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Research report

Human hydroxyindole-*O*-methyltransferase in pineal gland, retina and Y79 retinoblastoma cells

Marianne Bernard, Susan J. Donohue, David C. Klein *

Section on Neuroendocrinology, Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA

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Abstract

Hydroxyindole-*O*-methyltransferase (HIOMT, EC 2.1.1.4) was studied in extracts of human pineal gland, retina and Y79 retinoblastoma cells. HIOMT enzyme activity and immunoreactive protein (~ 42 kDa) were undetectable in the human retina; very low levels of HIOMT mRNA were detected using a highly sensitive RT-PCR/Southern blot method, as has been reported. Analysis of extracts of Y79 cells indicated that HIOMT enzyme activity, immunoreactivity (~ 42 kDa) and mRNA (~ 1.3 kb) were detectable at ~ 1/5–1/40 the levels found in the pineal gland. This unambiguously establishes that the HIOMT gene is expressed in Y79 cells. Kinetic analysis of Y79- and pineal-derived HIOMT indicates that the enzyme is generally similar in both tissues; one difference, however, is that substrate inhibition by *N*-acetylserotonin is greater with the Y79-derived enzyme. These studies show that Y79 cells represent a valid model to study the regulation of human HIOMT protein and mRNA; the differences detected may reflect the existence of tissue-specific regulatory mechanisms or differential patterns of expression of HIOMT isoforms.

Keywords: Hydroxyindole-*O*-methyltransferase; Melatonin; Pineal; Retina; Y79 retinoblastoma; Tissue-specific

1. Introduction

Hydroxyindole-*O*-methyltransferase (*S*-adenosyl-L-methionine:*N*-acetylserotonin-*O*-methyltransferase; EC 2.1.1.4; HIOMT) catalyzes the final step in the serotonin → *N*-acetylserotonin (NAS) → melatonin pathway [3,31]. This enzyme is consistently expressed at high levels in the pineal gland, the source of circulating melatonin [20,25,33]. HIOMT plays an essential role in the vertebrate circadian system by virtue of the important role melatonin plays in mediating photo-induced seasonal changes in physiology.

Relatively little is known about human HIOMT, in part reflecting the difficulty in obtaining tissue suitable for analysis. Recently, however, a cDNA-encoding human HIOMT was isolated, which made it possible to detect mRNA encoding this enzyme, to develop specific antisera [11], and to clone and characterize the human HIOMT gene [28].

In the report presented here, two related issues regarding the expression of the human HIOMT gene were addressed. The first is whether active enzyme is present in human retina. This is of special interest because melatonin receptors occur in the retina, and melatonin alters elements of retinal function in some vertebrates [5,12,22,23]. If melatonin does play an important role in human retinal function, the absence of local synthesis would make the retina totally dependent upon melatonin in the circulation. HIOMT is known to be present in non-mammalian vertebrate retina [4,25,33,34] and in the rat retina [7,8]. However, it has not been possible to obtain clear evidence that HIOMT is present in all mammalian retina. For example, HIOMT enzyme activity has never been reported in the bovine or human retina, and efforts to detect retinal mRNA [13] or immunocytochemical reactivity [16] have not been successful.

Interest in the issue of retinal expression of HIOMT has recently been stimulated by two developments. One is the retraction of earlier claims that HIOMT immunoreactivity is present in human and bovine retinae [35,36]. Another is a report that the presence of HIOMT mRNA in some human retina is detectable using a highly sensitive combined RT-PCR/Southern blot method [28]. This is consis-

* Corresponding author. Building 49, Room 5A38, National Institutes of Health, Bethesda, MD 20892, USA. Fax: (1) (301) 480-3526.

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tent with a preliminary report claiming that putative HIOMT PCR products can be generated from bovine retinal mRNA [9]. These observations point to the possibility that bovine and human retinae might contain enzymatically active HIOMT protein.

The second issue examined is whether the HIOMT gene is expressed in the Y79 retinoblastoma cell line or if the low levels of HIOMT-like activity in these cells [14] reflect the non-specific action of another enzyme. The Y79 cell line is of special interest because it is the only transformed melatonin-producing cell line available [24]; these cells express a number of genes typically found only in the pineal gland and retina [14,17,24]. The issue of whether Y79 cells express the HIOMT gene must be settled before these cells can be used as a model to study the regulation of human HIOMT gene expression.

The results of our studies indicate that HIOMT activity and immunoreactivity are undetectable in the bovine and human retina; and, that HIOMT gene products in Y79 cells are generally similar to those in the human pineal gland, although discrete differences are apparent.

2. Materials and methods

2.1. Tissue and cell culture

Human pineal glands and retinae were obtained through the National Disease Research Interchange (Philadelphia, PA). Pineal glands were delivered frozen on solid CO_2; the interval between death and freezing was 6–12 h. Human eyes were delivered on wet ice and the retinae were dissected and stored frozen (−70°C); the interval between death and placing tissue on wet ice was 2–4 h and the time on wet ice before freezing was 4–9 h. Bovine pineal glands (Biological Resources and Delivery Service, Gaithersburg, MD) were removed and placed on solid CO_2 within 10 min after death. Bovine eyes were removed within 10 min after death. The eyes were placed on ice and retinae were removed immediately or within 3 h. Rat tissue was obtained from females (Sprague–Dawley; Taconic Farms, Germantown, MD) sacrificed during the daytime.

Y79 retinoblastoma cells (American Type Tissue Culture; Rockville, MD) were maintained in suspension culture at 37°C (95% air, 5% CO_2) in RPMI 1640 medium (Biofluids, Rockville, MD) supplemented with 2 mM glutamine, 10% fetal calf serum (Biofluids), 100 U/ml penicillin and 100 mcg/ml streptomycin.

2.2. HIOMT enzyme assay

HIOMT activity was determined radiochemically, using NAS and [^{3}H-methyl]-*S*-adenosyl-L-methionine (SAM) [3,31]. The assay described as 'standard' is used routinely in this laboratory for analysis of rat HIOMT. The 'high sensitivity' assay was developed to detect HIOMT-like activity in Y79 cells [14]. The concentrations of NAS and SAM and the specific activity of [^{3}H]SAM used in these assays are given in the legends to Table 1, Table 2 and Fig. 1. Background was calculated as the sum of (a) radioactive products formed chemically during a minus-homogenate assay and (b) products formed during a minus-NAS with homogenate.

2.3. Protein assay

Proteins were measured using a dye-binding method [6] with BSA as standard.

