

# Role of oestradiol-17 $\beta$ in the regulation of synthesis and secretion of human chorionic gonadotrophin by first trimester human placenta

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## ABSTRACT

Inhibition of aromatase, a key enzyme in the biosynthesis of oestradiol-17 $\beta$ , by the addition of 1,4,6-androstatrien-3,17-dione resulted in a significant increase in the levels of immunoreactive human chorionic gonadotrophin (hCG) in the medium and tissue. This increase was partially reversed by the simultaneous addition of oestradiol-17 $\beta$ . These effects on the levels of immunoreactive hCG were also reflected by the increased levels of mRNA

specific for the  $\alpha$  and  $\beta$  subunits of hCG following the addition of the aromatase inhibitor. However, addition of tamoxifen resulted in a drastic decrease in the levels of both the messages. Based on these results, it is suggested that the synthesis of hCG is negatively modulated by oestradiol-17 $\beta$  in the human placenta.

*Journal of Molecular Endocrinology* (1993) **11**, 91–101

## INTRODUCTION

Human chorionic gonadotrophin (hCG) is a glycoprotein hormone produced by the human placenta (Talamantes & Ogren, 1988). It shares structural and functional homology with pituitary luteinizing hormone (LH) (Birken, 1984). Both positive and negative regulation of LH, respectively by gonadotrophin-releasing hormone (GnRH) and the gonadal steroids progesterone and oestradiol-17 $\beta$  (OE<sub>2</sub>), are well documented in the literature (McCann, 1974; Conn *et al.* 1987). It is possible that, as the placenta is also known to produce GnRH and steroids like progesterone and OE<sub>2</sub>, hCG may also be subject to similar regulation by GnRH and steroids. Using minced first trimester human placenta we have shown in an earlier study (Mathialagan & Rao, 1986) that GnRH stimulates the synthesis and secretion of hCG, thus providing support for the suggestion that hCG is also subject to positive modulation by GnRH, as in the case of pituitary LH. However, while it has been reported by Belleville *et al.* (1978) that the addition of progesterone or OE<sub>2</sub> had no effect on hCG secretion by first trimester human placenta, Maruo *et al.* (1986), using cDNA probes for the  $\alpha$  and  $\beta$  subunits of hCG and an in-situ hybridization technique,

have shown that the addition of progesterone (5–20  $\mu$ g/ml) to cultures of normal first trimester human placenta results in a decrease in the cellular levels of hCG  $\alpha$  and  $\beta$  mRNA. OE<sub>2</sub> at lower concentrations had no effect on the immunoreactive levels of the hormone in the medium. One of the main problems associated with studies of the role of OE<sub>2</sub> or progesterone in the regulation of the synthesis and secretion of hCG in the placenta is that placental tissue is already exposed to high concentrations of endogenous steroids and it is difficult to observe the effects of added steroids. In the present study we have examined the effect of OE<sub>2</sub> on the synthesis and secretion of hCG under conditions where the influence of endogeneous OE<sub>2</sub> is minimal.

## MATERIALS AND METHODS

First trimester human placentae (6–8 weeks) were collected from cases of medical termination of pregnancy from the local hospital. Placentae were collected after obtaining written consent from the subjects. The tissue was collected in cold Earl's balanced salt solution (EBSS; pH 7.4) and immediately brought to the laboratory on crushed ice and

processed at 4 °C. The tissue was washed extensively with cold EBSS until the washing liquid was clear, and villous tissue was separated by visual examination; a wet weight of 2–5 g was recorded. The villous tissue was finely minced with surgical scissors, washed twice with EBSS and suspended in a known volume of buffer. About 80–100 mg tissue (wet weight) were dispensed into 3 ml tubes. The total volume of incubation was 0.5 ml.

Tamoxifen (TMX) was a gift from Imperial Chemical Industries Ltd, London, U.K. Rabbit reticulocyte lysate (N.90 lysate) was obtained from Amersham International plc, Amersham, Bucks, U.K. [ $\alpha$ - $^{32}$ P]dCTP (3000 Ci/mmol) and [ $^{35}$ S]methionine (800 Ci/mmol) were obtained from Bhabha Atomic Research Center, Bombay, India. Unlabelled OE<sub>2</sub> and 1,4,6-androstatrien-3,17-dione (aromatase inhibitor; AI) were obtained from Steroloids Inc., Wilton, NH, U.S.A. EBSS was obtained from Hi-Media, Bombay, India.

Nitrocellulose membrane filters were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Whatman filter paper no. 3 was purchased from Whatman Ltd, Maidstone, Kent, U.K. Trizma base, formamide, oligo(dT) cellulose, potassium acetate, dithiothreitol and sodium dodecyl sulphate (SDS) were purchased from Sigma Chemical Co., St Louis, MO, U.S.A. DNA markers (1 kb ladder) were obtained from Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, MD, U.S.A. All other chemicals used were obtained locally and were of analytical grade. Clones for hCG  $\alpha$  subunit cDNA and  $\beta$  subunit cDNA cloned at the ampicillin PstI site in the pBR322 vector were obtained as a gift from Dr I. Boime (Department of Pharmacology, Washington University, St Louis, MO, U.S.A.). These were transformed in the HB 101 strain of *Escherichia coli*, and the 0.7 kb insert for  $\alpha$  and 1 kb insert for  $\beta$  hCG were isolated by digestion with PstI. These probes were labelled with [ $\alpha$ - $^{32}$ P]dCTP by nick translation. The specific activity of the probes was around  $5 \times 10^8$  c.p.m./ $\mu$ g.

#### Effect of addition of AI on the levels of OE<sub>2</sub> in the placental tissue and medium

Minced placental tissue (80–100 mg) was incubated in the presence or absence of different concentrations (5, 10, 15 and 30  $\mu$ M) of AI under an atmosphere of 95% oxygen and 5% carbon dioxide for 4 h. Following this, the tissue was separated by centrifugation (2000 g) and washed three times with EBSS, homogenized and extracted with ether. The ether extract was used for the estimation of OE<sub>2</sub> and progesterone by specific radioimmunoassays (RIAs).

