

# Retinoic acids promote the action of aromatase and 17 $\beta$ -hydroxysteroid dehydrogenase type 1 on the biosynthesis of 17 $\beta$ -estradiol in placental cells

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## Abstract

The biosynthesis of 17 $\beta$ -estradiol ( $E_2$ ) in human placenta involves the actions of aromatase and 17 $\beta$ -hydroxysteroid dehydrogenase type 1 (17HSD1). Aromatase, an enzyme complex comprised of P450aromatase (P450arom) and NADH-cytochrome P450 reductase, converts androgens to estrogens, whereas 17HSD1 catalyzes the reduction of estrone to  $E_2$ .

In the present study, the effects of retinoic acids (RAs) on P450arom and 17HSD1 expression in placental cells were investigated. Treatment with *all-trans*-RA (*at*-RA) or *9cis*-RA increased  $E_2$  production in JEG-3 chorio-carcinoma cells and cytotrophoblast (CTB) cells isolated from normal early placentas. Meanwhile, the activity of aromatase and expression of P450arom mRNA were induced by *at*-RA in JEG-3 cells. Northern blot analysis showed that the effect on P450arom mRNA expression occurs in a dose- and time-dependent fashion. Similar to *at*-RA and *9cis*-RA, Ro40-6055, the retinoic acid receptor  $\alpha$  (RAR $\alpha$ )-selective activator, increased the expression

of P450arom and 17HSD1 mRNA in JEG-3 cells. On the other hand, Ro41-5253 (Ro41), the RAR $\alpha$ -selective antagonist, blocked the stimulatory effect of RAs on P450arom expression. Surprisingly, Ro41 induced the activity and mRNA expression of 17HSD1 in JEG-3 cells, which is in contrast to the expected inhibitory effect and, moreover, remarkably potentiated the induction by *at*-RA and *9cis*-RA. However, reporter gene analysis revealed that the influence of Ro41 on the transcription of the HSD17B1 gene, which encodes 17HSD1, is considerably milder in JEG-3 cells, and it only additively enhanced the effect of *at*-RA. Finally, it was found that *at*-RA and *9cis*-RA increased the expression of P450arom and 17HSD1 mRNA in CTB cells, but to a lesser extent.

The data suggest that RAs may play a role in promoting the biosynthesis of  $E_2$  in the placenta. In addition, Ro41 has divergent effects on gene expression in JEG-3 cells.

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## Introduction

The placenta is a unique organ of the feto-maternal unit. It not only anchors the fetus physically to the uterus and co-ordinates the dialogue between the embryo and the maternal body, but it also acts as an endocrine unit for the production of the hormones needed by the maternal body and the fetal organs. In the human, the functions of the ovary gradually decline after fertilization and, concurrently, the placenta becomes the primary site of estrogen biosynthesis during pregnancy. By 7 weeks of gestation, nearly all estrogens in the circulation are produced by the placenta (Simpson & MacDonald 1981). Unlike the ovary, the human placenta is not capable of *de novo* estrogen synthesis from cholesterol because of the absence of the 17 $\alpha$ -hydroxylase/C<sub>17–20</sub>lyase, which hydroxylates C<sub>21</sub> steroids at position C<sub>17</sub>. Alternatively, it uses fetal-derived

androgens, such as dehydroepiandrosterone (DHEA) and DHEA sulfate, as precursor molecules to synthesize estrogens (Albrecht & Pepe 1990, Conley & Mason 1990).

The biosynthesis of estrogens in the human placenta occurs in trophoblast cells, involving the action of two steroidogenic enzymes: aromatase and 17 $\beta$ -hydroxysteroid dehydrogenase type 1 (17HSD1). Aromatase catalyzes the aromatization of androgens to estrogens, for example, androstenedione (A-dione) to estrone ( $E_1$ ) and testosterone to 17 $\beta$ -estradiol ( $E_2$ ), the active form of estrogens, while 17HSD1 is responsible for the conversion of  $E_1$  to  $E_2$ . Both enzymes are abundantly expressed in syncytiotrophoblast (STB) cells, the most active endocrine cell type in the placenta (Fournet-Dulguerov *et al.* 1987).

Aromatase is an enzyme complex that consists of two microsomal proteins: P450aromatase (P450arom)

and NADH-cytochrome P450 reductase. The non-steroidogenic cells transfected with the full-length P450arom cDNA are capable of aromatization of various C<sub>19</sub> androgens to estrogens (Corbin *et al.* 1988, Pompon *et al.* 1989). Also, the dynamic changes in the P450arom mRNA level correlate well with aromatase activity (Evans *et al.* 1987). On the other hand, the reductase component of the aromatase complex is only modestly regulated (Steinkampf *et al.* 1987). These results indicate that aromatase activity is primarily determined by P450arom.

P450arom is encoded by the CYP19 gene, which contains 10 exons and 9 introns (Means *et al.* 1989). Compared with the other members of the P450 gene superfamily, the CYP19 gene is exceptionally large, over 250 kb in size. In the human, P450arom is expressed in various tissues, such as the ovary, placenta and adipose tissue (Means *et al.* 1991, Jenkins *et al.* 1993, Kilgore *et al.* 1993, Mahendroo *et al.* 1993, Toda & Shizuta 1993). Selective use of distinct tissue-specific promoters and the subsequent post-transcriptional process using alternative polyadenylation signals at the 3'-ends of transcripts result in tissue-specific expression of a number of P450arom mRNA species (Simpson *et al.* 1994).

The gene encoding 17HSD1 is HSD17B1 (previously also called EDH17B2) containing two transcription start sites, which generate 1.3 and 2.3 kb mRNA species (LuuThe *et al.* 1990, Peltoketo *et al.* 1992). Of these two transcripts, only the 1.3 kb mRNA is evidently believed to be translated into 17HSD1 protein (Miettinen *et al.* 1996). Our recent studies demonstrated that the transcription of the HSD17B1 gene is controlled by a promoter, a silencer and an enhancer located in the 5'-flanking region (Piao *et al.* 1995, 1997a, Leivonen *et al.* 1999). The cell specificity of the enhancer determines the cell-specific expression of the HSD17B1 gene (Piao *et al.* 1995).

In addition to being nutritional signals, derivatives of vitamin A called retinoids have been suggested to affect the function of placental trophoblast cells (Matsuo & Strauss 1994). A number of studies have shown that retinoids regulate the biosynthesis of several placental hormones, such as progesterone, human chorionic gonadotropin (hCG) subunits, and placental lactogen (Kato & Braunstein 1991, Matsuo & Strauss 1994, Stephanou & Handwerger 1995, Guibourdenche *et al.* 1998a,b). Also, retinoids induce leptin synthesis and secretion in STB cells (Guibourdenche *et al.* 1998c). Our previous work demonstrated that retinoic acids (RAs) induce 17HSD1 expression in choriocarcinoma cells (Piao *et al.* 1997b). In addition, a recent report showed that they have a stimulatory effect on aromatase activity in breast cancer cells (Mu *et al.* 2000). These data prompted us to further examine whether RAs affect E<sub>2</sub> biosynthesis in placental cells. In the present study, we examined the effects of RAs on E<sub>2</sub> biosynthesis in cultured human cytotrophoblast (CTB) cells and the JEG-3 choriocarcinoma cell line derived from human trophoblast. JEG-3 cells retain the

capability of converting C<sub>19</sub> steroids to estrogens (Bahn *et al.* 1981) and have been widely used for the study of the production of placental hormones. We have focused on how RAs, either independently or in combination with other factors, affect the expression of P450arom and 17HSD1. Our data suggest that RAs may promote the biosynthesis of E<sub>2</sub> in the placenta.