2.4. Western blot analysis

Sample preparation

Tissues or Y79 cells were homogenized in 50 mM $NaPO_4$, pH 7.9. Ammonium sulfate precipitation was used to concentrate HIOMT protein as follows: homogenates were centrifuged at 100,000 × *g* for 1 h. The supernatant was made 37% ammonium sulfate by addition of a saturated solution of the salt. After 30 min (4°C), the protein precipitate was collected by centrifugation (30 min, 16,000 × *g*) and the ammonium sulfate concentration in the supernatant was adjusted to 68%. Precipitate was allowed to form on ice (30 min) and was collected by centrifugation (30 min, 16,000 × *g*). The pellet was resuspended in 50 mM $NaPO_4$, pH 7.9, and the solution was desalted by

Table 1
HIOMT activity in pineal and retina of selected mammals and in human Y79 cells

HIOMT activity (nmol/h/mg protein)			
	Pineal	Y79	Retina
(A) Standard assay (1 mM NAS; 0.1 mM [^{3}H]SAM, 25 Ci / mol)			
Human	4.3 ± 0.1	ND	ND
	(1400 dpm; 20 μg)	(0 dpm; 70 μg)	(32 dpm; 112 μg)
Bovine	20.3 ± 0.3		ND
	(5630 dpm; 20 μg)		(9 dpm; 36 μg)
Rat	3.9 ± 0.7		0.2 ± 0.01
	(4900 dpm; 92 μg)		(170 dpm; 86 μg)
(B) High sensitivity assay: (50 μM NAS; 2.5 μM [^{3}H]SAM, 500 Ci / mol)			
Human	4.1 ± 0.3	0.23 ± 0.01	ND
	(27,500 dpm; 24 μg)	(4550 dpm; 70 μg)	(3 dpm; 112 μg)
Bovine	9.4 ± 0.3		ND
	(52,400 dpm; 20 μg)		(8 dpm; 36 μg)
Rat	0.6 ± 0.005		ND
	(14,600 dpm; 92 μg)		(0 dpm; 86 μg)

HIOMT activity was assayed in 100,000 × *g* supernatants of pineal glands, retinae and Y79 cells. Two conditions of assay were used. A: standard assay: 1 mM NAS, 0.1 mM [^{3}H]SAM (25 Ci/mol). B: high sensitivity assay: 50 μM NAS, 2.5 μM [^{3}H]SAM (500 Ci/mol). Values were obtained from pools of tissue (human pineal and retina $n = 4$; bovine pineal $n = 4$; bovine retina $n = 3$; rat pineal $n = 18$; rat retina $n = 16$) assayed in triplicate and results are expressed as mean ± S.E.M. Mean dpm values corrected for background and amount of protein assayed are given in parentheses. dpm values < 50% of background were designated as non-detectable (ND)

Table 2
HIOMT activity in individual human pineal glands

NDRI ID	HIOMT activity (nmol/h/mg protein)	Age (yr)	Sex	Race	Postmortem time (h)
23912	41.1	61	M	C	8
26787	12.7	29	M	B	8
23915	12.5	84	M	C	6
26650	11.4	15	M	B	12
26641	8.6	43	M	C	11
26948	8.2	22	F	C	11
23560	7.8	67	M	C	3
26560	7.6	30	M	C	6
26999	4.5	83	M	C	12
26956	0.04	19	M	B	10

Ten human pineal glands were homogenized individually in 50 mM $NaPO_4$, pH 7.9, centrifuged at 100,000× g for 1 h (4°C), and HIOMT activity was assayed in supernatant (20 μg protein) with 50 μM NAS and 50 μM SAM (50 Ci/mol). NDRI, National Disease Research Interchange. M, male; F, female; B, black; C, Caucasian

sequential concentration and dilution on a size exclusion filter (Filtron, Northborough, MA; 10K, 7500 × g for 2.5–3 h). This procedure resulted in a 2–3.5-fold enrichment.

Electrophoresis and blotting

Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) [18] on 10% polyacrylamide gels and electrotransferred onto Immobilon-P membrane (Millipore, Bedford, MA) [32]. Blots were coated with 0.3% Tween-20 in phosphate-buffered saline (PBS).

Immunodetection

An anti-human HIOMT serum (139A) was used which had been raised in rabbits injected with 3 synthetic HIOMT peptides (HH1, HH2 and HH3) [11]. This serum was affinity-purified using the ~ 42-kDa HIOMT protein from the human pineal gland according to the procedure of Smith and Fischer [29], with the exception that the antibody was eluted with 0.1 M acetic acid, pH 2.85, containing 0.1% bovine serum albumin (BSA). It was determined that this immunopurified antiserum strongly recognizes HH1; it has not been possible to demonstrate that it recognizes HH2 or HH3. Samples of this immunopurified serum were blocked by incubation (24 h, 4°C) with HH1

