

Retinoic Acid Increases Hydroxyindole-*O*-Methyltransferase Activity and mRNA in Human Y-79 Retinoblastoma Cells

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Abstract: Hydroxyindole-*O*-methyltransferase (HIOMT) plays an important role as the final enzyme in the synthesis of melatonin. Here we present the first evidence that retinoic acid (RA) stereoisomers are potent regulators of HIOMT in the human retinoblastoma-derived Y-79 cell line. Treatment with all-*trans*-, 13-*cis*-, and 9-*cis*-RA induced a gradual 10-fold increase in HIOMT activity and mRNA, without changing the levels of mRNA encoding glyceraldehyde-3-phosphate dehydrogenase, actin, S-antigen, and interphotoreceptor retinoid-binding protein. These findings point to the possibility that RA may play a physiological role in the regulation of human HIOMT. **Key Words:** Hydroxyindole-*O*-methyltransferase—Melatonin—Y-79 retinoblastoma—Retinoic acid. *J. Neurochem.* **67**, 1032–1038 (1996).

Melatonin plays a central role in circadian biology and seasonal reproduction. It is synthesized on a rhythmic basis as follows: tryptophan → hydroxytryptophan → serotonin → *N*-acetylserotonin (NAS) → melatonin. The last enzyme in this pathway is hydroxyindole-*O*-methyltransferase (HIOMT; EC 2.1.1.4; Axelrod and Weissbach, 1960; Sugden et al., 1986). HIOMT is expressed at high levels in the pineal gland, the source of circulating melatonin (Axelrod and Weissbach, 1961; Axelrod et al., 1961), and is also found to a variable degree in the vertebrate retina (Baker et al., 1965; Quay, 1965; Cardinali and Rosner, 1971; Voisin et al., 1988). Melatonin synthesized in the vertebrate retina is thought to act as a local modulator, which influences pigment aggregation (Pang and Yew, 1979), rod shedding (Besharse and Dunis, 1983), dopamine release (Dubocovich, 1983), and retinomotor movements (Pierce and Besharse, 1985).

HIOMT is also expressed in the human retinoblastoma-derived Y-79 cell line, which expresses a set of genes typically found only in the pineal gland and retina (Kyritsis et al., 1985; Bogenmann et al., 1988; Pierce et al., 1989; Janavs et al., 1991; Bernard et al., 1995). This cell line is the only one available that expresses these genes and is a useful model to study their regulation.

Little is known about the factors regulating HIOMT expression in mammals. Here we examined the effects of retinoic acid (RA) on the expression of HIOMT in Y-79 cells. RA has been shown to induce the differentiation of tumor cell lines, and also to play a major role in normal development (De Luca, 1991; Linney, 1992). Studies on the rat retina have shown that RA triggers the differentiation of embryonic retinal cells into photoreceptors, as indicated by the induction of opsin and recoverin (Kelley et al., 1994). The results of our study demonstrate that RA stimulates the expression of the melatonin-synthesizing enzyme HIOMT.

EXPERIMENTAL PROCEDURES

Cell culture

Y-79 human retinoblastoma cells (American Type Tissue Culture, Rockville, MD, U.S.A.) were maintained in suspension culture at 37°C (95% air, 5% CO₂) in RPMI 1640 medium (Biofluids, Rockville, MD, U.S.A.) supplemented with 2 mM glutamine, 10% fetal calf serum (Biofluids), 100 U/ml penicillin, and 100 µg/ml streptomycin. The density of cells was 0.5–1 × 10⁶ cells/ml.

For experimental treatments, cells were cultured in RPMI 1640 supplemented with bovine serum albumin (1 mg/ml; BSA) and a mixture of insulin (10 µg/ml), transferrin (5 µg/ml), and sodium selenite (6.7 ng/ml) (ITS supplement, GibcoBRL, Gaithersburg, MD, U.S.A.). Unless otherwise indicated in the figure legends, Y-79 cells were cultured for 3 days in this medium before starting the pharmacological treatments. Retinol, 9-*cis*-retinal, all-*trans*-RA, 13-*cis*-RA, ergocalciferol, and cholecalciferol were from Sigma (St. Louis, MO, U.S.A.); 9-*cis*-RA was a gift from Hoffmann-

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Abbreviations used: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HIOMT, hydroxyindole-*O*-methyltransferase; IRBP, interphotoreceptor retinoid-binding protein; NAS, *N*-acetylserotonin; RA, retinoic acid; RARE, RA-responsive element; RXR, retinoid-X receptor; SDS, sodium dodecyl sulfate; SSC, saline-sodium citrate.