## Materials and Methods

### Chemicals and reagents

The isotopes [2,4,6,7-<sup>3</sup>H]E<sub>1</sub> (101 Ci/mmol), [2,4,6,7-<sup>3</sup>H]E<sub>2</sub> (98 Ci/mmol) and [1β-<sup>3</sup>H]A-dione (24 Ci/mmol) were obtained from Amersham Life Science (Little Chalfont, Bucks, UK). [<sup>3</sup>H]Acetyl coenzyme A (200 mCi/mmol, CAT assay grade) was from Du Pont NEN (Boston, MA, USA). [α-<sup>32</sup>P]dCTP (3000 Ci/mmol) was purchased from Yuhui Biomedical Engineering Inc. (Beijing, China). Epidermal growth factor (EGF), 8-bromo-adenosine-3',5'-monophosphate, cyclic, free acid (8Br-cAMP), N-[(1-(2,3-dioleoyloxy)propyl)]-N,N,-trimethyl-ammoniummethylsulfate (DOTAP) and chloramphenicol acetyl transferase (CAT) were the products of Boehringer Mannheim (Mannheim, Germany). Trypsin, DNase I, fetal calf serum (FCS), bovine serum albumin (BSA), *all-trans* RA (*at*-RA), interleukin-1β (IL-1β), bovine insulin, DHEA, A-dione, E<sub>1</sub> and E<sub>2</sub> were from Sigma Chemical Co. (St Louis, MO, USA). 12-O-tetradecanoylphorbol-13-acetate (TPA) was supplied by Calbiochem-Novabiochem Inc. (La Jolla, CA, USA). AMV reverse transcriptase, Taq DNA polymerase, RNasin and restriction enzymes were obtained from Promega Co. (Madison, WI, USA). TRIZol reagent, nick translation system and media for cell culture were purchased from Gibco BRL-Life Technologies (Grand Island, NY, USA). Plates for cell culture were obtained from Nunc Inter Med (Roskilde, Denmark). *9cis*-RA, the selective retinoic acid receptor (RARα) activator Ro40-6055 (Ro40) and inhibitor Ro41-5253 (Ro41) were gifts from F Hoffmann-La Roche Ltd (Basel, Switzerland). The antiserum against E<sub>2</sub> was raised in this laboratory and has been used in previous studies (Zhuang & Li 1991, Rong-Hao *et al.* 1996). The plasmid containing human P450arom cDNA was provided by Professor Olavi Pelkonen (Department of Pharmacology, University of Oulu, Finland).

### Cell culture

JEG-3 choriocarcinoma and T47D breast cancer cell lines were obtained from the American Type Culture Collection (Rockville, MA, USA). JEG-3 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS, 2 mM glutamine,

1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 µg/ml streptomycin sulfate. T47D cells were cultured in RPMI 1640 medium containing 10% FCS, 1 mM non-essential amino acids and 0.2 IU insulin/ml. Before each experiment, the cells were detached with 0.025% trypsin–0.02% EDTA. JEG-3 cells were applied to 24-well plates ( $2 \times 10^5$  cells/well) for  $E_2$  detection, 6-well plates ( $2.5 \times 10^5$  cells/well) for the measurement of aromatase and reductive 17HSD activities, and 10 cm plates ( $2.5 \times 10^6$  cells/plate) for Northern blot analysis. The cells were allowed to grow for 24 h and the media were then replaced with fresh ones containing 5% FCS treated twice with dextran-coated charcoal (DCC–FCS), followed by culturing for a further 4 h before treatment. After that, the cells were subjected to various stimuli in media with 5% DCC–FCS for the time indicated before the cells and media were collected for different assays.

The normal human placental tissues of 7–9 weeks of gestation were obtained from Beijing Haidian Hospital with the permission of the local ethics committee and the informed consent of the patients. The method for isolating and culturing CTB cells has been described in our recent studies (Xu *et al.* 2000, 2001). Briefly, the chorionic villi were collected from placental tissues and minced to 1 mm<sup>3</sup> in size, followed by digestion of the samples with 0.2% trypsin–10 units/ml DNase I at 7–9 °C for 1 h. The remaining undigested fragments were removed by filtration through a sieve (120 mesh). After gravity sedimentation through a BSA density gradient, the purified CTB cells were applied to type I collagen-coated 24-well plates ( $2 \times 10^5$  cells/well) for the detection of  $E_2$  biosynthesis, and 60 cm plates ( $2 \times 10^6$  cells/plate) for Northern blot analysis. The cells were cultured in FD medium (Ham's mixed nutrients F12:DMEM=1:1) containing 10 ng/ml EGF, 10 µg/ml insulin, 2 mM glutamine and 0.1% BSA for 36 h and then exposed to the same medium containing different stimuli for the time indicated.

#### *Analysis of the conversion of DHEA/A-dione to $E_2$ in cultured cells*

After treatment with various stimuli for 24 h, JEG-3 and CTB cells cultured in 24-well plates were washed twice with serum-free media (DMEM for JEG-3 cells and FD for CTB cells). Then, 1.0 ml of the same media containing 500 nM DHEA or A-dione was added to each well. The cells were incubated for 1 h at 37 °C in cell culture conditions. After that, the media were collected and  $E_2$  concentration was determined by radioimmunoassay as described previously (Zhuang & Li 1991).

#### *Measurement of aromatase and reductive 17HSD activities in cultured cells*

Aromatase activity was assessed by measuring the tritiated  $H_2O$  produced by the specific release of tritium from

[1 $\beta$ -<sup>3</sup>H]A-dione (Thompson & Siiteri 1974). In brief, JEG-3 cells, after exposure to various stimuli for the time indicated, were incubated in serum-free DMEM containing  $1.0 \times 10^5$  c.p.m. [1 $\beta$ -<sup>3</sup>H]A-dione and 0.2 µM cold A-dione for 1 h at 37 °C. The reaction was stopped by adding 0.5 ml 30% trichloroacetic acid to the media and then the plates were placed in an ice bath for 10 min. After that, the media were extracted with 5 ml diethyl ether–ethyl acetate (9:1) twice, for 15 min each time. A 900 µl aliquot of the aqueous phase was measured in a scintillation counter.

Reductive 17HSD activity was determined by calculating the conversion rate of  $E_2$  to  $E_1$ . After JEG-3 cells had been stimulated for 24 h, the media were removed from the 6-well plates and 2 ml serum-free medium containing 500 nM unlabeled  $E_1$  and  $2.5 \times 10^6$  c.p.m. of [2,4,6,7-<sup>3</sup>H] $E_1$  was added to each well. The cells were then incubated for 1 h at 37 °C in cell culture conditions and the subsequent steps followed the method described previously (Miettinen *et al.* 1996).

