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Role of 17β-Estradiol and Progesterone in the Regulation of Synthesis and Secretion of Chorionic Gonadotropin by the First Trimester Human Placenta

A. Jagannadha Rao,* K. S. S. Prasad, S. C. Sharma and V. S. R. Subbarayan

Department of Biochemistry and Center for Reproductive Biology and Molecular Endocrinology, Indian Institute of Science, Bangalore 560 012, India

The role of 17β-estradiol and progesterone in the regulation of synthesis of chorionic gonadotropin (CG) by first trimester human placenta has been studied using 1,4,6 androstatriene 3-17-dione to block the synthesis of 17β-estradiol or tamoxifen to block its action at receptor level and RU486 to block the action of progesterone at the receptor level. Results indicate that the synthesis of CG is negatively modulated by 17β-estradiol and positively modulated by progesterone as judged by the change in the levels of immunoreactive CG, α- and β-CG mRNA and *in vitro* translation, as well as biosynthesis of α- and β-CG and, finally, nuclear run off transcription.

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INTRODUCTION

Human chorionic gonadotrophin (hCG) is a glycoprotein hormone produced by the syncytiotrophoblast of the human placenta [1]. It consists of two dissimilar subunits, α and β, held together by non-covalent interactions [2]. The α-subunit of hCG is identical to the α-subunit of human pituitary LH while the β-subunit shares 82% sequence homology with the β-subunit of human pituitary LH [3]. Available evidence indicates that the main function of hCG is to maintain and stimulate the steroidogenic function of the corpus luteum of the fertile cycle [4]. Thus hCG shares both structural and functional homology with human pituitary LH, the regulation of which by gonadotrophin releasing hormone and gonadal steroids is well documented in literature [5, 6]. Considering this, we reasoned that the synthesis and secretion of hCG may also be subject to similar regulation by the two placental steroids, 17BE-estradiol (E_2) and progesterone. In the present study, we have examined the role of E_2 and progesterone (P_4) in the regulation of hCG using 1,4,6,androstatrien 3,17-dione, aromatase inhibitor (AI) to block the synthesis of E_2, tamoxifen (TMX) to block E_2 action and RU486 to block the action of P_4, respectively, at the receptor level.

MATERIALS AND METHODS

First trimester human placenta (6–12 weeks) was collected from cases of medical termination of pregnancy from the local hospital. The tissue was collected in cold Earl's balanced salt solution (EBSS), washed with cold EBSS and villous tissue was separated by visual examination. The villous tissue was finely minced, suspended in EBSS buffer and about 80–100 mg or 0.5–1 g of tissue (wet weight) was dispensed in a total volume of 0.5–5 ml or 3 ml and used for further studies.

Unlabelled E_2, P_4 and AI were obtained from Steroloids Inc., Wilton, U.S.A.; tamoxifen was a gift from Imperial Chemical Industries Ltd., London, U.K.; RU486 was generously provided by Rousell-Uclaff, France; rabbit reticulocyte lysate was obtained from Amersham International, U.K.; α-[^{32}P] dCTP 3000 Ci/mmol, γ [^{35}S] methionine (800 Ci/mmol) were obtained from Bhabha Atomic Research Centre, Bombay; nitrocellulose membrane filters were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A.; Trizma base, formamide, oligo-DT-cellulose, potassium acetate, dithiothrietol (DTT) and sodium

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*Correspondence to A. J. Rao.

dodecyl sulphate (SDS) were purchased from Sigma Chemical Co., St Louis, MO, U.S.A.; and 1 kb DNA markers were obtained from Bethesda Research Laboratories, Life Technologies Gaithesburg Inc., U.S.A. Highly purified human chorionic gonadotropin (13,000 IU/mg) α- and β-subunits of hCG were generously provided by NIAMDD, NIH, Bethesda, MD, U.S.A. All other chemicals used were obtained locally and were of analytical grade.

Clones for α-hCG subunit cDNA and β-hCG subunit cDNA cloned at the ampicillin pstI site in pBR322 vector, obtained as a gift from Dr I. Boime (Department of Pharmacology, Washington University, St Louis, MO, U.S.A.) were used in the studies on the role of E_2 while the α- and β-hCG cDNA probes cloned at the HindIII site in pBR322 vector, obtained from Dr J. Fiddes, California Biotechnology Inc., Mountain View, were used in studies on the role of P_4. These were transformed in *E. coli* and α-(621 bp) and β-(580 bp) hCG inserts were isolated and labelled by standard procedures with α-[^{32}P] dCTP by random priming method using a kit from Boeringer Mannheim, Germany.