La Roche (Nutley, NJ, U.S.A.). All drugs were dissolved in dimethyl sulfoxide (DMSO) and were stored as 50- μ l aliquots at -20°C . The final concentration of DMSO in the medium was 0.005%.

HIOMT assay

HIOMT activity was determined radiochemically by incubating (30 min; 37°C) a cell extract prepared from $\sim 10^6$ cells (~ 150 μg of protein) with 50 μM NAS and 2.5 μM S-[methyl- ^3H]adenosyl-L-methionine (500 Ci/mol; Axelrod and Weissbach, 1960; Sugden et al., 1986; Bernard et al., 1996). After 30 min of incubation at 37°C , the [^3H]melatonin formed was extracted in chloroform, as previously described (Sugden et al., 1986). The chloroform was taken to dryness, scintillation fluid (Bio-Safe II, RPI, Mount Prospect, IL, U.S.A.) was added, and radioactivity was determined by liquid scintillation on a β -counter (Beckman, Fullerton, CA, U.S.A.).

Protein assay

Protein was measured using a dye-binding method (Bradford, 1976) with BSA as standard.

Northern blot analysis of RNA

Extraction of mRNA. Total RNA was isolated using Trizol reagent as described by the manufacturer (GibcoBRL, Gaithersburg, MD, U.S.A.). Total RNA was fractionated on 1.5% agarose/0.66 M formaldehyde gel and subsequently transferred to nylon membrane by capillary blotting with $20\times$ saline-sodium citrate (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, U.S.A.; 120 mJ, 35 s).

Probes. The cDNA probes for human HIOMT, human S-antigen (arrestin), and human phosphodiesterase (G3PDH) (Clontech, Palo Alto, CA, U.S.A.) were used to monitor the quality of RNA preparations and to normalize the HIOMT signal. A 472-bp probe for human interphotoreceptor retinoid-binding protein (IRBP) was prepared by PCR amplification of human retinal cDNA using primers specific for the human IRBP-coding sequence (GenBank/EMBL Data Bank, accession no. M22453): 5' primer = CACCAACCTCTACCTCACTATCCCC (bp 3,670–3,694); 3' primer = TGGTAGAACTTGGGGTGGTATTTT (bp 4,141–4,117). A 327-bp probe for human opsin was prepared by PCR amplification of human retinal cDNA using primers specific for exons 4 and 5 of the human opsin gene (GenBank/EMBL Data Bank, accession no. K02281): 5' primer = GCAGCAGGAGTCAGCCACCA (bp 4,106–4,125, exon 4); 3' primer = CCTGGCTCGTCTCCG-TCTTG (bp 5,264–5,245, exon 5). The identity of PCR-generated probes was verified by partial sequencing; in all cases, these probes generated signals of predicted sizes on northern blots of human retina or pineal mRNA. Probes were labeled with [α - ^{32}P]dCTP (3,000 Ci/mmol) by random priming (Megaprime kit; Amersham, Arlington heights, IL, U.S.A.).

Analysis. Blots were incubated in prehybridizing solution (Quikhyb; Stratagene, La Jolla, CA, U.S.A.) for 15 min at 68°C , ^{32}P -labeled probe was added (10^5 cpm/ml), and the 68°C incubation was continued for 1.5 h. After hybridization, the blots were washed twice (15 min at room temperature) in $2\times$ SSC/0.1% sodium dodecyl sulfate (SDS) and once

TABLE 1. HIOMT activity is stimulated by vitamin A derivatives, but not by vitamin D