#### Estimation of hormones

hCG in the tissue homogenate and medium was estimated by plastic tube RIA as described previously (Murthy *et al.* 1989). Inter- and intra-assay coefficients of variation were 12.3 and 8.6% respectively, and the minimum detectable quantity of hCG was 2 ng/ml. The assay was specific for whole hCG, and assays were carried out in the presence of (0.01%) lima bean trypsin inhibitor. OE<sub>2</sub> and progesterone in the ether extract of the tissue homogenate and medium were estimated by specific RIAs as described previously (Jagannadha Rao *et al.* 1984). The inter- and intra-assay coefficients of variation were 10.3 ( $n=10$ ) and 6.5% ( $n=8$ ) respectively for progesterone and 8.66 ( $n=9$ ) and 5.2% ( $n=8$ ) respectively for OE<sub>2</sub>.

Total protein in the tissue homogenate was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard, and all values are expressed per mg protein.

#### Effect of AI, TMX or OE<sub>2</sub> on immunoreactive hCG levels in first trimester human placenta

Minced placental tissue in EBSS (1 g for the isolation of RNA or 80–100 mg for the estimation of hCG) were incubated in triplicate in 3 ml tubes or 25 ml conical flasks with or without AI (15  $\mu$ M), TMX (5  $\mu$ M) or OE<sub>2</sub> (10 nM–7.5  $\mu$ M) for 4 h at 37 °C under 95% oxygen and 5% carbon dioxide. Medium and tissue were separated by centrifugation at 2000 g at 4 °C.

#### Effect of the addition of AI with or without TMX or OE<sub>2</sub> on $\alpha$ and $\beta$ hCG mRNA levels in first trimester human placenta

Minced first trimester human placental tissue (1 g wet weight) was incubated in 10 ml EBSS in a 25 ml conical flask with or without 15  $\mu$ M AI (a concentration of AI at which the maximum decrease in OE<sub>2</sub> levels in the tissue was noticed after 4 h) for 30 min at 37 °C under 95% oxygen and 5% carbon dioxide. Following this, both groups of minced tissue were washed twice with 3 vols EBSS and used for further studies. While control tissue was incubated without any additions, AI-treated tissue was resuspended in a medium containing 15  $\mu$ M AI and incubated in the presence or absence of 10 nM OE<sub>2</sub> or 5  $\mu$ M TMX for 4 h under the conditions described above. Following incubation, the tissue and medium were separated by centrifugation and the tissue was processed for the isolation of total RNA according to the procedure of Boime *et al.* (1976). Total RNA was quantitated by monitoring the optical density at 260 nm. Poly(A)<sup>+</sup> RNA was

isolated by chromatography on oligo(dT) cellulose as described by Aviv & Leader (1972).

### Quantitation of mRNA

mRNA quantitation was performed by dot blot hybridization and also after resolving RNA by agarose gel electrophoresis.

#### *RNA dot blot hybridization*

Total cytoplasmic RNA (20 µg) from each group was immobilized on nitrocellulose filters and hybridized separately to nick-translated cDNA probes for  $\alpha$  (0.7 kb) and  $\beta$  (1 kb) hCG subunits according to the procedure described by Thomas (1980). The nitrocellulose filters were washed with  $2 \times$  SSC ( $1 \times$  SSC is 8.765 g NaCl, 4.41 g sodium citrate in one litre of double-distilled water, pH 7.0) four times at room temperature for 20 min each, followed by  $1 \times$  SSC four times at 65 °C for 20 min each and  $0.2 \times$  SSC three times at 65 °C for 20 min each. Filters were dried between sheets of Whatman no. 3 filter paper, air-dried and exposed to X-ray film for 2–5 days. The hybridization spots were subjected to laser beam densitometric scanning.

#### *Electrophoretic analysis of RNA*

Electrophoresis of RNA (20 µg) on 1% agarose gels containing 2.2 M formaldehyde was carried out according to the procedure described by Maniatis *et al.* (1982). RNA was transferred to nitrocellulose filters as described by Southern (1975). Following transfer, the nitrocellulose filters were baked in a vacuum oven at 80 °C and hybridized with nick-translated probe. Hybridization and washing conditions were the same as those used in the dot blot procedure. mRNA levels were quantitated by scanning the autoradiograms with a laser beam scanner (LKB Model 2001) and also by monitoring the radioactivity associated with the hybridization spots.

### In-vitro translation

Poly(A)<sup>+</sup> RNA (1–2 µg) was translated using rabbit reticulocyte lysate. Equal quantities of trichloroacetic acid-precipitable counts from individual samples were immunoprecipitated by a specific hCG anti-serum raised against highly purified hCG (CR 123, 13 000 IU/mg; a gift from NIAMDD, NIH, Bethesda, MD, U.S.A.) in the rabbit. The antiserum was highly specific to hCG, with minimal cross-reactivity with  $\alpha$  and  $\beta$  hCG subunits; it did not show any cross-reactivity with other glycoprotein hormones and thus was found to be suitable for immunoprecipitation studies. In

addition, minced placental tissue was incubated in the presence or absence of modulators with [<sup>35</sup>S]methionine. hCG was precipitated from the labelled proteins using the specific antiserum described above. The immunoprecipitate was analysed by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and the gels were processed for autoradiography and scanned in a LKB laser beam scanner.

#### *General*

The results (means  $\pm$  s.e.) of at least three separate observations presented here are from a representative experiment. However, each experiment was repeated at least three times with different batches of placental tissue. The gestational ages of the placentae were determined from the time of the last menstrual period, with the gestational age ranging from 6 to 10 weeks. Due to problems involved in collecting tissues of the same gestational age on a single day, placentae collected from gestations of between 6 and 10 weeks on the same day were pooled. Although the absolute values varied between experiments due to variability in the health of the subjects and their exact gestational ages, the pattern of response was comparable in each experiment. The results were analysed for statistical significance using Student's *t*-test and all *P* values below 0.05 were considered to be significant.