#### *RNA isolation and Northern blot analysis*

Total RNAs from cultured cells were isolated using TRIzol reagent according to the manufacturer's instruction. Fifteen micrograms of each sample were resolved on 1.0% agarose/formaldehyde gel and then transferred onto Hybond nylon membrane (Amersham International plc, Amersham, Bucks, UK) by pressure blotting. The subsequent steps followed a method described previously (Xu *et al.* 2000). The same membrane was separately probed by the <sup>32</sup>P-labeled 1.0 kb *EcoRI*–*Sad* fragment of human 17HSD1 cDNA (Piao *et al.* 1997b), 1.4 kb *Sad* fragment of human P450arom cDNA (Ghersevich *et al.* 1994) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The autoradiographic signals were quantified by densitometry analysis with a Molecular Dynamics 300A computing densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

#### *Transfection and CAT assay*

The construct pBL(–661/–392)CAT2 containing 17HSDB1 enhancer has been used in our previous study (Piao *et al.* 1995). The enhancer is located at the region from –661 to –392 in the gene, with respect to the transcription start site for the 1.3 kb 17HSD1 mRNA. Transient transfection experiments were performed as described previously (Piao *et al.* 1995). Briefly, the cells were applied to 6 cm plates in amounts of  $7.5 \times 10^5$  cells/plate (JEG-3) or  $1.5 \times 10^6$  cells/plate (T47D) and cultured for 24 h before transfection. Five micrograms of each plasmid construct were transfected into the cells using the transfection reagent DOTAP (5.0 µg/ml). After 18 h, the media were replaced and the cells were cultured for a further 48 h in the presence of various stimuli.

**Table 1** Summary of oligonucleotide primers used for RT-PCR

Gene	Primer sequence (5'→3')	Positions (nt)	Product size (bp)
RAR $\alpha$	Sense: CTGCCCGACACCTGGGGGC	123–141	160
	Antisense: GTGTGGTAGGGGTCGGTGG	264–282	
RAR $\beta$	Sense: TTCATGATTCGGGGCTGGGAAAAA	322–345	154
	Antisense: CGTTCCTCTAGTACAAAC	457–475	
RAR $\gamma$	Sense: GACTCTTGCGGCTGGTGC	434–452	162
	Antisense: GGGTTCCTCTACCGGAGAG	577–595	
RXR $\alpha$	Sense: ATGGACACCAACATTTCCTGC	76–97	268
	Antisense: GGGACCCGAAGTCGTGAC	325–343	
RXR $\beta$	Sense: ATGTCTGGGCGGCTCGCC	118–136	133
	Antisense: GCTGCCGGGACCGACCTAG	232–250	
RXR $\gamma$	Sense: GAGGCTCCCCTGGCCACAC	74–92	129
	Antisense: GGTGCTGAGACTCACGTC	184–202	

After transfection, the cells were collected and subjected to four freeze–thaw cycles (freezing in dry ice/ethanol for 5 min and thawing at 37 °C for 3 min) in 150  $\mu$ l 0.25 M Tris–HCl (pH 7.8). The treatment was followed by heat inactivation at 65 °C for 20 min, after which the CAT activity of the samples was measured by fluor diffusion assay (Neumann *et al.* 1987). Protein concentration was measured by a Bio–Rad protein assay system (Bio–Rad Laboratories, Richmond, CA, USA). CAT expression is described as ng/mg protein resulting from comparison of the CAT activity in samples with the CAT standard curve.

#### Reverse transcription-polymerase chain reaction (RT-PCR) analysis

The primers used for RT-PCR analysis of retinoid X receptors (RXRs) and RARs are summarized in Table 1. Total RNA (1.0  $\mu$ g) of JEG-3 cells was reversed transcribed using an oligo(dT) primer in a total volume of 20  $\mu$ l. For PCR amplification, 2  $\mu$ l of RT products was added to a mixture (50  $\mu$ l) containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.25 mM dNTPs, 1  $\mu$ M sense and antisense primers, and 5 U Taq DNA polymerase. The reaction was performed in 30 sequential cycles at 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s, using the GenAmp PCR System 2400 (Perkin-Elmer Corporation, Norwalk, CT, USA).

#### Statistics

All experiments were done at least twice, and statistical significance was determined by analysis of variance or Student's *t*-test where appropriate.

## Results

#### Actions of RAs on E<sub>2</sub> biosynthesis in cultured JEG-3 and CTB cells

JEG-3 and CTB cells were treated with 100 nM *at*-RA or *9cis*-RA for 24 h, and the capacity of treated cells to

convert DHEA to E<sub>2</sub> was examined by measuring the E<sub>2</sub> concentration in the media. In JEG-3 cells, both *at*-RA and *9cis*-RA stimulated E<sub>2</sub> production over 2.2-fold (Fig. 1A). In CTB cells, *at*-RA and *9cis*-RA caused 2.0- and 2.8-fold increases in E<sub>2</sub> synthesis respectively. Also, the conversion of A-dione to E<sub>2</sub> in JEG-3 cells was increased over 2.0-fold by the same administration of *at*-RA or *9cis*-RA (Fig. 1B).

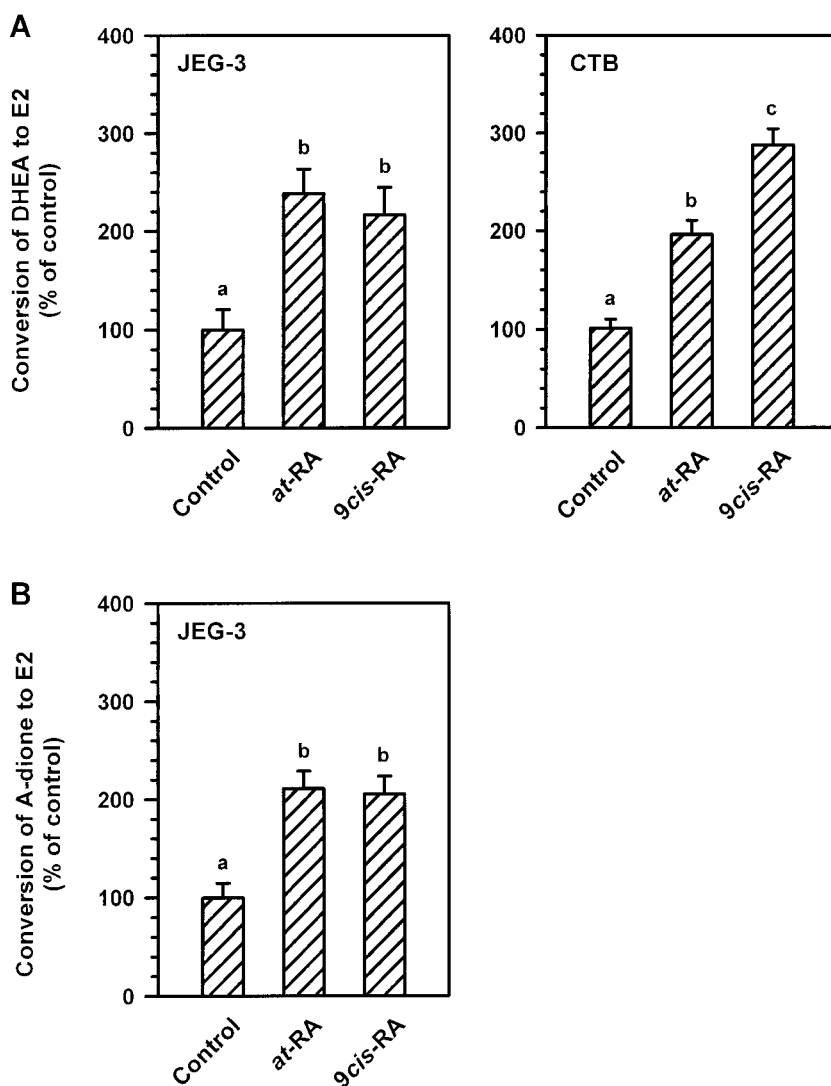
#### Induction of aromatase activity by *at*-RA, 8Br-cAMP, TPA and IL-1 $\beta$ in JEG-3 cells