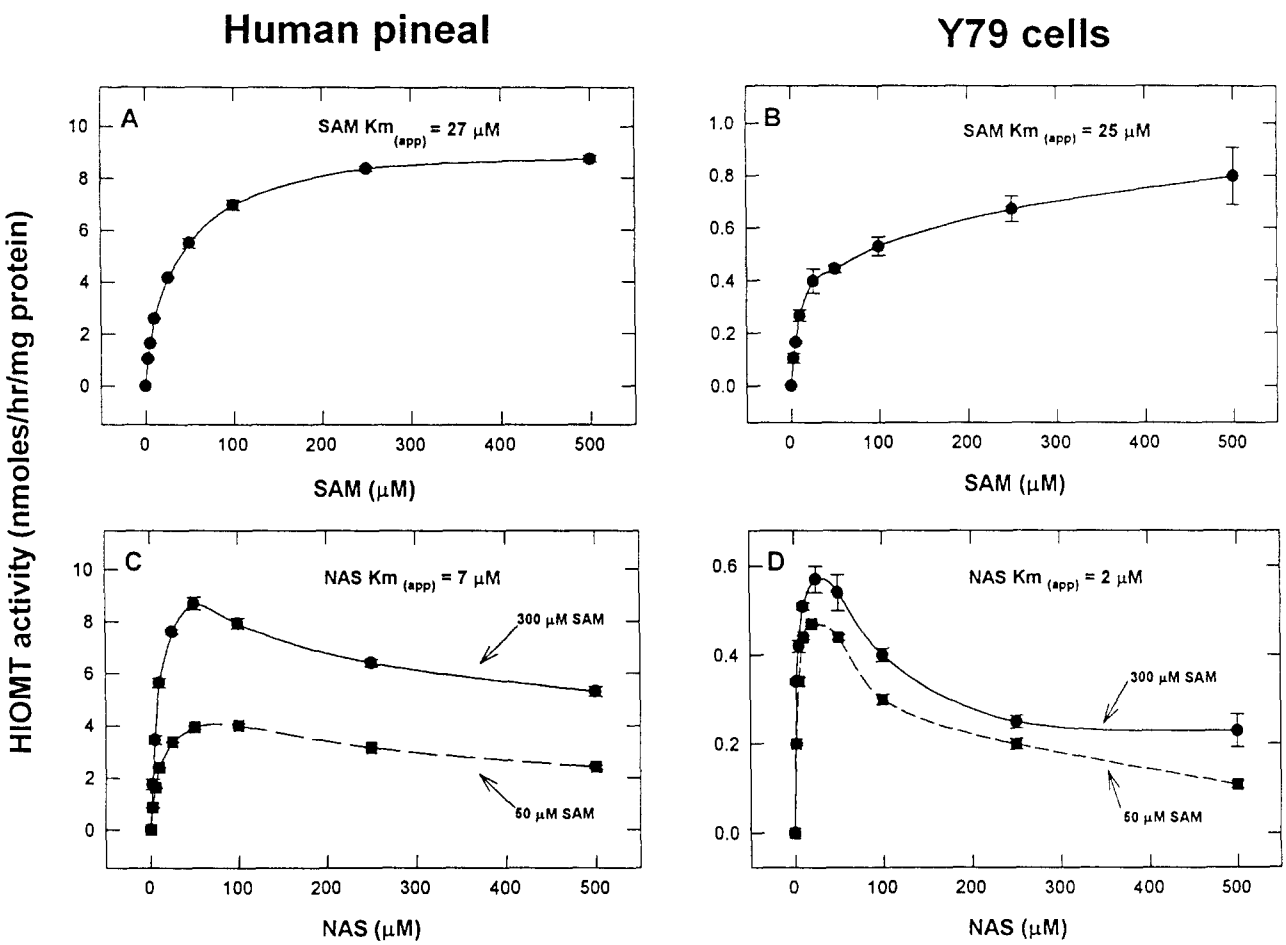

Fig. 1. HIOMT kinetics in human pineal and Y79 cells. HIOMT activity was assayed with 50 μg of human pineal protein (pool of 4 glands) or 170 μg of Y79 cells protein (10^6 cells). A,B: increasing concentrations of SAM (50 Ci/mol) in the presence of 50 μM NAS; C,D: increasing concentrations of NAS in the presence of either 50 μM or 300 μM SAM (50 Ci/mol). Results are expressed as the mean ± S.E.M. (n = 3). For further technical details, see Materials and methods.

or with a mixture of the peptides HH1, HH2 and HH3. The final concentration of each peptide was 10 μM.

After incubation with the immunopurified serum, blots were washed with TBS containing 0.05% Tween-20 and the antigen–antibody complexes were detected with goat-antirabbit IgG conjugated to horseradish peroxidase (1:20,000 in TBS containing 0.05% Tween-20 and 0.05% thimerosal). Protein bands were visualized by an enhanced chemiluminescence method (ECL Western blotting detection reagents, Amersham; Arlington Heights, IL).

Quantitation

Densitometric analysis of the Western blots was performed with Quantity ONE software package (PDI, Huntington Station, NY) on a Sun Sparcstation (Sun Microsystems).

2.5. Northern blot analysis of RNA

Extraction of RNA

Total RNA was isolated using RNA STAT-60 reagent as described by the manufacturer (Tel-Test 'B'; Friendswood, TX). Total RNA was fractionated on 1.5% agarose/0.66 M formaldehyde gel and subsequently transferred to nylon membrane by capillary blotting with 20 × SSC (3 M NaCl and 0.3 M sodium citrate). RNA was cross-linked to the membrane in a UV oven (Stratagene, La Jolla, CA; 120 mJ, 35 s).

Probes

The probe used for detection of HIOMT mRNA on Northern blots was 5′ HIOMT, a 473-bp 5′ fragment of the human HIOMT cDNA (bases 28–500) [11]; this does not include the truncated LINE-1 sequence found in one isoform of HIOMT mRNA [11]. A 2-kb probe for the human β-actin and a 1.1-kb probe for the human glyceraldehyde-3-phosphate-dehydrogenase (G3PDH; Clontech, Palo Alto, CA) were used to monitor the quality of RNA preparations and to normalize the HIOMT signal. A 704-bp probe for human S-antigen was prepared by PCR amplification of human retinal cDNA, using primers corresponding to the rat coding sequence of S-antigen cDNA (GenBank/EMBL Data Bank, accession number M60737): 5′ primer = GAGGACATTGATGTGATTGGCCTG (bp 238–261; 83% identity with the human sequence); 3′ primer = GTGTCCTCATGCTTTATCTTCCC (bp 941–919; 87% identity with the human sequence). A 436-bp probe for human phosducin was prepared by PCR amplification of human retinal cDNA, with primers corresponding to the rat coding sequence of phosducin cDNA (GenBank/EMBL Data Bank, accession number M33530): 5′ primer = TCCTCAGACAAATGTCCTCTCCTC (bp 237–260; 87% identity with the human sequence); 3′ primer = CCTTTGTATACGAGCAATGTCGGG (bp 672–649; 76% identity with the human sequence). A 1.5-kb probe for bovine HIOMT was prepared from clone B-18 [10,13]. Probes were labeled with ^{32}P-dCTP (3000 Ci/mmol) by random priming (Megaprime kit; Amersham, Arlington Heights, IL).

Analysis of blots

Blots were incubated in prehybridizing solution (Quikhyb; Stratagene, La Jolla, CA) for 15 min at 68°C, ^{32}P-labeled probe was added (10^6 cpm/ml of either HIOMT, β-actin, G3PDH, S-antigen or phosducin probe) and the 68°C incubation was continued for 1.5 h. After hybridization, the blots were washed twice (15 min each at room temperature) in 2 × SSC/0.1% SDS and once (30 min at 42°C for HIOMT, S-antigen and phosducin probes or 60°C for β-actin and G3PDH probes) in 0.1 × SSC/0.1% SDS. Blots were exposed overnight to a PhosphorImager screen and band intensities were measured by integration of area under the peaks of interest (Image Quant; Molecular Dynamics, Sunnyvale, CA). Typically, blots were first hybridized with 5′ HIOMT probe and subsequently stripped (twice 15 min in water $\leq$ 100°C) before hybridization with β-actin probe. In some cases, blots were stripped again and probed with another ^{32}P-labeled cDNA.

2.6. RT-PCR analysis

cDNA synthesis and PCR

cDNA was synthesized as described by Rodriguez and Chader [27]. PCR amplification of HIOMT was performed using primers 5′ HIOMT (ATGGGATCCTCAGAGGACCAGG, bp 1–22) [11] and 3′ HIOMT (CTGGAAGTCTCTGAAGCCAGC, bp 1074–1054). The final composition of the PCR reaction was 10 mM Tris, pH 8.4, 0.01% gelatin, 50 mM KCl, 1 mM $MgCl_2$, 0.67 mM each dATP, dCTP, dGTP and dTTP, 2.5 U DNA polymerase, Taq (Boehringer Mannheim, Indianapolis, IN), 0.5 μM of each primer and 2 μl of cDNA. The reaction (final volume 50 μl) was covered with mineral oil (50 μl). The cycling parameters were 94°C for 5 min, (67°C for 1 min, 72°C for 1 min, 94°C for 45 s) 30 cycles, 67°C for 1 min, 72°C for 15 min, hold (4°C).

Electrophoresis and analysis

PCR products were resolved on 2.5% NuSieveGTG:SeakemGTG (FMC Bioproducts, Rockland, ME; 3:1) agarose gels made with 40 mM Tris-acetate containing 2 mM EDTA and 0.5 μg/ml ethidium bromide. After the gel was washed for 10 min in 0.2 N HCl (depurination) and rinsed in water, double-stranded DNA was denatured by 2 washes (15 min each) in 1.5 M NaCl and 0.5 M NaOH. The gel was subsequently neutralized in 3 M NaCl and 0.5 M Tris, pH 7.5, for 30 min, and DNA was transferred to nylon membrane (Nytran; Schleicher and Schuell, Keene, NH) by capillary blotting with 20 × SSC. DNA was cross-linked to the membrane (120 mJ for 35 s) and the Southern blot was incubated with the ^{32}P-labeled 5′ HIOMT probe. The hybridization protocol was

as described for Northern blots, except that the second wash was at 60°C.