Treatment	Concentration (μM)	HIOMT activity (pmol/h/ 10^6 cells)	Fold increase
None		4.3 ± 0.5	1.0
All- <i>trans</i> -Retinol (vitamin A)	0.3	5.9 ± 0.5	1.4
	1	6.0 ± 0.1	1.4
9- <i>cis</i> -Retinal	0.3	9.3 ± 0.5	2.2
	1	13.1 ± 1.3	3.0
All- <i>trans</i> -RA	0.3	41.9 ± 2.4	9.7
	1	40.6 ± 2.7	9.4
13- <i>cis</i> -RA	0.3	47.0 ± 6.1	10.9
	1	46.4 ± 3.8	10.8
9- <i>cis</i> -RA	0.3	49.8 ± 3.8	11.6
	1	48.1 ± 3.7	11.2
Ergocalciferol (vitamin D ₂)	0.3	3.7 ± 0.3	0.9
	1	3.5 ± 0.2	0.8
Cholecalciferol (vitamin D ₃)	0.3	4.2 ± 0.3	1.0
	1	3.7 ± 0.3	0.9

Y-79 cells were incubated for 3 days with the compounds listed below. HIOMT activity was measured in extracts of 10^6 cells (~ 150 μg of protein) using a radioenzymatic assay. Data are expressed as mean \pm SEM ($n = 3$) values. For further details, see Experimental Procedures.

(30 min at 60°C) in $0.1\times$ SSC/0.1% SDS. Blots were exposed overnight to a PhosphorImager screen, and band intensities were determined by integration of area under the peaks (Image Quant; Molecular Dynamics, Sunnyvale, CA, U.S.A.). Typically, blots were first hybridized with 5'-HIOMT probe and subsequently stripped (2×15 min in $\sim 100^{\circ}\text{C}$ water) before hybridization with G3PDH. In some cases, blots were stripped and subsequently incubated with another probe.

RESULTS

Effects of RA and related compounds on HIOMT expression

The relative effects of RA and six related compounds (0.3 and 1 μM) on HIOMT activity were determined (Table 1). Neither all-*trans*-retinol (vitamin A), ergocalciferol (vitamin D₂), nor cholecalciferol (vitamin D₃) increased HIOMT activity. A two- to three-fold stimulation was obtained with 9-*cis*-retinal, a stereoisomer of the natural vitamin A derivative 11-*cis*-retinal. In contrast, HIOMT activity increased ~ 10 -fold after a 3-day treatment with all-*trans*-RA, 13-*cis*-RA, or 9-*cis*-RA. This did not reflect an increase in cell number, because treatment with RA did not change the doubling rate.¹

Concentration dependence

Concentration-dependency analysis (Fig. 1) revealed that all-*trans*-RA, 13-*cis*-RA, and 9-*cis*-RA had similar stimulatory effects on both HIOMT mRNA

¹ We did not find that RA suppressed proliferation of Y-79 cells, as reported (Kyritsis et al., 1984, 1986). This may reflect the 50- to 100-fold lower concentrations of RA we used.