## RESULTS

### **Effect of the addition of AI on OE<sub>2</sub> and progesterone levels in the placental tissue**

A dose-dependent decrease in tissue OE<sub>2</sub> levels was seen following the addition of AI. Although the maximum decrease was seen with 30 µM AI (Table 1), as the inhibition at 15 µM (71.5%) was not significantly different from that seen at 30 µM (76.8%) in all studies 15 µM was used. However, the addition of AI had no effect on the levels of progesterone, thus establishing the specificity of the inhibitor at the concentration used.

### **Effect of the addition of AI, TMX or OE<sub>2</sub> on immunoreactive hCG levels in the tissue and medium of minced first trimester human placentae**

Analysis of the tissue homogenate and incubation medium for immunoreactive hCG indicated a significant increase in the levels of hCG in the tissue and a marginal increase in the medium after the addition of AI (Fig. 1). This increase was partially reversed when OE<sub>2</sub> was added along with AI,

TABLE 1. Effect of the addition of aromatase inhibitor on the levels of oestradiol-17 $\beta$  and progesterone in minced placenta: oestradiol and progesterone were estimated by specific radioimmunoassays

Aromatase inhibitor ( $\mu\text{M}$ )	Oestradiol (ng/g tissue)	Progesterone (ng/g tissue)
Control	2.8 $\pm$ 0.31	125 $\pm$ 2.5
5	1.0 $\pm$ 0.12**	120 $\pm$ 15.0
10	1.3 $\pm$ 0.20**	128 $\pm$ 3.0
15	0.8 $\pm$ 0.07**	118 $\pm$ 10.0
30	0.65 $\pm$ 0.10**	113 $\pm$ 17.0

Results are means  $\pm$  S.E. of triplicate experiments.  
\*\* $P$  = 0.01 compared with control (Student's  $t$ -test).

although the reversal was much more evident in the medium (Fig. 1a and b). However, OE<sub>2</sub> alone was not effective in suppressing the levels of hCG in either tissue or medium.

In a separate experiment, the effect of the addition of higher concentrations of OE<sub>2</sub> on hCG levels in the tissue and medium was examined (Fig.

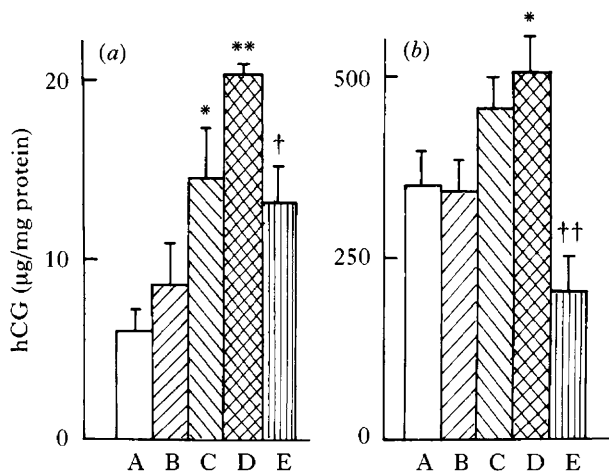


FIGURE 1. Effect of the addition of aromatase inhibitor (AI) on immunoreactive levels of human chorionic gonadotrophin (hCG) in the tissue and medium of first trimester human placenta. Each value is the mean  $\pm$  S.E. of three observations. Minced first trimester human placenta was incubated with no additions (A), 10 nM oestradiol-17 $\beta$  (OE<sub>2</sub>) (B), 1  $\mu\text{M}$  AI (C), 10  $\mu\text{M}$  AI (D) or 10 nM OE<sub>2</sub> and 10  $\mu\text{M}$  AI (E) for 4 h at 37 °C under 95% O<sub>2</sub> and 5% CO<sub>2</sub>. hCG was quantitated both in medium and tissue by plastic tube radioimmunoassay. For experimental details refer to the Materials and Methods; (a) hCG levels in tissue, (b) hCG levels in medium. \* $P$  < 0.05, \*\* $P$  < 0.001 compared with control; † $P$  < 0.02, †† $P$  < 0.01 compared with group treated with 10  $\mu\text{M}$  AI alone.

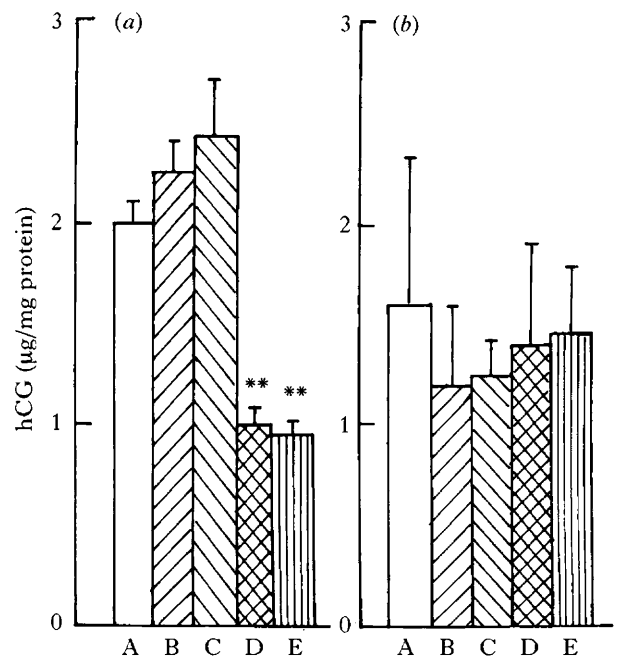


FIGURE 2. Effect of the addition of oestradiol-17 $\beta$  (OE<sub>2</sub>) on immunoreactive levels of human chorionic gonadotrophin (hCG) in minced first trimester human placenta and medium. Each value is the mean  $\pm$  S.E. of three observations. Minced first trimester human placenta was incubated with or without OE<sub>2</sub> (A, 0  $\mu\text{M}$ ; B, 0.074  $\mu\text{M}$ ; C, 0.74  $\mu\text{M}$ ; D, 3.7  $\mu\text{M}$ ; E, 7.4  $\mu\text{M}$ ) for 4 h at 37 °C under 95% O<sub>2</sub> and 5% CO<sub>2</sub>. hCG was quantitated both in medium and tissue by plastic tube radioimmunoassay. For experimental details refer to the Materials and Methods; (a) hCG levels in tissue, (b) hCG levels in medium. \*\* $P$  < 0.001 compared with control.