Since aromatase is involved in E<sub>2</sub> biosynthesis, aromatase activity was analyzed after the treatment of JEG-3 cells with 0.1–1000 nM *at*-RA for 48 h. Increased aromatase activity (2.0-fold) was detected after treatment with 0.1 nM *at*-RA, and the activity was further enhanced dose-dependently, reaching 3.8-fold at 1000 nM. On the other hand, when the cells were stimulated with 100 nM *at*-RA, significantly increased aromatase activity (4.0-fold) could be observed at 6 h, and the induction remained for up to 48 h (Fig. 2).

In addition to *at*-RA, 8Br-cAMP, TPA (the protein kinase C activator) and IL-1 $\beta$  increased aromatase activity to different extents in JEG-3 cells. Furthermore, simultaneous administration of *at*-RA with 8Br-cAMP or TPA potentiated the stimulatory effect of *at*-RA on aromatase activity, while IL-1 $\beta$  merely enhanced the induction in an additive manner (Fig. 3).

#### Effect of *at*-RA, 8Br-cAMP and TPA on the expression of P450arom mRNA in JEG-3 cells

To elucidate whether the transcription of the CYP19 gene is affected, JEG-3 cells were treated for 6–48 h with different doses of *at*-RA, ranging from 0.1 to 1000 nM, and P450arom expression was determined by Northern blot analysis. Similar to the effect of *at*-RA on aromatase activity, an increased expression of P450arom mRNA was



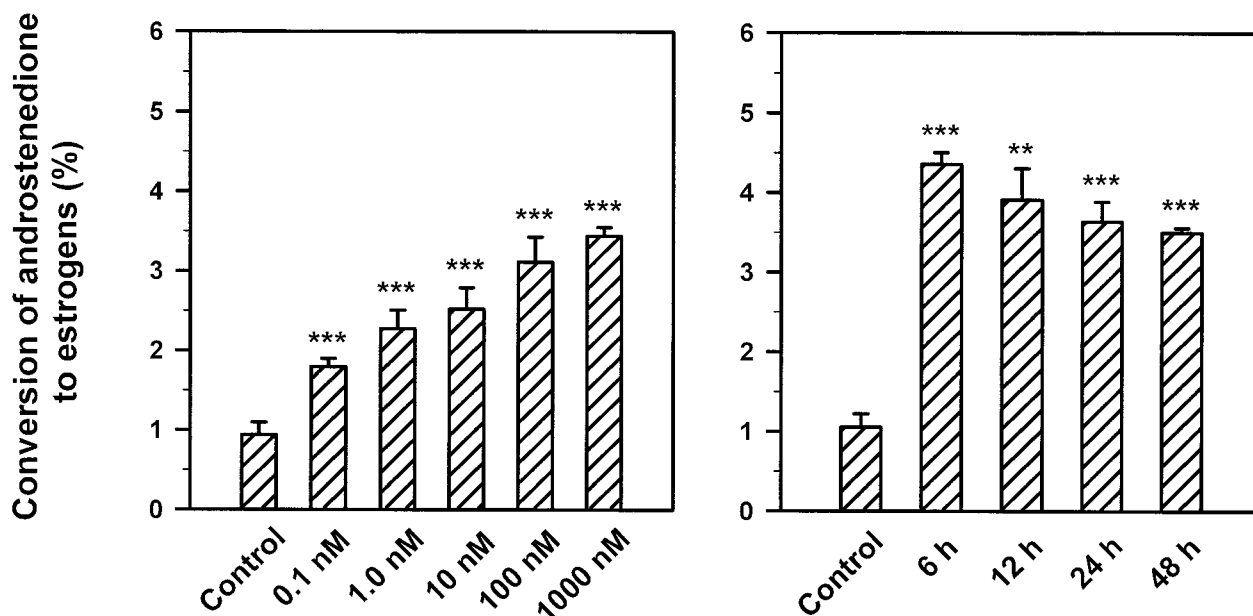
**Figure 1** Action of RAs on  $E_2$  biosynthesis in JEG-3 and CTB cells. Bars without the same letter are significantly different from each other ( $P < 0.05$ ). (A) JEG-3 cells and CTB cells were treated with 100 nM *at*-RA or 9*cis*-RA for 24 h, after which the cells were incubated with 500 nM DHEA for 1 h in cell culture conditions. The data represent the means  $\pm$  s.d. of four (JEG-3) or three (CTB) independent experiments, with quadruplet samples in each. (B) JEG-3 cells were stimulated with 100 nM *at*-RA or 9*cis*-RA for 24 h and then incubated with 500 nM A-dione for 1 h in cell culture conditions. The data represent the means  $\pm$  s.d. of three independent experiments with duplicate samples in each.

observed after treatment of the cells with 0.1 nM *at*-RA, and the stimulation was further increased with the elevation of the *at*-RA concentration tested, reaching a 4.8-fold maximal value compared with the control level (Fig. 4, left panel). Unlike the influence on the enzyme activity, the induction on P450arom mRNA expression appeared to be time-dependent. Treatment of cells with 100 nM *at*-RA caused a 1.4-fold increase in the mRNA level at 6 h, and the stimulation continued to rise in

parallel with the time-course, reaching 2.7-fold at 48 h (Fig. 4, right panel).

The influence of *at*-RA on CYP19 gene expression in JEG-3 cells was also examined in the presence of 8Br-cAMP and TPA. The expression of 3.0 and 2.4 kb P450arom mRNA was remarkably increased by treatment of the cells with 20 nM TPA and especially 1.5 mM 8Br-cAMP. Both compounds further enhanced the effects of *at*-RA (Fig. 5).





**Figure 2** Effect of *at*-RA on aromatase activity in JEG-3 cells. Aromatase activity was examined after the cells had been treated with 0.1–1000 nM *at*-RA for 48 h (left panel), or 100 nM *at*-RA for 6–48 h (right panel). The data show the means  $\pm$  S.D. of three independent experiments with duplicate samples in each. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

#### Effects of the selective RAR $\alpha$ agonist Ro40 and antagonist Ro41 on the expression of P450arom and 17HSD1 mRNA in JEG-3 cells

The effects of the selective RAR $\alpha$  agonist Ro40 and antagonist Ro41 on P450arom and 17HSD1 expression in JEG-3 cells were investigated by Northern analysis for further elucidation of the regulatory mechanisms of RAs (Fig. 6). Treatment of cells with 100 nM Ro40 for 24 h resulted in a stronger induction of P450arom expression than treatment with 100 nM *at*-RA or 9*cis*-RA. This stimulation, as expected, was blocked by simultaneous administration of 1.0  $\mu$ M Ro41, which also decreased the effects of *at*-RA and 9*cis*-RA. Unexpectedly, in addition to being induced by *at*-RA (9.6-fold), 9*cis*-RA (8.5-fold) and Ro40 (6.8-fold), 17HSD1 expression was also increased by Ro41 (3.6-fold). Furthermore, Ro41 in combination with *at*-RA and 9*cis*-RA caused 28- and 24-fold induction respectively. On the other hand, co-treatment with Ro40 and Ro41 resulted in an additive increase in 17HSD1 expression.