2.7. Sequencing of B′ HIOMT PCR product

Human pineal HIOMT PCR products were generated and resolved as described above. Individual bands were identified on the ethidium bromide-stained gel under ultraviolet light. The cDNA band corresponding to form B′ was excised, electroeluted (2 h at 100 V in 100 mM Tris, pH 8.3, 2 mM EDTA, and 100 mM boric acid), and cloned into the PCR 3 vector using the eukaryotic TA cloning kit (Invitrogen, San Diego, CA). Clones were selected at random and tested for the presence of a ~ 940-bp insert, using PCR primers described above. The size of the insert was estimated by comparison to standards and to the 4 HIOMT PCR products. HIOMT DNA extracted from a clone containing the a ~ 940-pb insert (Plasmid Midi Kit; Qiagen, Chatsworth, CA) was sequenced (Sequenase version 2.0 kit; United States Biochemical, Cleveland, OH).

3. Results

3.1. Enzyme analysis

HIOMT activity in human tissues was initially analysed using a standard assay developed for analysis of the rat pineal enzyme (Table 1A). For comparative purposes, enzyme activity was also determined in bovine and rat pineal glands and retinae (Table 1A). With this assay, rat and human pineal HIOMT values were similar, while the

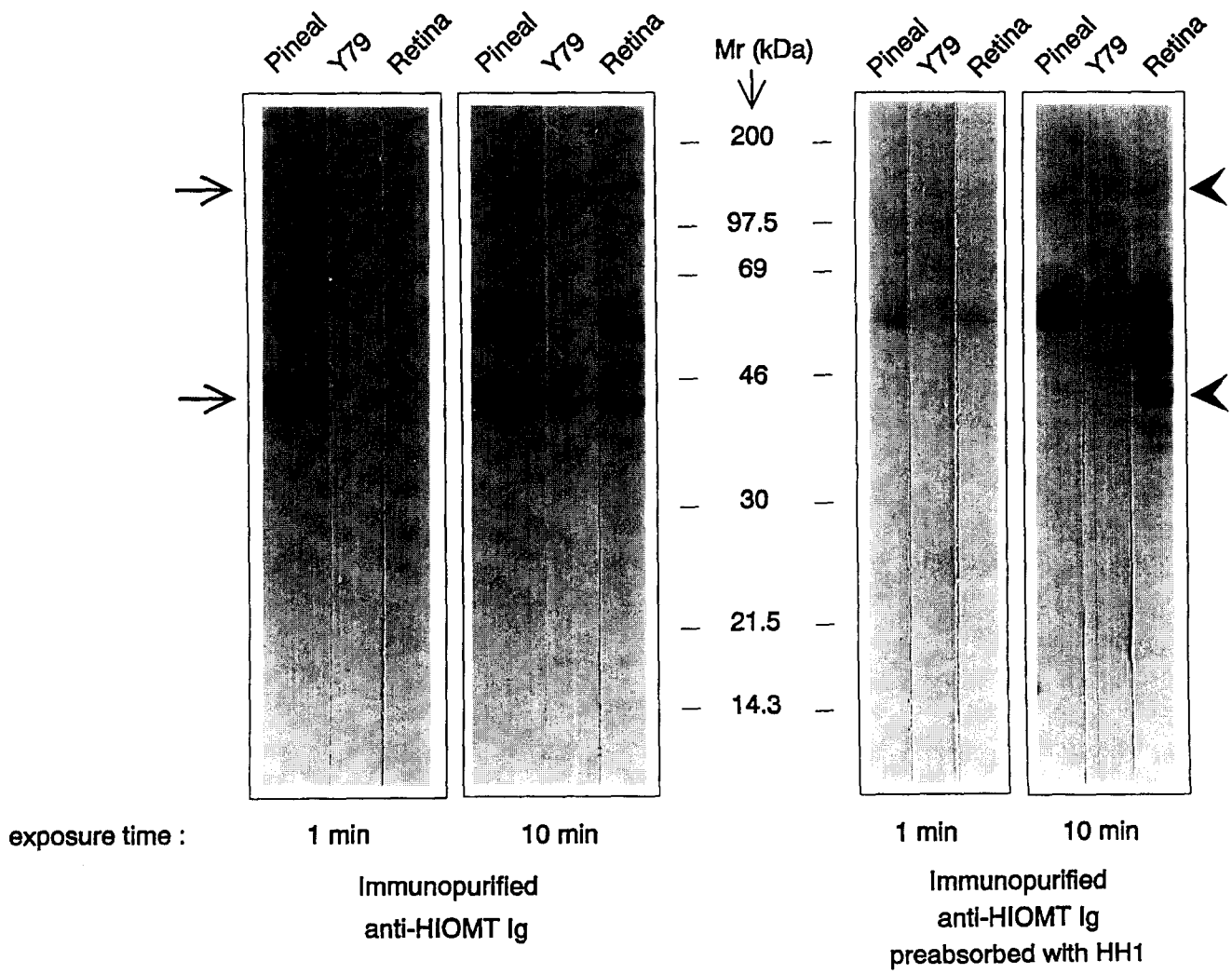

Fig. 2. Immunodetection of HIOMT in human tissues. Proteins were precipitated with ammonium sulfate (37/68% cut) and resolved by SDS–PAGE on 10% polyacrylamide gels. Samples containing 20 μg of pineal proteins and 40 μg of Y79 or retina proteins were applied. Proteins were resolved by SDS–PAGE and transferred to Immobilon-P membrane. HIOMT was detected with affinity-purified anti-HIOMT immunoglobulins solution diluted 1:10 (left panel, arrows). Specificity of the detection was tested with the same solution of affinity-purified anti-HIOMT immunoglobulins, preabsorbed with HH1 peptide (right panel, arrowheads). Antigen–antibody complexes were detected by enhanced chemiluminescence (ECL) method using goat-antirabbit immunoglobulins conjugated to peroxidase, diluted 1:20,000. After incubation with ECL reagents, the blot was exposed to X-ray film for the indicated times. Similar results were obtained in three experiments. For further technical details, see Materials and methods.

bovine value was ~ 5-fold greater. Enzyme activity was detected at very low levels in the rat retina with the standard assay (Table 1A). However, HIOMT activity in human and bovine retinae and in Y79 cells was < 50% of the background value with this assay, making unambiguous identification of the enzymatic product impractical. Activity at or below this level was regarded as undetectable.

A high sensitivity assay was also used. This contains 1/20 the concentration of NAS (50 μM) and 1/40 the concentration of SAM (2.5 μM). In addition, the specific activity of [^{3}H]SAM is 20-fold greater (500 vs. 25 Ci/mol). With this assay, HIOMT activity was detected in all tissues in which it had been detected with the standard assay (Table 1B). However, rat pineal enzyme activity was markedly lower when measured with the high sensitivity assay because of the concentration of substrates used and the K_m values of the rat enzyme [30]. With the high sensitivity assay, HIOMT activity was detectable in Y79 cells at ~ 5% of the human pineal value but was undetectable in retinal extracts (Table 1B).