2). A significant decrease in tissue levels was seen only at 3.7 and 7.4  $\mu\text{M}$  OE<sub>2</sub> (Fig. 2a). In contrast, TMX was found to be effective even at low concentrations (0.1  $\mu\text{M}$ ) in suppressing hCG levels in tissue (Fig. 3a), while a decrease in the hCG levels in the medium (Fig. 3b) could be seen only at high concentrations (1 and 5  $\mu\text{M}$ ).

#### Effect of the addition of AI, TMX, OE<sub>2</sub>, AI+OE<sub>2</sub> or AI+TMX on the levels of $\beta$ and $\alpha$ hCG subunit specific mRNAs

Addition of AI resulted in an increase in the levels of  $\beta$  hCG subunit mRNA by 110% over the control levels (Fig. 4, panel I). In contrast, the addition of TMX, which was used as an antagonist to block the action of OE<sub>2</sub>, resulted in a decrease of 70% in the levels of hCG  $\beta$  mRNA. While OE<sub>2</sub> had no discernible effect at concentrations of 10 and

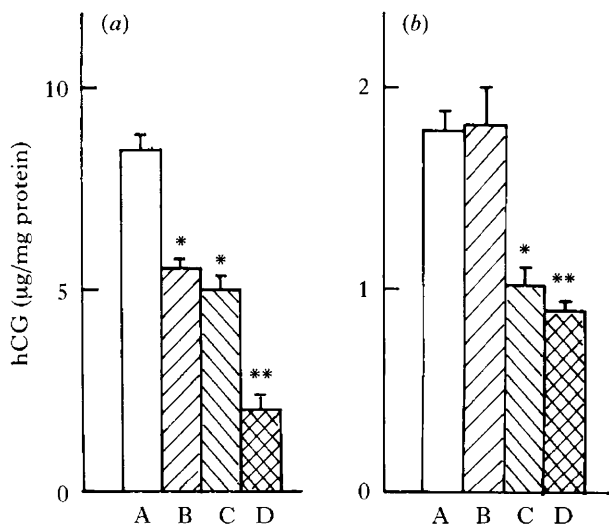


FIGURE 3. Effect of the addition of tamoxifen (TMX) on immunoreactive levels of human chorionic gonadotrophin (hCG) in minced first trimester human placenta and medium. Each value is the mean  $\pm$  S.E. of three observations. Minced first trimester human placenta was incubated with or without TMX (A, 0  $\mu$ M; B, 0.1  $\mu$ M; C, 1  $\mu$ M; D, 5  $\mu$ M) for 4 h at 37 °C under 95% O<sub>2</sub> and 5% CO<sub>2</sub>. hCG was quantitated both in medium and tissue by plastic tube radioimmunoassay. For experimental details refer to the Materials and Methods; (a) hCG levels in tissue, (b) hCG levels in medium. \* $P$ <0.002, \*\* $P$ <0.001 compared with control.

100 nM, it exerted a marginal (28%) inhibitory effect at a concentration of 500 nM. It may be pertinent to point out that TMX is known to have both agonistic and antagonistic effects and was perhaps acting in the present case as an agonist exerting effects that were similar to the addition of high concentrations of OE<sub>2</sub>.

The results of the studies carried out on the effect of the addition of TMX and OE<sub>2</sub> along with AI are presented in Fig. 4, panel II. It can once again be seen that the inhibition of oestrogen synthesis in minced first trimester human placenta by the addition of AI resulted in an increase in the levels of  $\beta$  hCG subunit mRNA levels by 78% over the control values. Addition of OE<sub>2</sub> with AI maintained the levels of  $\beta$  mRNA at control levels, while the addition of TMX with AI resulted in a drastic decrease in the levels of  $\beta$  hCG subunit mRNA.