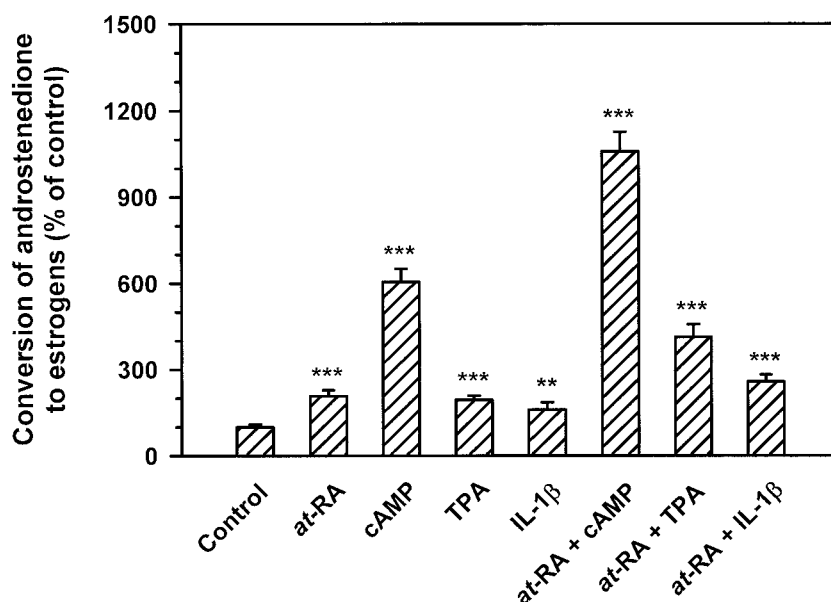
#### Effects of Ro40 and Ro41 on reductive 17HSD activity in JEG-3 cells

To confirm the stimulatory influence of Ro40 and Ro41 on 17HSD1 expression, the reductive 17HSD activity of cultured JEG-3 cells was determined after the same treatment of the cells as for mRNA analysis. Stimulation with 100 nM *at*-RA, 9*cis*-RA and Ro40 as well as 1  $\mu$ M

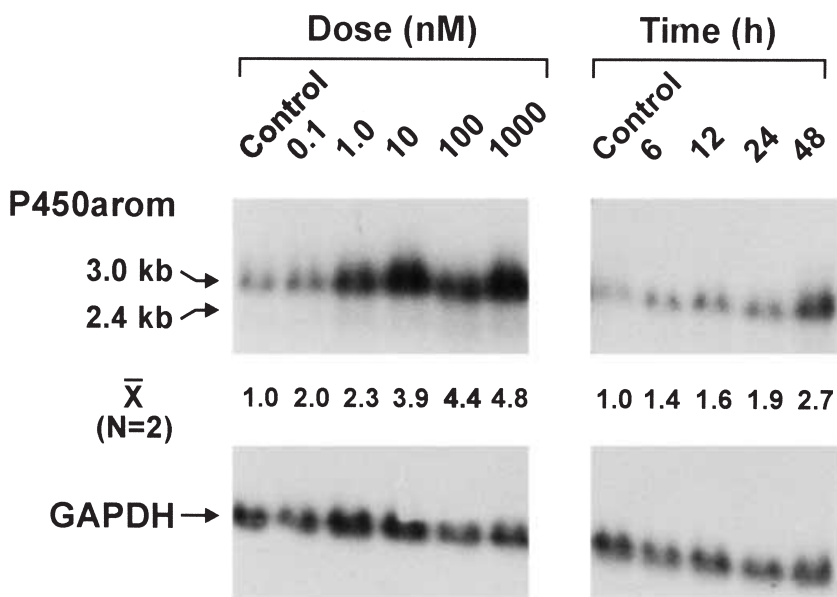
Ro41 led to 2.5-, 2.5- 2.3- and 1.7-fold increases in reductive 17HSD activity respectively. After the cells had been treated with 1  $\mu$ M Ro41 in combination with 100 nM *at*-RA, 9*cis*-RA and Ro40, the induction was potentiated to 5.4-, 5.2- and 3.4-fold respectively (Fig. 7).

#### Effect of Ro41 on HSD17B1 enhancer

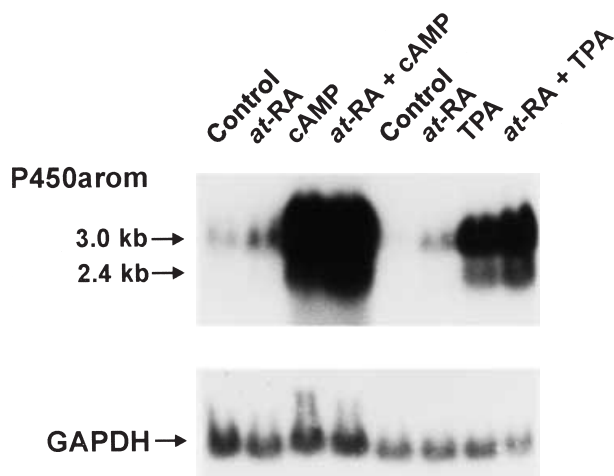
Reporter gene analysis, using the construct pBL(-661/-392)CAT2 and the control vector pBLCAT2, was conducted to further understand how Ro41 affects the transcription of the HSD17B1 gene. In the construct pBL(-661/-392)CAT2, the HSD17B1 enhancer containing an RA response element (RARE) at the position from -512 to -479 is linked to a thymidine kinase (TK) promoter, whereas pBLCAT2 merely has a TK promoter (Piao *et al.* 1995). Reporter gene analysis showed that the TK promoter alone has almost no activity and cannot be induced by *at*-RA treatment in both T47D and JEG-3 cells. When it was connected with the HSD17B1 enhancer, however, the TK promoter displayed significant transcriptional activity (Fig. 8). In T47D cells, Ro41 (1  $\mu$ M) did not affect pBL(-661/-392)CAT expression, but blocked the effect of *at*-RA (100 nM) remarkably. In JEG-3 cells, treatment with 100 nM *at*-RA and 1  $\mu$ M Ro41 enhanced pBL(-661/-392)CAT expression to 260% and 130% respectively. After the cells were stimulated with both *at*-RA and Ro41, the CAT expression was additively increased to 300% (Fig. 8).



**Figure 3** Interaction of *at*-RA with various factors on aromatase activity in JEG-3 cells. Aromatase activity was examined after the cells had been incubated for 24 h with (1) vehicle, (2) 100 nM *at*-RA, (3) 1.5 mM 8Br-cAMP (cAMP), (4) 20 nM TPA, (5) 25 pg/ml IL-1 $\beta$ , (6) 100 nM *at*-RA+1.5 mM 8Br-cAMP, (7) 100 nM *at*-RA+20 nM TPA, and (8) 100 nM *at*-RA+25 pg/ml IL-1 $\beta$ . The data show the means  $\pm$  range of two independent experiments with duplicate samples in each. \*\* $P$ <0.01, \*\*\* $P$ <0.001.