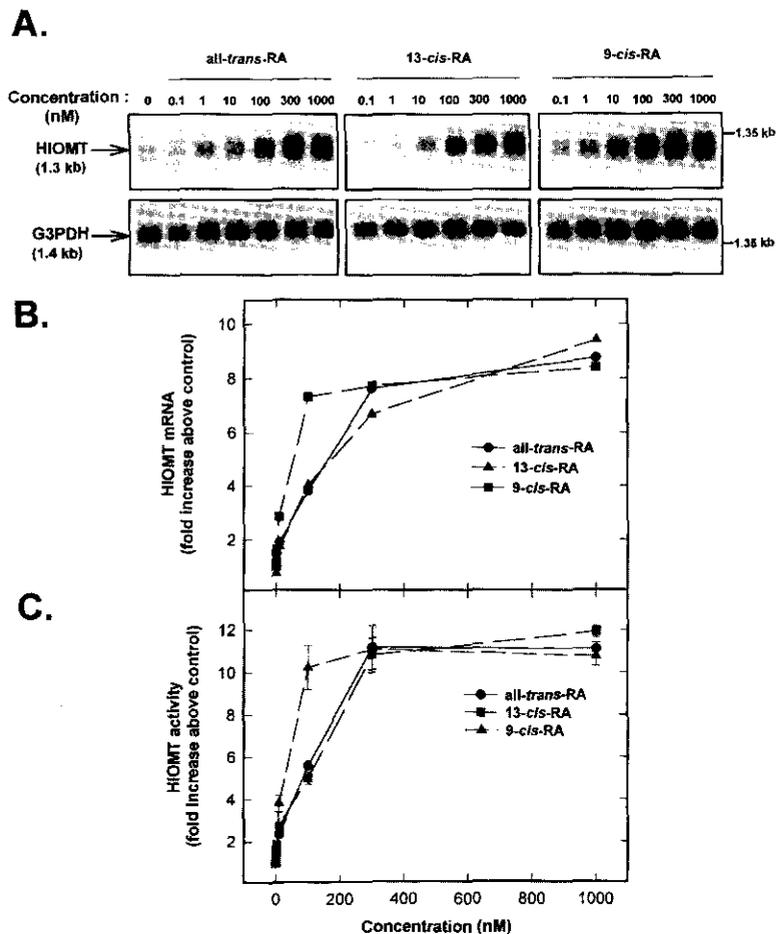


FIG. 1. RA increases the levels of HIOMT mRNA and enzyme activity in a concentration-dependent manner. Y-79 cells were incubated for 3 days with the indicated concentrations of all-trans-RA, 13-cis-RA, or 9-cis-RA. **A:** Representative northern blot analysis of HIOMT and G3PDH mRNAs (10 μ g of total RNA per lane). **B:** HIOMT mRNA normalized to G3PDH mRNA. Data are mean values of duplicate determinations and are expressed as values of fold increase above the control (untreated cells). Individual values were within 25% of the mean. **C:** HIOMT enzyme activity. The conditions of the assay were as described in Table 1. Data are mean \pm SEM ($n = 3$) values and are expressed as values of fold increase above the control. Similar results were obtained in a second experiment. For further details, see Experimental Procedures.

levels (Fig. 1A, B) and enzyme activity (Fig. 1C). The range of effective concentrations (100–300 nM) was similar to that reported for other systems (Linney, 1992). Autoinhibition was not observed for these stereoisomers in the concentration range of 0.1–2 μ M (Fig. 1; unpublished results). The 9-cis-RA isomer was consistently found to be \sim 2.5-fold more effective than all-trans-RA or 13-cis-RA at concentrations of <0.3 μ M, which do not produce maximal stimulation (Fig. 1). In addition, 9-cis-RA (300 nM) appeared to be \sim 2-fold more effective than all-trans-RA or 13-cis-RA in increasing HIOMT mRNA levels in four of five experiments (Figs. 2–5). The effects of maximal concentrations of all-trans-RA and 9-cis-RA on either HIOMT activity or HIOMT mRNA did not appear to be additive (data not shown), suggesting that they may act through the same mechanism.

Temporal characteristics

Time course analysis indicated that maximal stimulation of HIOMT mRNA and HIOMT activity by all-trans-RA, 13-cis-RA, and 9-cis-RA occurred gradually over the course of a 3-day treatment (Fig. 2). In other studies, we found that HIOMT mRNA did not continue to increase during an additional 3 days of

treatment (unpublished data). The increase in HIOMT activity lagged behind that in HIOMT mRNA (Fig. 2C). The rate of increase in HIOMT mRNA was faster with 9-cis-RA than with all-trans-RA or 13-cis-RA (Fig. 2B, also see Fig. 3), but this was not correlated with a faster increase in HIOMT activity (Fig. 2C). This suggests that the rate of increase in active HIOMT protein, after RA treatment, might be limited at the translational or posttranslational level.

The gradual response to RA raised the possibility that the stimulation of HIOMT expression might be due to the induction of another factor that would, in turn, activate the HIOMT gene. If this were the case, a 24-h treatment with all-trans-RA or 9-cis-RA might be sufficient to induce such a hypothetical regulatory protein and consequently stimulate HIOMT expression. However, when this was tested, we found that the continued presence of all-trans-RA or 9-cis-RA was required to produce maximal stimulation; removal of either agent after 1 day of treatment reversed their stimulatory effects (Fig. 3).