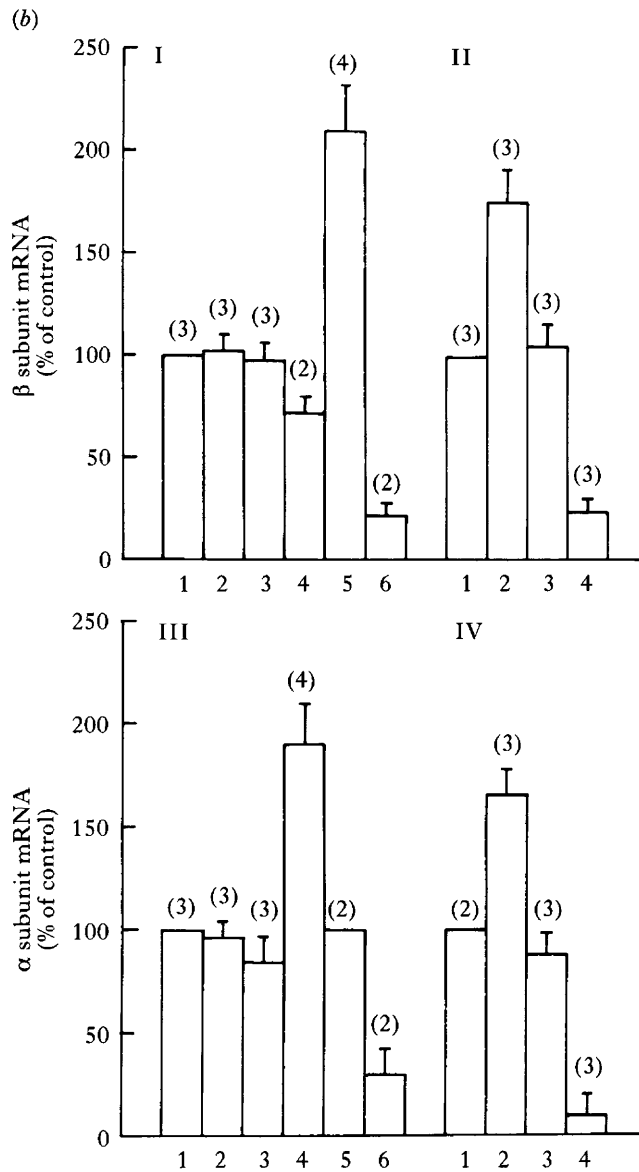
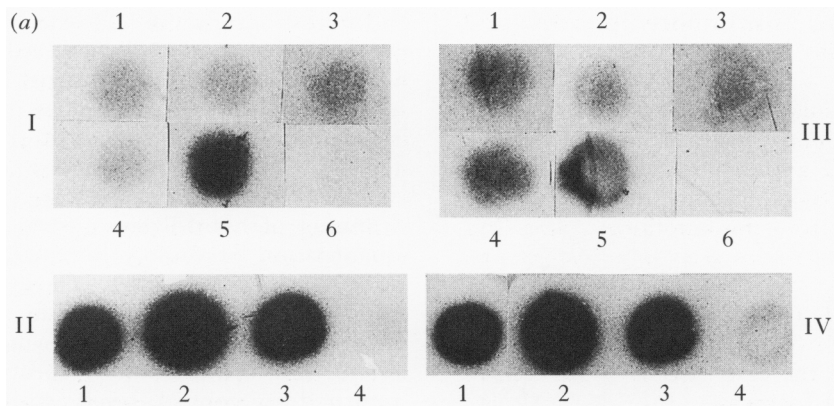
It can also be seen from the results presented in Fig. 4, panel III, that, as was the case for  $\beta$  hCG mRNA levels, the addition of AI resulted in an increase of over 90% in the levels of the  $\alpha$  message and TMX caused a drastic decrease (70%).

Interestingly, the effect of AI on the levels of  $\alpha$  mRNA was also partially reversed by the addition of OE<sub>2</sub> along with AI (Fig. 4, panel IV). However, the addition of TMX along with AI completely reversed the stimulatory effect of AI and decreased the levels to below basal values.

These results are further supported by the scanning of hybridization spots as well as by the quantitation of radioactivity in the hybridization spots (data not shown). Thus, while there were 230 and 310% increases in the radioactivity recovered in the spots following the addition of AI for  $\beta$  and  $\alpha$  subunits respectively, these values returned to control levels with the addition of OE<sub>2</sub>. However, with TMX, the amount of radioactivity recovered decreased drastically in the cases of both  $\alpha$  and  $\beta$  subunits. It can also be seen that the effect of TMX was much more pronounced when compared with OE<sub>2</sub>, in that it drastically decreased  $\beta$  hCG mRNA well below control levels. However, the addition of OE<sub>2</sub> resulted in levels of  $\beta$  hCG subunit mRNA which were near control values.

### Northern blot analysis

The effects of the addition of AI, TMX or OE<sub>2</sub> on the levels of  $\beta$  hCG subunit mRNA, as judged by Northern blot hybridization analysis, are presented in Fig. 5a and b. It is evident that, based on the DNA size markers, the signal seen in the control lane is of 1.05 kb, which corresponds to the size of mRNA for  $\beta$  hCG reported in the literature (Ringler *et al.* 1989). It can also be seen that addition of AI resulted in a very clear increase in the levels of mRNA specific for  $\beta$  hCG; this increase was nearly 88% greater than the control, as assessed by scanning (Fig. 5b). The addition of OE<sub>2</sub> alone had no discernible effect, while the addition of TMX resulted in a drastic decrease (over 80%) in the level of  $\beta$  hCG mRNA. As expected, the addition of OE<sub>2</sub> along with AI prevented the increase induced by AI, while addition of TMX along with AI not only prevented the increase but also resulted in a decrease in the level of  $\beta$  hCG message. These conclusions are evident not only from the intensity of the signals but also from scanning the autofluorogram (Fig. 5b). Analysis of 28S and 18S RNAs before transfer to nitrocellulose revealed no differences (data not shown) in the quantities of RNA loaded from various groups. This establishes that the observed differences in the levels of mRNA are not due to differences in the quantities of RNA loaded.



### In-vitro translation, immunoprecipitation and SDS-PAGE analysis

In addition, RNA isolated in the above experiment was translated *in vitro*, the product was immunoprecipitated using hCG antiserum and the precipitate was subjected to SDS-PAGE and autofluorography. It can also be seen from the autofluorograms (Fig. 6a and b) that the increases seen in the levels of  $\alpha$  and  $\beta$  hCG mRNA following the addition of AI were also reflected in the translation product, namely  $\alpha$  and  $\beta$  hCG (62% increase in the case of  $\alpha$  and 50% increase in the case of  $\beta$  over the control; Fig. 6, panel I). A decrease of nearly 50% was observed following the addition of TMX (Fig. 6, panel II), indicating a decrease in the levels of both  $\alpha$  and  $\beta$  hCG mRNA. Results of studies carried out using [ $^{35}$ S]methionine to monitor the synthesis of hCG in the presence or absence of modulators and immunoprecipitation of radioactive hCG using antiserum to whole hCG are also supportive of the negative modulation of hCG by OE<sub>2</sub> (Fig. 6, panel III).