Effect of addition of E_2 and/or AI or TMX, P_4 and/or RU486 to first trimester human placental (FTHP) minces

Effect on immunoreactive hCG levels in the tissue. Placental minces were incubated in triplicate in 3 ml tubes with or without E_2 and/or TMX or AI, and P4 and/or RU486 for 2 to 4 h at 37°C under 95% O_2 and 5% CO_2 in a total volume of 0.5 ml. Medium and tissue were separated by centrifugation and hCG was estimated in the medium and tissue by specific solid phase RIA [7]. hCG in each sample was initially calculated as μg/mg tissue protein and expressed as percent increase or decrease (change) over the untreated sample which was considered as 100%.

Effect on α- and β-hCG mRNA levels. First trimester placental minces (0.5–1 g wet weight) were incubated in a 10 ml conical flask as described above. Following the incubation, tissue and medium were separated by centrifugation and tissue was processed for isolation of total RNA according to the procedure of Boime *et al.* [8]. Total RNA was quantitated by monitoring optical density at 260 nm. Poly A$^+$ RNA was isolated by chromatography on oligo dT cellulose as described by Aviv and Leader [9]. RNA (30 μg) was subjected to electrophoresis on 1% agarose gels containing 2.2 M formaldehyde and RNA was transferred to nitrocelluse filters as described by Southern [10]. The filters were baked in a vacuum oven at 80°C and processed for hybridization with cDNA probes for α- and β-hCG labelled by random priming method. The nitrocellulose filters were washed with 2 × SSC (1 × SSC is 8.765 g NaCl, 4.41 g sodium citrate in 1 l of double distilled water, pH 7.0) four times at room temperature for 20 min each followed by 2 × SSC four times at 65°C for 20 min each and 0.2 × SSC three times at 65°C for 20 min each. Filters were exposed to X-ray film for 3–5 days after drying.

In vitro translation of Poly A$^+$ RNA isolated from AI (15 μM) or TMX (5 μM) treated FTHP minces

Poly A$^+$ RNA (1–2 μg) was translated using rabbit reticulocyte lysate. Equal quantities of trichloro acetic acid (TCA) precipitable radioactivity from individual samples was immunoprecipitated by a specific hCG antiserum raised against highly purified hCG in the rabbit. The antiserum was highly specific to hCG and did not show any cross-reactivity with other glycoprotein hormones and thus was found to be suitable for immunoprecipitation studies.

Effect of addition of P_4 and RU486 on in vitro biosynthesis of α-hCG subunit and AI or E_2 or TMX on β hCG subunit by FTHP

FTHP minces were incubated with or without P4 and RU486 or AI of E_2 or TMX under an atmosphere of 95% O_2 and 5% CO_2 in the presence of 30 μCi of [^{35}S]methionine for 3 h at 37°C. At the end of incubation, tissue and medium were separated by centrifugation and tissue was extensively washed with EBSS containing 1 mg unlabelled methionine (1 mg/ml) and TCA precipitable radioactivity was determined in tissue. Equal quantities of TCA precipitable radioactivity were processed for immunoprecipitation with specific antiserum to α- and β-hCG (raised in rabbits) as described by Ramsey *et al.* [11]. Immunoprecipitated products were resolved on 10% SDS–PAGE and subjected to autoflurography.

Effect of addition of E_2 and or AI or TMX on α- and β-hCG gene transcription

Human placental villous minces were incubated with or without E_2 (10 nM) and or AI (15 μM) or TMX (5 μM) for 4 h as described earlier. Nuclei were isolated from the villi according to the procedure of Mulvihill and Palmiter [12]. For transcription 200 μg of DNA equivalent of nuclei were again incubated with E_2 (10 nM) and or TMX (5 μM) or AI (15 μM) in 150 μl reaction mixture (16% glycerol, 20 mM HEPES (pH 7.6), 150 mM NaCl, 5 mM magnesium acetate, 1 mM manganese chloride, 2 mM DTT, 0.4 mM of ATP, GTP and CTP 100 μCi of [^{32}P]UTP (3000 Ci/mmol) 40 units of RNase inhibitor per reaction, 2 mM creatine phosphate and 0.45 units of creatine phosphokinase for 30 min at 26°C. Following this, radioactive RNA was isolated from the nuclei by hot phenol extraction and used for hybridization with linearized α- and β-hCG cDNAs in pBR 322 as described by Piechazyk *et al.* [13], while PBR 322 alone without insert was used as control. After exposing the filters to X-ray film, the hybridization spots in the filter were cut out and radioactivity was determined in a liquid scintillation counter.

