*, 1997). Thus, the current uncovering of a local mechanism regulating serotonin synthesis and its transformation to NAS in hamsters further underlines the potential significance of this pathway in the rodent skin.

In conclusion, we provide evidence for the expression of a cutaneous serotonin biosynthetic pathway in hamster skin. This novel system is also endowed with being able to process further the biogenic amine into molecule(s) with additional biologic activities.

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