### DISCUSSION

Over the years it has become well established that pituitary gonadotrophins are subject to negative regulation by gonadal steroids, and these conclusions have been confirmed by studies using molecular biological approaches (Counis *et al.* 1983; Nilson *et al.* 1985; Papavasiliou *et al.* 1986; Gharib *et al.* 1987, 1990). It is pertinent to point out that, in the whole animal model, the effects of added OE<sub>2</sub> can be studied in the relatively complete absence of the influence of endogenous steroids by performing castration or ovariectomy. In the case of human placenta, in which the same cell, namely the syncytiotrophoblast, produces hCG as well as OE<sub>2</sub>, the influence of endogenous steroids cannot be completely eliminated. In fact, it has been suggested that, as the placenta is already exposed to large quantities of OE<sub>2</sub>, the effects of added steroids which are seen are over and above those due to

endogenous hormone (Joel *et al.* 1961). In the present study, the influence of endogenous OE<sub>2</sub> has been eliminated as far as possible by the use of the AI; the inhibition of the enzyme aromatase by this compound to the extent of 81% is well documented in the literature (Schwarzel *et al.* 1973). In our studies, a clear dose-dependent decrease in OE<sub>2</sub> concentration in the placenta was observed following the addition of AI. This suggests that the placenta is capable of synthesizing OE<sub>2</sub> from endogenous substrate even though no precursors have been provided in the medium. It should be noted that Wunsch *et al.* (1986) have recently reported that term placental cells produce increased quantities of OE<sub>2</sub> over a period of 96 h in the absence of any added precursor. Although a culture of purified cells would have been the ideal system, in view of the very low yield of purified cells from first trimester human placenta (6–10 weeks, weighing only 5–10 g), we have restricted our studies to a minced system. We have established the functional viability of the minced tissue in an earlier study (Mathialagan & Rao, 1986), for a period of 4–6 h under in-vitro conditions.

The results of the present study using the AI show that the synthesis of both  $\alpha$  and  $\beta$  subunits is under the negative control of OE<sub>2</sub>. This conclusion is based on the results obtained from monitoring the effects of added AI and TMX on the levels of immunoreactive hCG, and  $\alpha$  and  $\beta$  specific mRNA, as judged by dot and Northern blot hybridization analysis, in-vitro translation of the isolated mRNA, and finally by monitoring the biosynthesis of hCG.

Although we considered monitoring actin as a housekeeping gene, there are several reports of actin mRNA varying with OE<sub>2</sub>, and thus it is suggested that it cannot be used as a true control (Hsu & Frankel, 1987; Fabienne L'Horset *et al.* 1990). However, in all the studies equal quantities of RNA were loaded (data not shown) and most of the experiments were repeated at least three times. It should be noted that the synthesis of the  $\beta$  CG subunit is rate-limiting in the synthesis of hCG by the human placenta (McQueen *et al.* 1978), and thus we feel that the estimation of immunoreactive total

**FIGURE 4.** Effect of the addition of oestradiol-17 $\beta$  (OE<sub>2</sub>), aromatase inhibitor (AI), tamoxifen (TMX), AI+OE<sub>2</sub> or AI+TMX on  $\beta$  (panels I and II) and  $\alpha$  (panels III and IV) hCG subunit mRNA levels in first trimester human placenta (FTHP). (a) Dot blot hybridization: representative autofluorogram. Minced FTHP was incubated with OE<sub>2</sub>, AI or TMX for 4 h. Total RNA was isolated and  $\alpha$  and  $\beta$  hCG subunit specific mRNA was quantitated by dot blot hybridization. Panel I: 1) control, 2) 10 nM OE<sub>2</sub>, 3) 100 nM OE<sub>2</sub>, 4) 500 nM OE<sub>2</sub>, 5) 15  $\mu$ M AI, 6) 5  $\mu$ M TMX. Panel II: 1) control, 2) 15  $\mu$ M AI, 3) 15  $\mu$ M AI+10 nM OE<sub>2</sub>, 4) 15  $\mu$ M AI+5  $\mu$ M TMX. Panel III: 1) control, 2) 100 nM OE<sub>2</sub>, 3) 500 nM OE<sub>2</sub>, 4) 15  $\mu$ M AI, 5) control, 6) 5  $\mu$ M TMX. Panel IV: 1) control, 2) 15  $\mu$ M AI, 3) 15  $\mu$ M AI+10 nM OE<sub>2</sub>, 4) 15  $\mu$ M AI+5  $\mu$ M TMX. (b) Graphic representation of scans of the corresponding autofluorograms in (a). Each bar represents the mean  $\pm$  s.e. of the number of observations (*n*) indicated. The area of the control group is taken as 100% and values for treated groups are expressed as percentages of the control value.