HIOMT activity varied widely between individual human pineal glands (Table 2), from undetectable levels to values 10-fold higher than the mean of a pool of glands (Table 1), as has been reported for HIOMT immunoreactivity [11]. This limited analysis provides no reason to suspect that such variation is a function of postmortem interval, age, race or sex (Table 2).

The kinetic characteristics of HIOMT enzyme were determined in human pineal extracts (pool of 4 glands) and Y79 cells. The V_{max} value of HIOMT, measured with [NAS] = 50 μM and [SAM] = 50 or 300 μM, was 10–14-fold higher in the human pineal than in Y79 retinoblastoma cells (Fig. 1). The apparent K_m values for SAM were similar in both preparations (Fig. 1A,B). The apparent K_m of Y79 HIOMT for NAS was ~ 1/3 that of the human pineal, using the peak enzyme activity value as the V_{max} (Fig. 1C,D). Substrate inhibition of human pineal and Y79 HIOMT by NAS was observed, as previously described with chicken and bovine HIOMT [15,20]. Substrate inhibition by NAS was more pronounced in Y79 cells than in the human pineal gland (Fig. 1C,D).

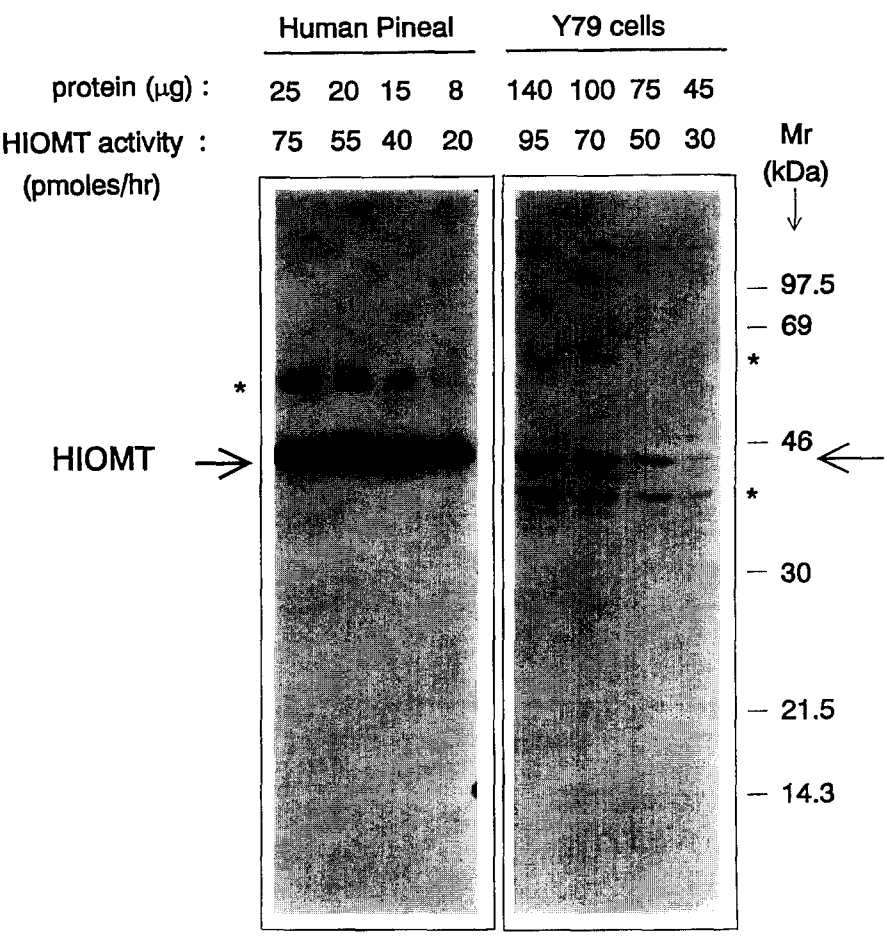

Fig. 3. Comparison of HIOMT-specific activity between human pineal and y79 cells. Proteins were sequentially precipitated with ammonium sulfate (37/68% cuts) and the final precipitate was assayed for HIOMT activity with 50 μM NAS and 50 μM SAM (25 Ci/mol). Decreasing amounts of precipitated proteins from human pineal and Y79 cells, corresponding to the indicated levels of HIOMT activity, were separated by SDS–PAGE on 10% polyacrylamide gels and transferred to PVDF membrane. HIOMT was immunodetected on the Western blot as indicated in Fig. 2. Non-specific bands are indicated by asterisks. Similar results were obtained in 4 independent experiments. For further technical details, see Materials and methods.

3.2. Western blot analysis

Previous studies have established that HIOMT protein is detectable by Western blot techniques in extracts of human pineal glands [11]. In contrast, it was not possible to generate a signal with extracts of human cortex, cerebellum, retina, lung, spleen, heart, thymus, thyroid, liver or Y79 cells using similar (~ 80 μg/lane) amounts of protein; overloading (~ 200 μg/lane) resulted in uninterpretable non-specific patterns (unpubl. obs.).

To enhance the possibility of detecting HIOMT in retina and Y79 cells, ammonium sulfate precipitation was used to obtain HIOMT-enriched preparations; and proteins were detected with an affinity-purified anti-HIOMT antiserum. HIOMT protein was identified as a strong ~ 42-kDa band in human pineal protein and as a weak band of similar size in Y79 extracts (Fig. 2). Preabsorbtion of the antiserum with synthetic HIOMT peptides prevented detection of these ~ 42-kDa signals, indicating these represent specific signals. Densitometric analysis indicated that immunoreactive HIOMT protein in pools of human pineal glands is 15–40 × greater than in Y79 cells. A weak ~ 42-kDa signal was detected in human retinal preparations (Fig. 2). However, this and several other signals were found to be non-specific, i.e. they were also detected using preabsorbed serum. Based on this, it is not possible to