**Figure 4** Dose- and time-effect of *at*-RA on P450arom mRNA expression in JEG-3 cells. The cells were treated with 0.1–1000 nM *at*-RA for 48 h (left panel), or 100 nM *at*-RA for 6–48 h (right panel). Northern blot analysis was performed using total RNA isolated from the treated cells. The positions of the 3.0 kb and 2.4 kb P450arom transcripts are indicated by the arrows. The positions below the P450arom signals represent the intensity ratio (3.0 kb P450arom/GAPDH) compared with the values (defined as 1.0) of the control samples. Two independent experiments were done with similar results and representative figures are shown.



**Figure 5** Effects of *at*-RA, 8Br-cAMP and TPA on P450arom mRNA expression in JEG-3 cells. The cells were treated for 24 h with vehicle, 100 nM *at*-RA, 1.5 mM 8Br-cAMP (cAMP), and 100 nM *at*-RA+1.5 mM 8Br-cAMP in one experiment (left panel), as well as with vehicle, 100 nM *at*-RA, 20 nM TPA and 100 nM *at*-RA+20 nM TPA in another (right panel). Northern blot analysis was performed after treatment using total RNA isolated from the treated cells. Two independent experiments were done with similar results, and representative data are shown. Because the strong expression caused the overlap of some hybridization signals, densitometry analysis was not performed.

#### RAR and RXR transcripts in JEG-3 cells

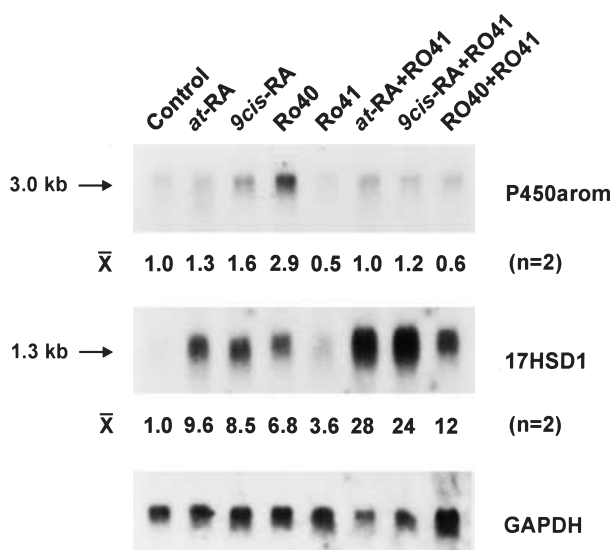
In order to further investigate the possible mechanisms of RAs, especially the profile of retinoid receptors, RT-PCR analysis was performed using total RNAs isolated from JEG-3 cells as the template and the primer set specific to each subtype of RXR and RAR. As shown in Fig. 9, RAR $\alpha$  and RXR $\alpha$  were found whereas other members of RXR and RAR could not be detected.

#### Effects of RAs on the expression of 17HSD1 and P450arom mRNA in cultured CTB cells

Finally, the expression of 17HSD1 and P450arom in CTB cells was also examined. Upon stimulation with 100 nM *at*-RA or 9*cis*-RA for 24 h, the expression of the 3.0 kb mRNA of P450arom in CTB cells was increased to 130% by *at*-RA and 170% by 9*cis*-RA. 17HSD1 expression was also elevated by the same treatment, but to a lesser extent (Fig. 10).

#### Discussion

Estrogens play a crucial role during human pregnancy (Loriaux *et al.* 1972). Even though it is well known that the placenta is the major site of estrogen production in pregnant women, the primary factor(s) affecting estrogen biosynthesis are difficult to define because of the



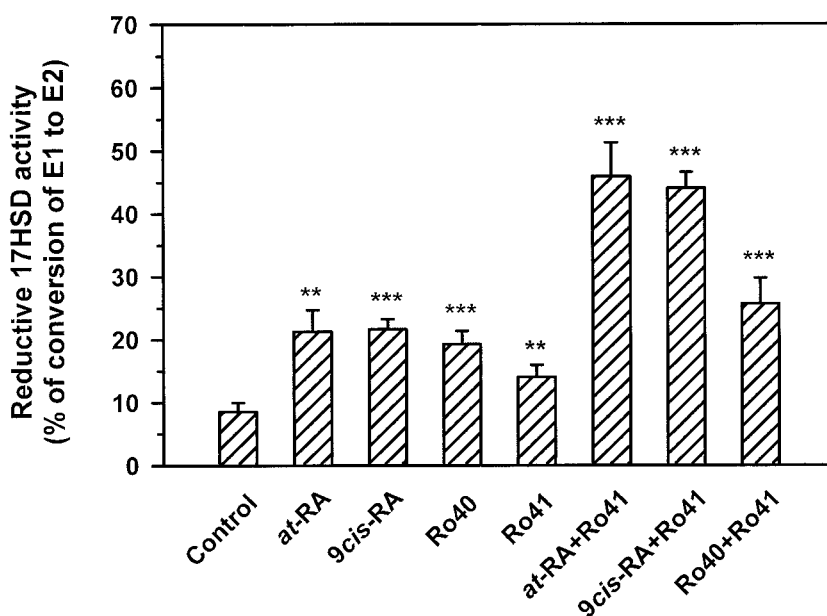
**Figure 6** Influence of the selective RAR $\alpha$  antagonist (Ro41) on RA-induced P450arom and 17HSD1 mRNA expression in JEG-3 cells. The cells were treated for 24 h with (1) vehicle, (2) 100 nM *at*-RA, (3) 100 nM 9*cis*-RA, (4) 100 nM Ro40, (5) 1.0  $\mu$ M Ro41, (6) 100 nM *at*-RA+1.0  $\mu$ M Ro41, (7) 100 nM 9*cis*-RA+1.0  $\mu$ M Ro41, and (8) 100 nM Ro40+1.0  $\mu$ M Ro41. Northern blot analysis was performed using total RNA isolated from the treated cells. The same membrane was probed sequentially with cDNA probes for 17HSD1, P450arom, and GAPDH. The positions of P450arom and 17HSD1 transcripts are indicated by the arrows. The numbers below the P450arom and 17HSD1 signals represent the intensity ratio (3.0 kb P450arom/GAPDH and 17HSD1/GAPDH) compared with the values (defined as 1.0) of the control samples. Two independent experiments were done with similar results and representative data are shown.

complexity of the placental endocrine environment. The present study showed that RAs promote E<sub>2</sub> production not only in JEG-3 cells but also in CTB cells isolated from normal placenta. These results indicate that RAs may act as regulatory factors of E<sub>2</sub> biosynthesis in the placenta.

P450arom and 17HSD1 play essential roles in placental E<sub>2</sub> biosynthesis. We recently demonstrated that RAs stimulate 17HSD1 expression in JEG-3 cells, and the effect is mediated by an RARE in the enhancer region of the HSD17B1 gene, which encodes 17HSD1 (Piao *et al.* 1995, 1997b). In the present work, it was revealed that aromatase is also subject to regulation of RAs in JEG-3 cells at the levels of both enzyme activity and P450arom mRNA expression. The data further demonstrate that RAs play a role in regulating key steroidogenic enzymes in choriocarcinoma cells.