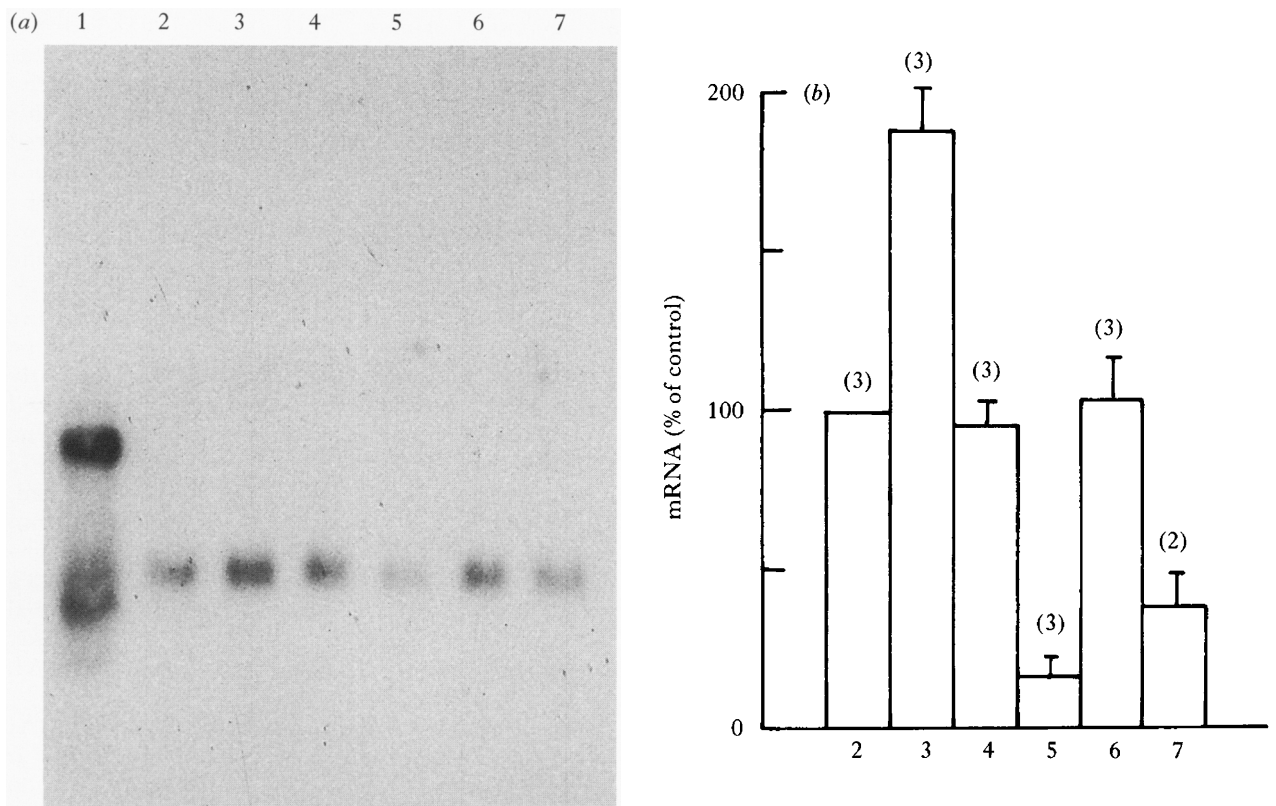


FIGURE 5. Effect of the addition of aromatase inhibitor (AI), oestradiol-17 $\beta$  (OE<sub>2</sub>) and tamoxifen (TMX) on  $\beta$  human chorionic gonadotrophin (hCG) subunit mRNA levels and OE<sub>2</sub> and TMX on AI-stimulated  $\beta$  hCG subunit mRNA levels. (a) Northern analysis: representative autoradiogram. Minced first trimester human placental tissue was incubated with OE<sub>2</sub>, TMX, AI, AI+OE<sub>2</sub> or AI+TMX for 4 h. Total RNA was isolated, separated by denatured formaldehyde agarose gel electrophoresis and Northern analysis was carried out. For experimental details refer to the Materials and Methods. Lane 1, 1 kb ladder (lower band 1.01 kb, upper band 1.63 kb); lane 2, control; lane 3, AI (15  $\mu$ M); lane 4, OE<sub>2</sub> (10 nM); lane 5, TMX (5  $\mu$ M); lane 6, AI (15  $\mu$ M)+OE<sub>2</sub> (10 nM); lane 7, AI (15  $\mu$ M)+TMX (5  $\mu$ M). (b) Graphic representation of a scan of the corresponding autoradiogram in (a). Each bar represents the mean  $\pm$  s.e. of the number of observations (*n*) indicated. The area of the control group is taken as 100% and the values of treated groups are expressed as percentages of the control value.

hCG would be adequate compared with the estimation of  $\alpha$  and  $\beta$  CG subunits separately.

The earlier report of Belleville *et al.* (1978) and the recent work of Maruo *et al.* (1986) indicating that the addition of OE<sub>2</sub> has no effect on hCG synthesis or on the levels of its  $\alpha$  and  $\beta$  mRNAs could result from interference by endogenous steroids. However, the addition of low concentrations of OE<sub>2</sub> was not very effective in decreasing the level of immunoreactive hCG. These experiments were carried out with the assumption that if hCG is under the negative control of endogenous OE<sub>2</sub>, then the addition of OE<sub>2</sub> should be much more effective in the inhibition of immunoreactive hCG levels. However, as can be seen from the results presented in Fig. 1, while the effects of AI could be reversed by the simultaneous addition of OE<sub>2</sub>, OE<sub>2</sub> was not

effective in suppressing hCG levels by itself, although at very high concentrations (3.7 and 7.4  $\mu$ M) it was partially effective (Fig. 2).

In contrast, TMX was effective in the presence or absence of AI over a range of concentrations (0.1–5.0  $\mu$ M, Fig. 3). In view of this, similar studies on the effect of addition of TMX and OE<sub>2</sub> together were not carried out as TMX was very effective independently, even at a concentration as low as 0.1  $\mu$ M, and it was felt that TMX would override the effects of OE<sub>2</sub> and still act as an agonist. In fact, initially the purpose of using TMX was to block the action of OE<sub>2</sub> at the receptor level, and to examine whether we could observe effects similar to those observed following the addition of AI. However, we consistently observed only an agonistic effect. Although traditionally TMX is used as an oestrogen