Studies on mRNA stability

To determine whether all-trans-RA or 9-cis-RA increases the levels of HIOMT mRNA by enhancing the

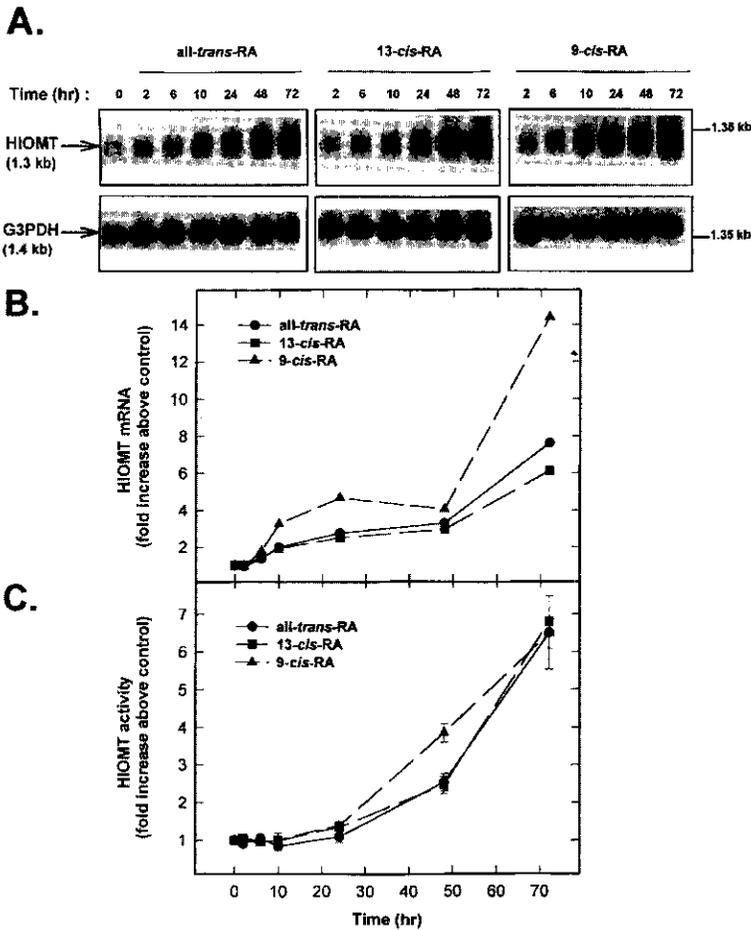
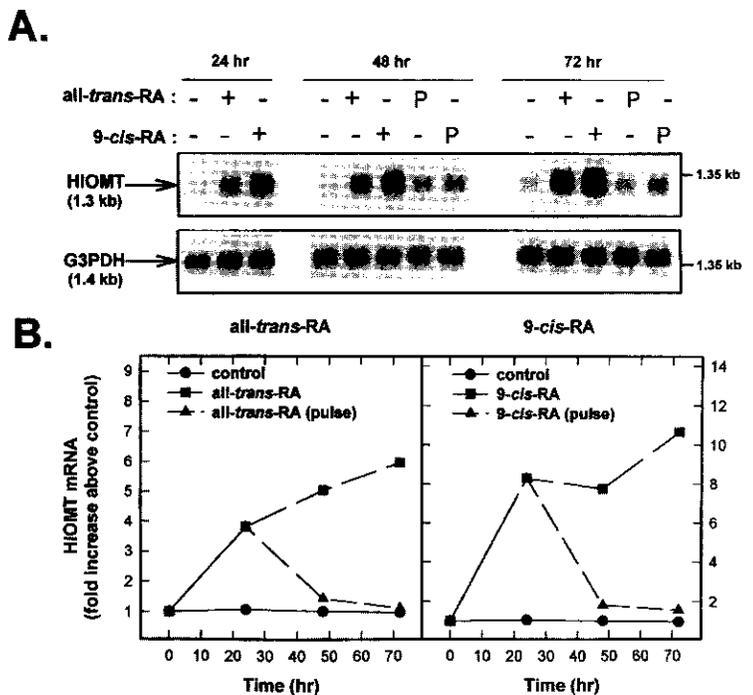


FIG. 2. Time course of the increase in HIOMT mRNA and enzyme activity induced by RA. Y-79 cells were incubated for the indicated times with 300 nM all-trans-RA, 13-cis-RA, or 9-cis-RA. **A:** Representative northern blot analysis of HIOMT and G3PDH mRNAs (10 μg of total RNA per lane). **B:** HIOMT mRNA normalized to G3PDH mRNA. Data are mean values of duplicate determinations and are expressed as values of fold increase above the control (time point zero). Individual values were within 15% of the mean. **C:** HIOMT enzyme activity. The conditions of the assay were as described in Table 1. Data are mean ± SEM (n = 3) values and are expressed as values of fold increase above the control. For further details, see Experimental Procedures.