The expression of the CYP19 gene in the placenta and choriocarcinoma cells is controlled by a distal promoter I.1 located adjacently upstream of exon I.1 in the gene (Means *et al.* 1991). Recently, an imperfect palindromic sequence 5'-AGGTCATGCCCC-3', situated at the region from -183 to -172 within the promoter has been identified to be functional as an RARE. This sequence binds the





**Figure 7** Effect of RAs, Ro40 and Ro41 on reductive 17HSD activity in cultured JEG-3. Reductive 17HSD activity was detected after the cells had been treated for 24 h with (1) vehicle, (2) 100 nM *at*-RA, (3) 100 nM *9cis*-RA, (4) 100 nM Ro40, (5) 1.0  $\mu$ M Ro41, (6) 100 nM *at*-RA+1.0  $\mu$ M Ro41, (7) 100 nM *9 cis*-RA+1.0  $\mu$ M Ro41, and (8) 100 nM Ro40+1.0  $\mu$ M Ro41. The results are presented as means  $\pm$  s.d. of three independent experiments with duplicate samples in each. \*\* $P$ <0.01, \*\*\* $P$ <0.001.

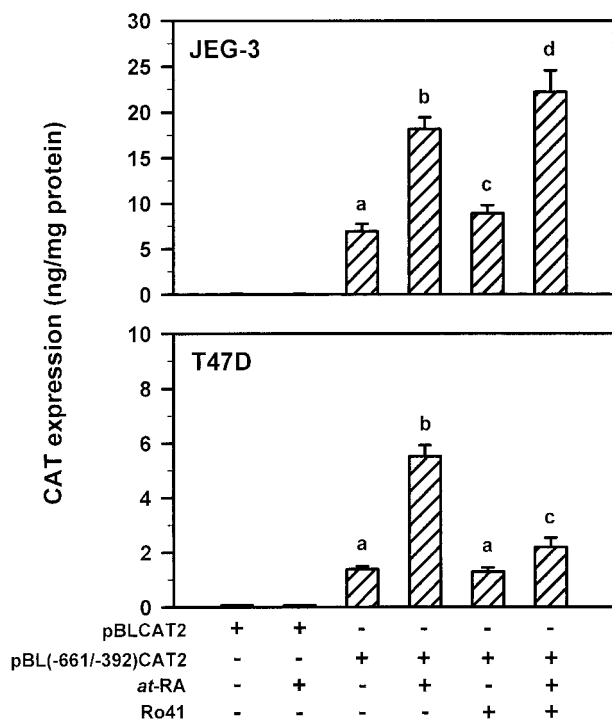
RXR $\alpha$ /VDR $\alpha$  (vitamin D receptor  $\alpha$ ) heterodimer and mediates transactivation of promoter I.1 by RXR- and VDR-selective agonists in JEG-3 cells (Sun *et al.* 1998). In the bandshift experiment, the complex formed by this element and nuclear extracts isolated from JEG-3 cells was not recognized by the specific antibody against RAR $\alpha$ , indicating that the sequence is not bound by the RXR $\alpha$ /RAR $\alpha$  heterodimer. On the other hand, the RAR-selective ligand TTNBP induced the promoter activity as well as the aromatase activity, indicating that RXR/RAR heterodimer(s) are involved in regulating the expression of the CYP19 gene in JEG-3 cells (Sun *et al.* 1998). In the present study, RT-PCR analysis showed that JEG-3 cells express only RXR $\alpha$  and RAR $\alpha$ . It was also found that *at*-RA, the ligand for RARs, as well as Ro40, a specific ligand for RAR $\alpha$ , induced P450 $\text{arom}$  mRNA expression and, further, this induction was blocked by Ro41, the selective inhibitor for RAR $\alpha$ . Collectively, it is indicated that another element, which can interact with RXR $\alpha$ /RAR $\alpha$  heterodimer, may exist in the regulatory region in the CYP19 gene.

In this study, we observed that a short-period stimulation (6 h) with *at*-RA resulted in a maximal increase in aromatase activity in JEG-3 cells. On the other hand, the influence on the mRNA expression of P450 $\text{arom}$  appeared to be time-dependent, reaching the highest value at 48 h as examined. Also, the extent of induction on enzyme activity was higher than that on mRNA

expression. Thus, the action of RAs on aromatase in choriocarcinoma cells may occur at translational or post-translational levels, in addition to increasing the transcription of the CYP19 gene or stabilization of P450 $\text{arom}$  transcripts.

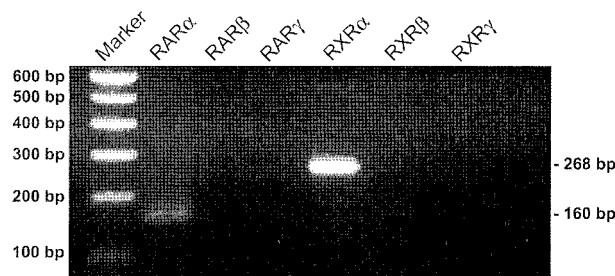
P450 $\text{arom}$  expression in choriocarcinoma cells is regulated by the protein kinase A and protein kinase C pathways (Ritvos & Voutilainen 1992). Also, IL-1 $\beta$  stimulated the aromatase activity in CTB cells (Nestler 1993). The present study demonstrated that the induction of RAs on aromatase activity is enhanced by cAMP, TPA and IL-1 $\beta$  in JEG-3 cells. In addition, both cAMP and TPA elevated the RA-induced expression of P450 $\text{arom}$  mRNA. It should be noted that, compared with retinoids, cAMP and TPA appeared to be more potent inducers for P450 $\text{arom}$  expression. However, retinoids are nutritional molecules exclusively available in the placenta and therefore have the potential to play a significant role in the biosynthesis of E $_2$ . The above data, together with our recent finding that a number of factors, such as EGF, TPA, cAMP and calcium ion, interact with RAs in regulating the action of 17HSD1 in JEG-3 cells (Piao *et al.* 1997b), indicate that E $_2$  biosynthesis in trophoblastic cells may be controlled by complicated mechanisms.

Apart from aromatase and 17HSD1, 3HSD1 also participates in placental E $_2$  biosynthesis by catalyzing the conversion of DHEA to A-dione. Thus, the effect of RAs on the expression of 3HSD1, if any, may also count for