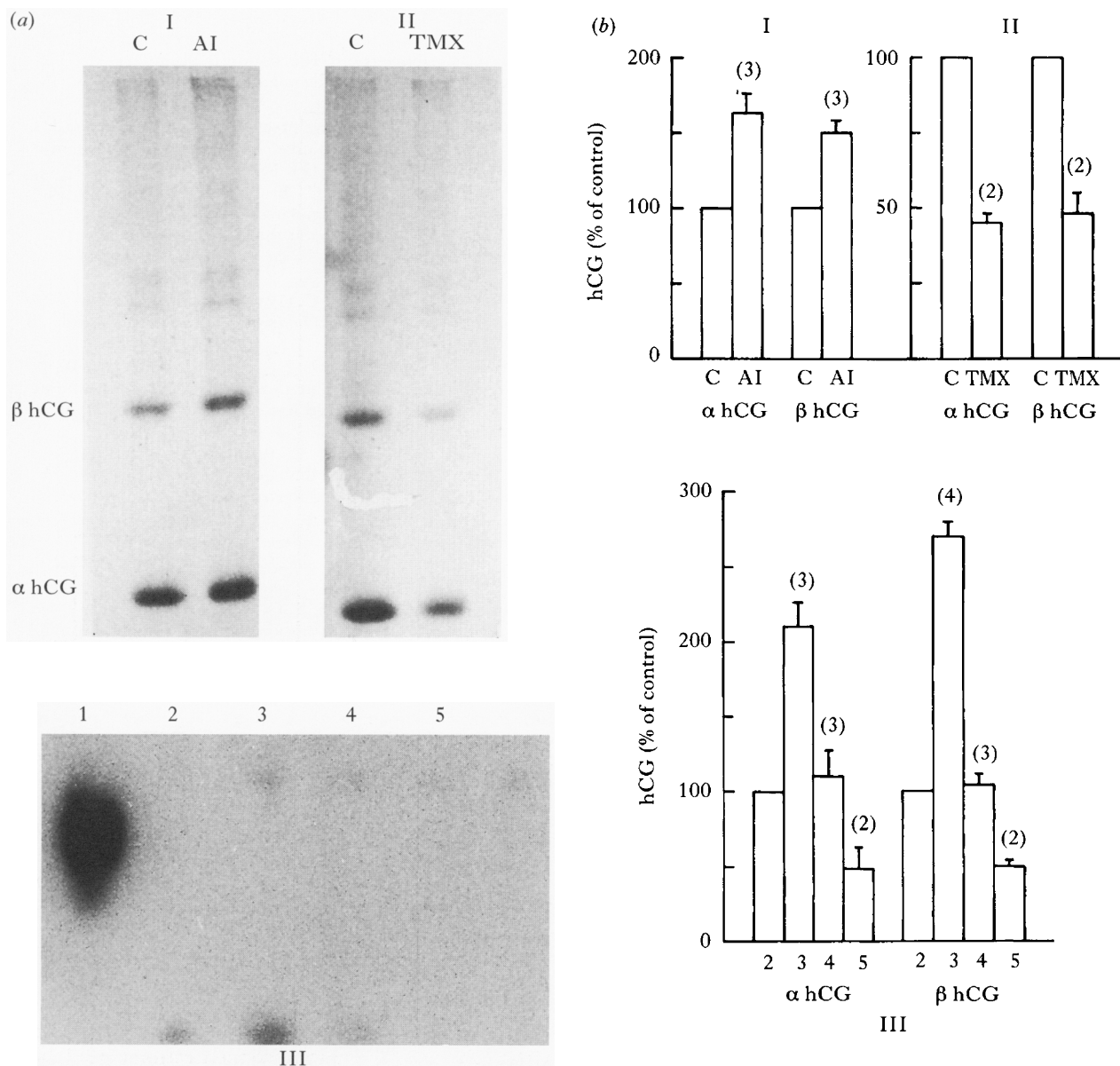


FIGURE 6. Effect of the addition of aromatase inhibitor (AI) or tamoxifen (TMX) on the levels of  $\alpha$  and  $\beta$  human chorionic gonadotrophin (hCG). (a) Panels I and II: representative autofluorograms are shown of SDS-PAGE analysis of in-vitro translated and immunoprecipitated products (C is control). Poly(A)<sup>+</sup> RNA was isolated from each group and translated *in vitro* using rabbit reticulocyte lysate. The product was immunoprecipitated by a specific antiserum to whole hCG. Panel III: an autofluorogram of SDS-PAGE analysis of the in-vitro biosynthesis of hCG using [ $^{35}$ S]methionine, followed by immunoprecipitation of the in-vitro translated product by a specific antiserum to whole hCG. Minced first trimester human placenta was incubated with AI or TMX for 4 h. Lane 1, [ $^{125}$ I]-labelled  $\beta$  subunit; lane 2, control; lane 3, AI (15  $\mu$ M); lane 4, oestradiol-17 $\beta$  (10  $\mu$ M); lane 5, TMX (5  $\mu$ M). (b) Graphic representation of the scans of the corresponding autofluorograms in (a). Each bar represents the mean  $\pm$  s.e. of the number of observations (*n*) indicated. The area of the control group is taken as 100% and the values of treated groups are expressed as percentages of the control value.

receptor antagonist it is known to behave both as an agonist and an antagonist depending on the dose and the type of tissue used (Furr & Jordan, 1984). It

is possible that in the present situation TMX may have been acting as an agonist, thus exerting a negative influence on the synthesis of hCG. In fact,

in an earlier study (Sharma *et al.* 1990), we have demonstrated that TMX does act as an agonist in the first trimester human placenta. However, its complete range of actions in the placenta is yet to be evaluated. Although we have not tested it at a concentration lower than 0.1 µM, it is possible that at even lower concentrations it may behave as an antagonist, resulting in effects similar to those seen with the AI. Our studies have also revealed (data not shown) that TMX is not toxic to the minced tissue at the concentration used, as judged by the incorporation of [<sup>3</sup>H]thymidine into DNA.

The results of the present study thus permit us to suggest that the synthesis and secretion of hCG subunits are under the negative control of OE<sub>2</sub>, although it is possible that the other major placental steroid, progesterone, may also have a role in the regulation of the synthesis of hCG. In fact, the inverse relationship between the declining levels of hCG and the rising levels of placental steroids during human pregnancy provides additional support for such a suggestion.

#### ACKNOWLEDGEMENTS

The authors wish to thank Dr I. Boime, Washington Medical University School, St Louis, MO, U.S.A., for providing clones for the α and β hCG subunits and the Department of Science and Technology, New Delhi, India, for financial assistance. A. J. R. is grateful to the Rockefeller Foundation for his Biotechnology Career Fellowship and P. P. wishes to thank CSIR for awarding him the position of Pool Officer. The authors also wish to thank Prof. G. Padmanabhan, Department of Biochemistry, Indian Institute of Science, Bangalore, India, for advice during the course of this work and the Superintendent and Staff of the Family Planning Clinic of K. C. General Hospital, Bangalore, India, for permitting the collection of placentae.

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REVISED MANUSCRIPT RECEIVED 23 October 1992