FIG. 3. The stimulation of HIOMT mRNA expression requires the continuous presence of RA. Y-79 cells were stimulated for 24 h with 300 nM of all-trans-RA or 9-cis-RA. The treatment was either extended for an additional 48 h or cells were transferred to control medium for 48 h (pulse). **A:** Representative northern blot analysis of HIOMT and G3PDH mRNAs (20 μg of total RNA per lane). The letter "P" identifies the cells exposed to the pulse treatment. **B:** HIOMT mRNA normalized to G3PDH mRNA. Data are mean values of duplicate determinations and are expressed as values of fold increase above the control (before starting the pulse treatment). Individual values were within 30% of the mean. The same result was obtained in a second experiment. For further details, see Experimental Procedures.

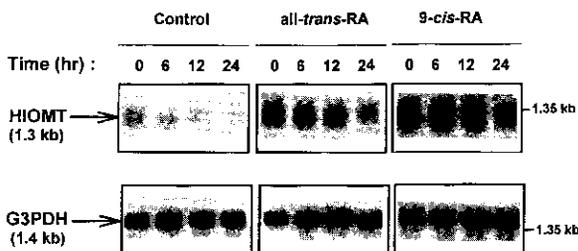


stability of HIOMT mRNA, we measured the disappearance of the HIOMT transcript in control and RA-treated Y-79 cells, after actinomycin D treatment (Fig. 4). The decrease in HIOMT mRNA was found to be the same in control cells and in cells treated with all-*trans*-RA or 9-*cis*-RA, indicating that these agents do not act by stabilizing HIOMT mRNA.

Selectivity of the RA effect

Previous studies have indicated that RA promotes the expression of opsin and recoverin in embryonic rat retinal cells (Kelley et al., 1994). This raises the possibility that RA influences the general differentiation of Y-79 cells by promoting the expression of a variety of pineal/retinal-specific genes. However, this does not appear to be the case because treatment with all-*trans*-RA or 9-*cis*-RA, which elevates HIOMT expression, did not induce expression of opsin, phosducin

A.



B.

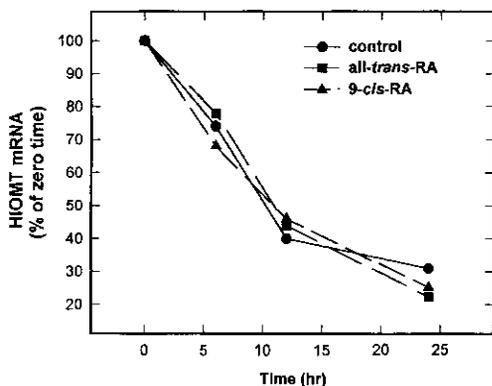


FIG. 4. All-*trans*-RA and 9-*cis*-RA do not influence the rate of disappearance of HIOMT mRNA. Y-79 cells were incubated for 3 days with 300 nM of either all-*trans*-RA or 9-*cis*-RA. After this treatment, transcription was blocked with actinomycin D (5 μ g/ml) for the indicated times, without removing RA if present. **A:** Representative northern blot analysis of HIOMT and G3PDH mRNAs (20 μ g of total RNA per lane). **B:** HIOMT mRNA normalized to G3PDH mRNA. Data are mean values of duplicate determinations and are expressed as percentages of the value before starting the actinomycin D treatment (zero time). Individual values were within 20% of the mean. Because of the greater efficiency of 9-*cis*-RA over all-*trans*-RA, different Y scales have been used for the two panels. The same result was obtained in a second experiment. For further details, see Experimental Procedures.