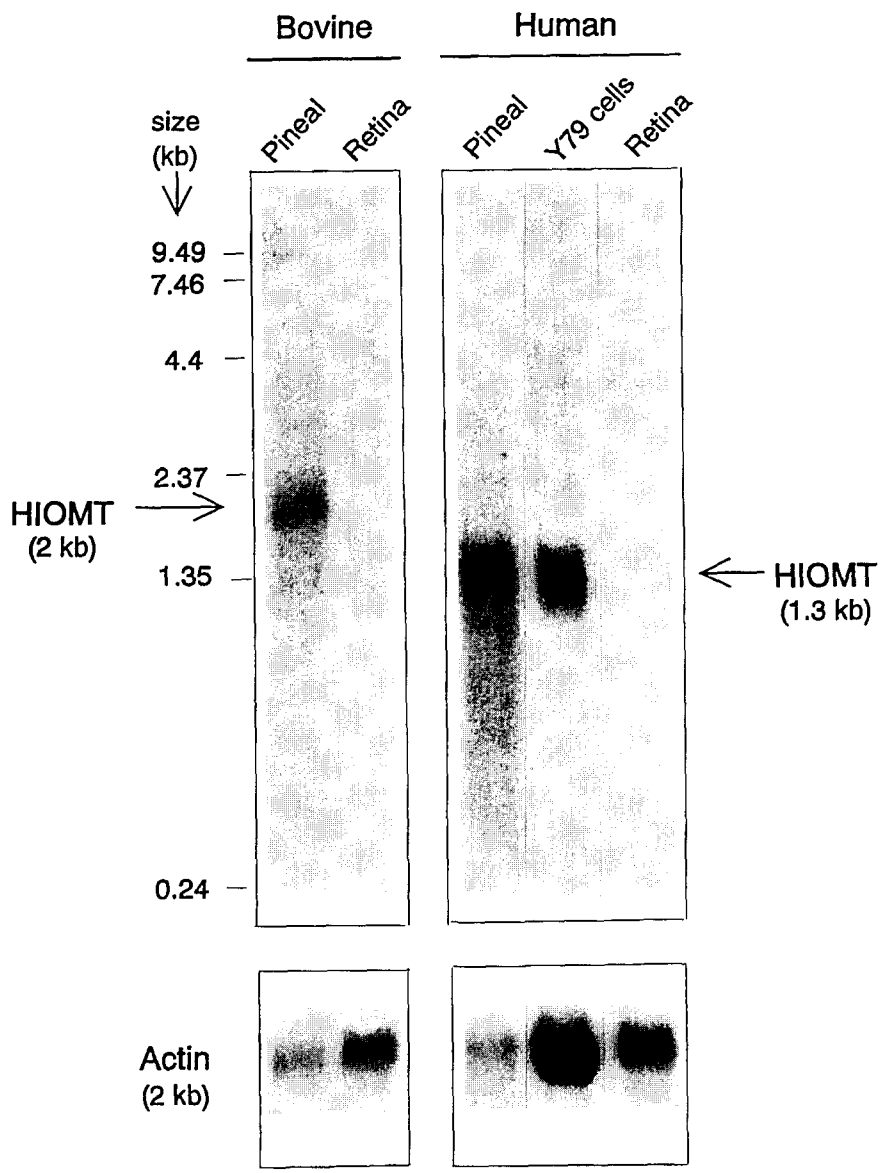

Fig. 4. Northern blot detection of HIOMT mRNA in bovine and human tissues. Total RNA (20 μg per lane) was fractionated on a 1.5% agarose/0.66 M formaldehyde gel and subsequently transferred to nylon membrane by capillary blotting in 20 × SSC. The Northern blot was successively hybridized with a probe corresponding to the 5′ region of human HIOMT cDNA (top panel) and with a cDNA probe encoding for human β-actin (bottom panel). Both probes were labeled with [^{32}P]dCTP by the random priming method and incubated with the blot (10^6 cpm/ml) for 1 h 30 min at 68°C in hybridizing solution (Quikhyb, Stratagene). Washes were performed as indicated in Materials and methods. After each hybridization, the blot was exposed overnight to a PhosphorImager screen. The band size was estimated from the RNA ladder (left of top panel). For further technical details, see Materials and methods.

conclude that HIOMT protein is present in the human retina.

In human retinal and Y79 cell preparations, the affinity-purified anti-HIOMT antiserum detected another protein of higher molecular weight (~ 130 kDa), which was not detected by the preabsorbed serum (Fig. 2). This ~ 130-kDa signal was not present in the human pineal gland nor in any other tissue (data not shown). The lack of correlation of the abundance of this signal with HIOMT activity, the ~ 42-kDa HIOMT signal, or HIOMT mRNA suggests that it is not due to HIOMT expression, but to an unidentified protein with a common epitope. The limited tissue expression of this protein suggests it might reflect a retina-specific protein.

3.3. Comparison of HIOMT enzyme activity and immunoreactivity in human pineal and Y79 cells

A range of ammonium-sulfate precipitated pineal and Y79 protein was analysed by radioenzyme assay and samples containing similar levels of enzyme activity were analysed by Western blot. Pineal extracts were found to contain > 3-fold more immunoreactive protein than samples of Y79 extracts containing the same amount of enzyme activity (Fig. 3). This suggests that pineal HIOMT might have a lower specific activity than Y79 HIOMT or that a greater percentage of HIOMT molecules in pineal extracts are inactive.

3.4. RNA analysis

Ethidium bromide staining indicated that RNA extracted from human and bovine pineal glands and retinae was partially degraded but that RNA from Y79 cells was intact, as judged by the compactness of the bands of ribosomal RNA.

Northern blot analysis using a human HIOMT cDNA probe detected a strong 1.3-kb signal in human pineal and in Y79 cells mRNA (Fig. 4). A 2.0-kb signal was detected in the bovine pineal gland (Fig. 4), consistent with previous reports [13]. This band was also seen using a bovine cDNA probe (data not shown). In contrast, a signal was not detected in extracts of 4 human or 2 bovine retinae. In all cases, an actin signal was detectable (Fig. 4). In addition, hybridization of the same Northern blots with cDNA probes encoding S-antigen and phosducin allowed to detect very strong signals in the human retina and weaker signals in the pineal gland (data not shown). These observations provide evidence that mRNA in retinal extracts was sufficiently intact to detect species encoding selected photoreceptor-related proteins.

The relative amount of HIOMT mRNA in the pineal gland and in Y79 cells was estimated by analysing a range of total RNA by Northern blot (Fig. 5). HIOMT signals of approximately equal strength were generated by ~ 2 μg of total human pineal RNA and ~ 10 μg of total Y79 RNA. Based on this, it would appear that the human pineal gland contains at least 5-fold more HIOMT mRNA. This estimate could be adjusted upward ~ 5-fold by normalization to mRNA-encoding actin (Fig. 5) or G3PDH (data not shown), both of which are 5-fold more abundant in Y79 mRNA. The lower amounts of actin and G3PDH in pineal extracts are consistent with partial degradation.

In previous PCR studies, 3 forms of HIOMT mRNA (A–C) resulting from alternative splicing, appeared to be present in the human pineal [11,28]. In the present study,

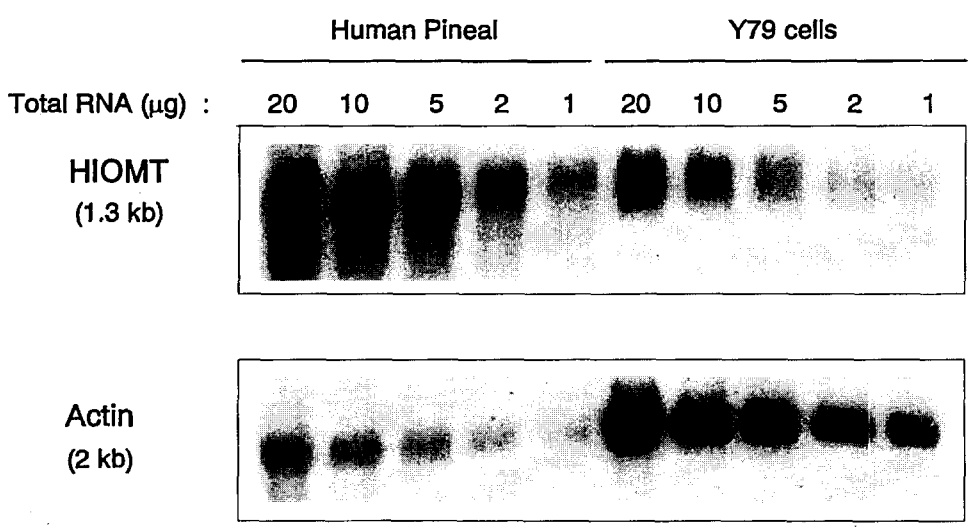

Fig. 5. Comparison of HIOMT mRNA levels between human pineal and Y79 cells. Decreasing amounts of total RNA (20 to 1 μg) from human pineal and Y79 cells were fractionated on a 1.5% agarose/0.66 M formaldehyde gel and subsequently transferred to nylon membrane by capillary blotting in 20 × SSC. The Northern blot was successively hybridized with a probe corresponding to the 5′ region of human HIOMT cDNA (top panel) and with a cDNA probe encoding for human β-actin (bottom panel), as indicated in the legend for Fig. 4. After each hybridization, the blot was exposed overnight to a PhosphorImager screen and the intensity of mRNA bands was estimated by integration of the area under the peaks. For further technical details, see Materials and methods.