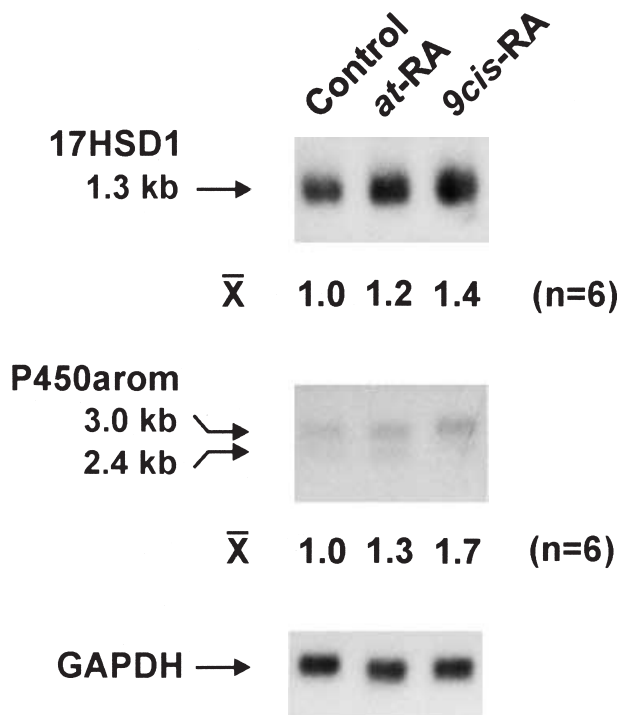


**Figure 8** Effects of at-RA and Ro41 on HSD17B1 enhancer activity. The cells were transfected with the reporter gene vector pBLCAT2 or the construct pBL(-661/-392)CAT2 for 18 h. After that, the cells transfected with pBL(-661/-392)CAT2 were treated with 100 nM at-RA, 1 μM Ro41 and 100 nM at-RA + 1 μM Ro41 for 48 h. Then the cells were collected and their CAT activities were examined. The results represent the means ± S.D. from four independent experiments, duplicate samples were analyzed in each. Bars without the same letter are significantly different from each other ( $P < 0.05$ ).

increased production of E<sub>2</sub> from DHEA. However, the conversion of A-dione to E<sub>2</sub>, which does not involve the action of 3 HSD1, was similarly induced by RAs in JEG-3 cells, implying that aromatase and 17HSD1 are the main targets for the action of RAs, at least in JEG-3 cells.



**Figure 9** RT-PCR analysis of RXRs and RARs in JEG-3 cells. Gel photograph shows the result of RT-PCR amplification, which generated the cDNA fragment of RARα (160 bp) and RXRα (268 bp), using total RNA isolated from JEG-3 cells as the template. Three independent experiments were done with same results and representative data are shown.



**Figure 10** Effects of RAs on 17HSD1 and P450arom mRNA expression in primary cultured CTB cells. Cultured CTB cells were stimulated with 100 nM at-RA or 9cis-RA for 24 h. The same membrane was subjected to hybridization with cDNA probes for 17HSD1, P450arom and GAPDH. The positions of P450arom and 17HSD1 transcripts are indicated by the arrows. The numbers below the P450arom and 17HSD1 signals represent the average intensity ratio (3.0 kb P450arom/GAPDH and 17HSD1/GAPDH) compared with the values (defined as 1.0) of the control samples. Six independent experiments were done with similar results and representative data are shown.

Ro41 has been identified to act as a selective antagonist for RARα (Apfel *et al.* 1992), but this does not readily explain the effect of Ro41 on 17HSD1 expression in JEG-3 cells. In a previous report, Ro41 was demonstrated to inhibit the stimulation of at-RA on 17HSD1 expression in breast cancer T47D cells (Reed *et al.* 1994). Correspondingly, this study also showed that Ro41 blocks the activation of at-RA on the transcription of the HSD17B1 gene in the same cells. In contrast, Ro41 induced the expression and activity of 17HSD1 in JEG-3 cells and, moreover, potentiated the effects of RAs. However, the same phenomenon was not identically observed in reporter gene analysis showing that, although Ro41 was able to increase the enhancer activity in JEG-3 cells, the extent was noticeably less than its influence on the abundance of 17HSD1 mRNA. Moreover, the combined effect of Ro41 and at-RA on HSD17B1 enhancer in JEG-3 cells was only additive, not as synergistic as was found in mRNA analysis of 17HSD1. Therefore, the effect of Ro41 on the expression of 17HSD1 in JEG-3 cells seems

to occur at both transcriptional and post-transcriptional levels, especially the mRNA stabilization. Alternatively, Ro41 may activate a subtype of retinoid receptors, which is targeted to an unknown regulatory area in the HSD17B1 gene rather than the RARE located at the region from -512 to -479. Nevertheless, this phenomenon is probably specific to JEG-3 cells, because Ro41 did block the stimulatory effect of *at*-RA on 17HSD1 expression in breast cancer cells (Reed *et al.* 1994).

Accumulating evidence suggests that retinoids regulate the production of key placental hormones and steroidogenic enzymes in JEG-3 cells (Kato & Braunstein 1991, Matsuo & Strauss 1994, Stephanou & Handwerger 1995, Piao *et al.* 1997b, Guibourdenche *et al.* 1998a, Tremblay *et al.* 1999). It was recently reported that RXR $\alpha$  and RAR $\alpha$  are expressed in JEG-3 cells, and RXR $\alpha$  mediates the biological effects of retinoids on cell function, i.e. hCG secretion. On the other hand, neither Western blot nor Northern blot analyses could detect the presence of RXR $\beta$ , RXR $\gamma$ , RAR $\beta$  and RAR $\gamma$  in the JEG-3 cells (Guibourdenche *et al.* 1998a). In agreement with these data, our RT-PCR analysis revealed that only RAR $\alpha$  and RXR $\alpha$  are present in JEG-3 cells. Meanwhile, we found that RAR $\alpha$  is also a mediator of the action of retinoids on JEG-3 cells, since Ro40, the selective ligand for RAR $\alpha$  and *at*-RA, the ligand for RARs, stimulated the expression of P450arom and 17HSD1 mRNA. It can also be speculated that the effects are mediated by RXR $\alpha$ /RAR $\alpha$ , which is the only form of heterodimer available in JEG-3 cells.

It is agreed that STB cells, which are differentiated from CTB cells, are mainly responsible for hormone production in the placenta. Correspondingly, P450arom and 17HSD1 are exclusively expressed in STB cells *in vivo* (Fournet-Dulguerov *et al.* 1987). However, cultured STB cells isolated directly from the placenta cannot be used as a model for *in vitro* studies because of their lack of viability under cell culture conditions. On the other hand, *in vitro* cultured CTB cells possess some features of STB cells, such as the ability to produce hCG, E<sub>2</sub> and progesterone (Kliman *et al.* 1991, Zhuang & Li 1991). They could therefore be employed as a cell model to investigate the mechanisms of steroidogenesis in the placenta. In this study, it was clearly shown that CTB cells cultured *in vitro* express both P450arom and 17HSD1, and are capable of producing E<sub>2</sub> from DHEA. *In situ* hybridization and immunohistochemical staining also confirmed 17HSD1 expression in CTB cells during the first trimester (our unpublished data).

In the human placenta, RAR $\alpha$  and RXR $\alpha$  are the major forms of retinoid receptors expressed in trophoblastic cells, and RXR $\alpha$  has been proved to be involved in regulating the production of hCG and leptin (Roulier *et al.* 1994, Guibourdenche *et al.* 1998b,c). In line with these observations, the present study demonstrated that *at*-RA, the ligand for RARs and especially *9cis*-RA, the ligand for

RXR $\alpha$ s, can promote E<sub>2</sub> production as well as the expression of P450arom and 17HSD1 mRNA in cultured CTB cells. On the other hand, the extent of the induction of RAs on P450arom and 17HSD1 expression is less than that on the conversion of DHEA to E<sub>2</sub> in CTB cells, suggesting that RAs may also act on the translational or post-translational levels of these two enzymes.

In brief, the results of the present study demonstrate that RAs promote the biosynthesis of E<sub>2</sub> in placental cells by increasing the expression of aromatase and 17HSD1 gene, but the mechanisms of the divergent effects of Ro41 on gene expression in JEG-3 cells remain to be elucidated.

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