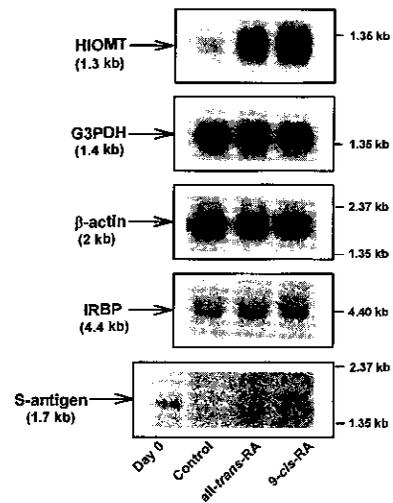


FIG. 5. Selectivity of the stimulatory effect of RA on HIOMT expression. Y-79 cells were incubated for 3 days with 300 nM of all-*trans*-RA or 9-*cis*-RA. The levels of mRNA encoding HIOMT, G3PDH, β -actin, IRBP, and S-antigen were determined by northern blot analysis (20 μ g of total RNA per lane). Day 0: before transferring the cells in serum-free medium. Similar results were obtained in two individual experiments. For further details, see Experimental Procedures.

(unpublished observations), or S-antigen,² nor did this alter expression of mRNA encoding IRBP, G3PDH, and β -actin levels (Fig. 5).

DISCUSSION

The present study identifies for the first time a specific factor that acts *in vitro* to regulate expression of the HIOMT gene and increase HIOMT activity. The effect of RA does not appear to reflect a global change in gene expression in Y-79 cells, because it does not increase the expression of mRNAs encoding photoreceptor proteins, like S-antigen, IRBP, opsin, and phosducin, and it did not affect the expression of actin and G3PDH. Accordingly, from the available data, it appears that RA acts selectively on the expression of HIOMT.

The results of our study indicate that the stereoisomers of RA do not act by increasing the stability of the HIOMT transcript in Y-79 cells. In accordance with this, it appears likely that the effect of RA reflects a stimulation of HIOMT gene transcription. The gradual nature of this stimulation has also been observed for other genes (i.e., laminin; Hosler et al., 1989), which are known to be directly regulated by RA at the transcriptional level.

Our studies indicate that 9-*cis*-RA is twice as potent

² Serum deprivation reduces S-antigen mRNA in Y-79 cells (Bernard et al., 1996) as seen in the experiment presented in Fig. 5. Opsin and phosducin mRNAs were undetectable in control Y-79 cells and in cells treated with 300 nM RA.

as all-*trans*-RA and 13-*cis*-RA in increasing HIOMT mRNA and enzyme activity in Y-79 cells. This potency profile does not unambiguously identify the receptor(s) that mediate(s) this response. The following are two categories of RA receptors that have been shown to mediate the effects of RA: RA receptors (RAR α , β , and γ ; de The et al., 1987; Giguere et al., 1987; Petkovitch et al., 1987; Krust et al., 1989), which can be transactivated by all-*trans*-RA or 9-*cis*-RA (Allenby et al., 1993, 1994), and retinoid-X receptors (RXR α and β ; Mangelsdorf et al., 1990; Leid et al., 1992), which bind 9-*cis*-RA almost exclusively (Heyman et al., 1992; Levin et al., 1992). Although in our study 9-*cis*-RA is slightly more potent than the other isomers tested, it appears unlikely that RXRs are the only receptors involved, because all-*trans*-RA would need to be isomerized into 9-*cis*-RA to be active. Therefore, the difference between the EC₅₀ of 9-*cis*-RA and all-*trans*-RA would probably be greater. Alternatively, the higher potency and efficiency of 9-*cis*-RA may reflect a greater stability of this stereoisomer or the fact that, unlike all-*trans*-RA, it does not bind to cellular RA-binding proteins (Boylan and Gudas, 1991; Allenby et al., 1993).

The finding that RA increases the concentration of HIOMT mRNA in Y-79 cells points to the possibility that RA-responsive elements (RAREs; Linney, 1992) are present in the 5'-noncoding region of the human HIOMT gene. Although consensus RARE elements are not known to be present in the human HIOMT gene (Rodriguez et al., 1994), this does not exclude the existence of such elements in uncharacterized regions of the gene. Alternatively, it is possible that RA does not act directly on the HIOMT gene, but induces the expression of another factor that stimulates HIOMT expression. The results of our study did not completely rule out this possibility.

The finding that RA stimulates the expression of human HIOMT in Y-79 cells is of special interest, because it raises the possibility that RA might also play a role in the regulation of HIOMT gene expression in the normal human pineal and/or retina.

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