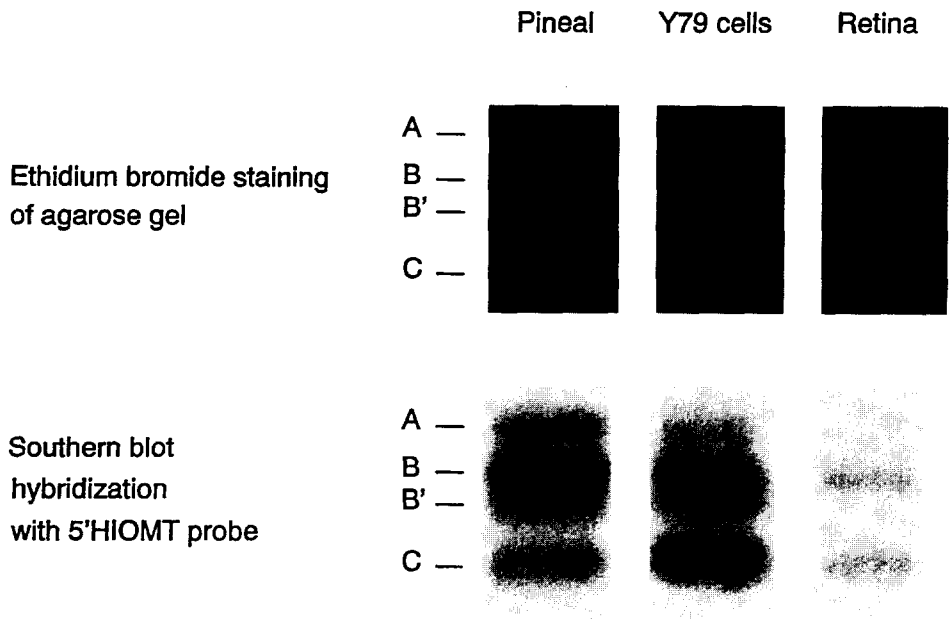

Fig. 6. PCR analysis of HIOMT mRNA in human tissues. cDNA was synthesized by reverse transcription with poly(A +) RNA of human pineal, retina and Y79 cells, and HIOMT mRNA was amplified by PCR using primers located in 5′ (bp 1–22) and 3′ (bp 1074–1054) of human HIOMT cDNA. PCR products were run on a 2.5% NuSieveGTG:SeakemGTG (3:1) agarose gel containing 0.5 μg/ml ethidium bromide and were visualized under UV light (top panel). The DNA was subsequently transferred to nylon membrane and the Southern blot was hybridized with the 5′ HIOMT cDNA probe and exposed overnight to a PhosphorImager screen (bottom panel). The bands size, estimated by comparison with a double-stranded DNA ladder, is: A = 1074 bp, B′ = 990 bp, B′ = 936 bp, C = 849 bp. For further technical details, see section 2.

an additional form (B′) was detected when PCR analysis was performed with primers that flank the region of HIOMT mRNA subject to alternative splicing (Fig. 6). This probably reflects improved electrophoretic resolution. Sequencing of this new PCR product revealed that it differs from form B in that it lacks the last 54 bp of the 3′ portion of exon 3 [11,28]. This new HIOMT isoform did not seem to be a PCR artifact because it was detected in all 5 individual pineals examined as well as in 2 unrelated pools of glands. Ethidium bromide staining revealed that the 4 HIOMT mRNA isoforms are present in Y79 cells, but not in human retina (Fig. 6). However, a more sensitive Southern blot analysis of the PCR products with the 5′ HIOMT probe revealed that 3/4 forms of HIOMT mRNA are present in the retina (Fig. 6), as has been reported [28].

The relative abundance of the 4 forms of HIOMT mRNA appeared to differ somewhat between tissues (Fig. 6). Form B was dominant in the human pineal gland, based on the analysis of 5 glands. In contrast, form C was dominant in each of 5 pools of Y79 cells examined (Fig.

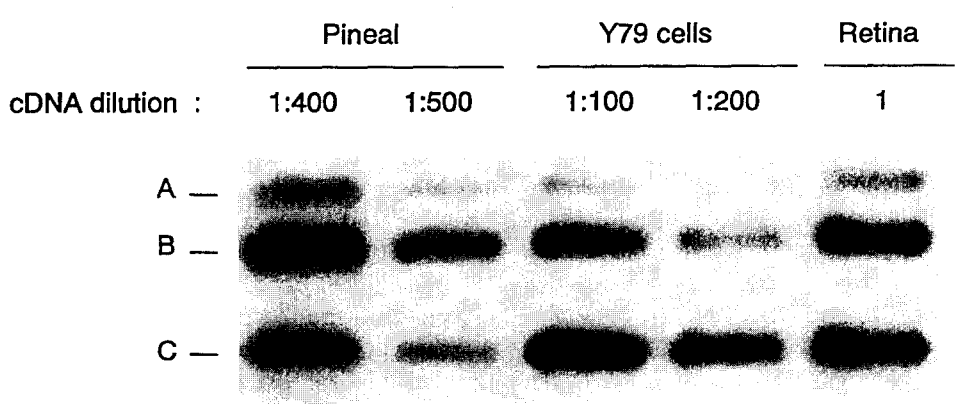

Fig. 7. Comparison of HIOMT mRNA levels in human pineal, retina and Y79 cells by Southern blot analysis of PCR products. cDNA was synthesized by reverse transcription with poly (A +) RNA of human pineal, retina and Y79 cells, and was diluted as indicated, before being used for PCR amplification. PCR was performed as described in legend to Fig. 6. Products were run on a 2.5% NuSieveGTG:SeakemGTG (3:1) agarose gel, transferred to nylon membrane and hybridized with the 5′ HIOMT cDNA probe. The Southern blot was exposed overnight to a PhosphorImager screen. Intensity of the different bands was measured by integration of the area under each peak. For further technical details, see section 2.

6). Forms B and C were nearly equally dominant in the human retina; A was weaker and B′ was undetectable (Fig. 6).

As indicated above, HIOMT mRNA could be identified on Northern blots with only 2 μg of total RNA from Y79 cells (Fig. 5), and was undetectable with 20 μg of total RNA from normal human retina (Fig. 4), indicating it is at least 10-fold more abundant in Y79 cells. To obtain another estimate of the relative abundance of HIOMT mRNA, PCR amplification of HIOMT was performed with a range of dilutions of cDNA. PCR products were subsequently detected on Southern blot by hybridization with the 5′ HIOMT probe. The intensity of the signal generated by undiluted retinal cDNA was the same as that generated by a 1:400 dilution of human pineal cDNA and a 1:100 dilution of Y79 cells cDNA (Fig. 7). Based on this, it appears that the amount of HIOMT mRNA present in the human pineal gland may be 400-fold greater than that in the retina and 4×; than that in Y79 cells, which is consistent with the Northern blot estimate without normalization to the actin signal. However, as indicated above, these differences are likely to be distorted by partial degradation of mRNA from human pineal and retina, and should be interpreted with caution. It should be noted that form B′ was not detectable by PCR amplification with diluted cDNA (Fig. 7), possibly reflecting a physical difference of this isoform leading to greater instability or lower amplification.

Taken together, these observations indicate that the average relative abundance of HIOMT mRNA in these tissues is pineal > Y79 > > > retina. In addition, all 3 contain multiple isoforms of mRNA-encoding HIOMT, the relative abundance of which differs among these tissues.

Table 3
Summary of results

	Pineal	Y79 cells	Retina
HIOMT protein			
Enzyme activity [1]			
V_{max} *	~ 8	0.6	NA
K_m (SAM)	27 μM	25 μM	NA
K_m (NAS)	7 μM	~ 2 μM	NA
Western blot [2]			
Molecular weight	~ 42 kDa	~ 42 kDa	ND
Immunoreactive Protein/mg tissue	15–40	1	ND
HIOMT mRNA			
Northern blot			
Size of transcript [3]	1.3 kb	1.3 kb	ND
Relative amount [4]	5–25	1	ND
PCR [5]			
Relative abundance			
of isoform A	+ + +	+ +	+
B	+ + + +	+ + +	+
B′	+ + +	+ + +	+ /-
C	+ + +	+ + + +	+

Values given are taken from data presented in figures and text. Ranges of values for human pineal HIOMT represent pools and do not reflect large tissue-to-tissue variation which exists
[1] From Fig. 1. [2] From Fig. 2. [3] From Fig. 4. [4] From Fig. 5. [5] From Fig. 6. * nmol/h/mg protein. ND, not detectable; NA, not analysed because enzyme activity was not detected.

4. Discussion

4.1. Characteristics of human pineal HIOMT

Our studies have revealed that human pineal HIOMT is generally similar to chicken, bovine and rat pineal HIOMT, although some differences exist in K_m values. In most cases, HIOMT exhibits substrate inhibition by NAS. This effect is strongest with HIOMT from chicken [20], and is seen with Y79 HIOMT to a greater degree than with human pineal HIOMT. The basis of this substrate inhibition is not clear. It is questionable whether it has any physiological significance, because inhibition occurs above physiological concentrations of NAS [19,21]. However, although substrate inhibition may have no physiological significance, it is of practical importance in detecting HIOMT activity. For example, it is clear that optimal conditions for the detection of Y79 HIOMT are not provided by the standard assay used for rat HIOMT. Rather, an assay with lower NAS is required to minimize inhibition.

Comparison of 10 individual human pineal glands revealed two interesting features of HIOMT. First, it appears that there is significant variation on a gland-to-gland basis, as first indicated by Western blot analysis [11]. This may be linked to the unexplained large individual-to-individual variation in melatonin production seen among human subjects [1,2]. However, the small number of samples obtained and the lack of data on the corresponding circulating melatonin makes it impossible to draw a firm conclusion about this.

The second feature of human HIOMT which is apparent from this analysis is that the enzyme appears to be relatively stable in the gland after death, because there was no strong correlation between HIOMT activity levels and postmortem delay. Previous studies have also indicated that this enzyme is stable in Y79 cells [14] and in the chicken pineal gland (M. Bernard, unpubl. obs.).

4.2. HIOMT in human retina

One unequivocal indication that the human HIOMT gene is expressed in retina comes from one of the most sensitive techniques available to detect mRNA, i.e. Southern blot analysis of RT-PCR products. This was first demonstrated by Rodriguez et al., [28] and confirmed here. All other techniques used failed to detect expression of the HIOMT gene in the retina (Table 3). One might argue that the reason why HIOMT activity and the ~ 42-kDa immunoreactive signal were not detected is that the enzyme

is unstable and was degraded. However, as discussed above, HIOMT appears to be relatively stable. One might also argue that human retinal mRNA was nearly completely degraded. However, this does not appear to be the case, based on the analysis of other species of mRNA in retinal extracts. Accordingly, it seems that the HIOMT gene is expressed at extremely low levels in the human retina, as also seems to be the case in the bovine retina [9]. When taken together, the low levels of HIOMT mRNA and the undetectable levels of HIOMT protein place in doubt the capacity of the human and bovine retinae to synthesize melatonin.

Absence or very low expression of HIOMT in the retina would indicate that any melatonin-regulated aspect of retinal function would be partially or fully dependent upon pineal-derived melatonin [26]. If this were the case, then it might be of special importance to provide melatonin to individuals who have low circulating melatonin.

4.3. Expression of HIOMT gene in Y79 cells

Comparative analysis of HIOMT in human pineal and Y79 cell extracts revealed that the 4 isoforms of HIOMT mRNA found in the human pineal gland are also present in the Y79 cells. In addition, Western blot analysis indicated that both tissues contain ~42-kDa HIOMT immunoreactive protein; and, kinetic analysis revealed that kinetic characteristics of HIOMT are similar in both tissues (Table 3). Accordingly, it appears that Y79 cells represent a valid model to study human HIOMT expression.

It is important to note that some differences were also apparent. First, all indices of HIOMT gene expression were distinctly lower in the Y79 cells. This may reflect the existence of an important regulatory link that is absent from Y79 cells. Second, the Y79 enzyme clearly exhibits greater NAS-dependent substrate inhibition. Third, it appears that specific activity of Y79-derived HIOMT is higher. However, as discussed in Results, this might be due to the presence of inactive HIOMT molecules in the human pineal gland. This might arise from postmortem inactivation of the enzyme or might be a reflection of the in vivo situation. The possibility of actual differences in specific activity is not unreasonable, and should be investigated in greater detail. It might reflect differences in posttranslational processing of HIOMT or in the level of expression of each HIOMT mRNA isoform in Y79 cells and the human pineal. Such differential expression of HIOMT isoforms might also contribute to individual-to-individual differences in HIOMT activity.

It is puzzling that the level of HIOMT expression is higher in the Y79 retinoblastoma cell line than in the retina. This observation suggests that factors promoting differentiation for melatonin synthesis are expressed differently in the retinoblastoma cells as compared with normal receptor cells, resulting in a cell which is more pineal-like in character. This raises the possibility that Y79 cells have the potential to be induced to express HIOMT at higher levels. Future research might lead to the identification of agents which control HIOMT expression in Y79 cell. This could also point to a regulatory role for such agents in the mechanisms involved in the neural and developmental regulation of HIOMT in the pineal gland.

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