General

The results (means ± SE) 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 12 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 12 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 blocking the synthesis or action of E_2 or P4 on immunoreactive CG levels in the tissue and the medium of FTHP minces

Analysis of the levels of immunoreactive hCG in the tissue homogenate and incubation medium revealed a significant increase in the levels of hCG in the tissue and a marginal increase in the medium (data not provided) following addition of AI (Fig. 1, Column 3) and this increase was partially reversed by simultaneous addition of E_2 (Fig. 1, Column 4). Also E_2 at high concentration (7.4 μM) inhibited hCG levels (Fig. 1, Column 2) compared to control (Fig. 1, Column 1). Interestingly, addition of TMX resulted in a drastic decrease in tissue hCG levels (Fig. 1, Column 5). Addition of RU486 also resulted in a significant increase in hCG levels in tissue (Fig. 1, Column 6). However, this could not be reversed by simultaneous addition of P_4 (Fig. 1, Column 7) and addition of P_4 alone resulted in an increase in hCG levels (Fig. 1, Column 8).

Northern blot analysis of RNA

It can be seen from the results presented in Fig. 2 that following addition of AI a significant increase in the message for both α- [Fig. 2(A), Lane 1] and β-hCG [Fig. 2(B), Lane 3] was observed over the corresponding controls [Fig. 2(A), Lane 3 and Fig. 2(B), Lane 1]. This could be partially reversed by simultaneous addition of E_2 [Fig. 2(A and B), Lane 4]. Addition of TMX caused a drastic decrease in the level of α-hCG message level [Fig. 2(A), Lane 2].

Addition of RU486 resulted in a significant increase in the message for both α- and β-hCG [Fig. 3(A and B), Lane 2]. Interestingly in both cases, this increase could not be reversed with simultaneous addition of P_4 [Fig. 3(A and B), Lane 4] and addition of P_4 alone resulted in an increase in the level of both α- and β-hCG messages [Fig 3(A and B), Lane 3].

In vitro translation

Also RNA isolated from AI and TMX treated samples was translated *in vitro*, the product was immunoprecipitated using hCG antiserum and the precipitate was subjected to SDS–PAGE and autoflurography. It is evident from the autofluorogram [Fig. 4(A)] that the increase seen in the level of α- and β-hCG mRNA by Northern blot analysis following addition of AI was also reflected in the translation product. A decrease of nearly 50% in the level of both α- and β-hCG mRNA was seen following addition of TMX [Fig. 4(B)].

Effect on in vitro biosynthesis of α- and β-hCG

The effect of addition of P_4 and or RU486 on the biosynthesis of α-hCG and AI and TMX on β-hCG by the first trimester human placental minces was monitored using [^{35}S]methionine. The radioactive α- and β-hCG were immunoprecipitated with specific antisera

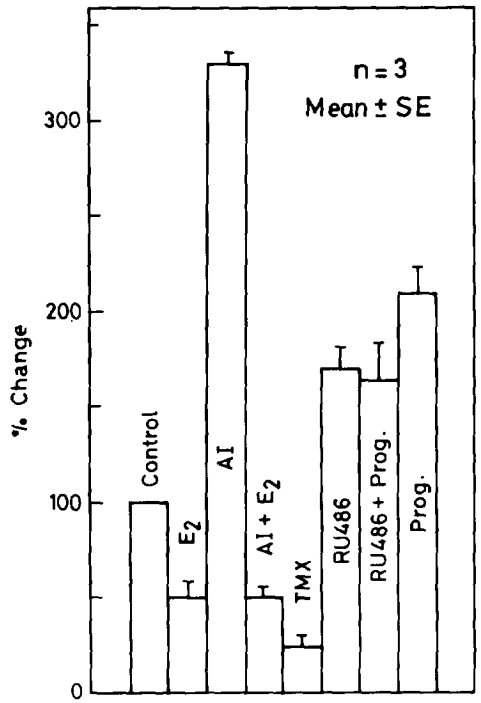

Fig. 1. Effect of addition of E_2 or AI, or TMX or RU486 or P_4 on immunoreactive hCG levels in the tissue of first trimester human placenta. Each value is the mean ± SE of three observations at 120 min. First trimester human placental minces were incubated with: no addition (Column 1); 7.4 μM E_2 (Column 2); 15 μM AI (Column 3); 15 μM AI + 7.4 μM E_2 (Column 4); 5 μM TMX (Column 5); 0.1 μM RU486 (Column 6); 63 μM P_4 (Column 7); 0.1 μM RU486 + 63 μM P_4 at 37°C under 95% O_2 and 5% CO_2 (Column 8). hCG was estimated in the tissue by plastic tube radioimmunoassay. *Significantly different from control $P < 0.001$.

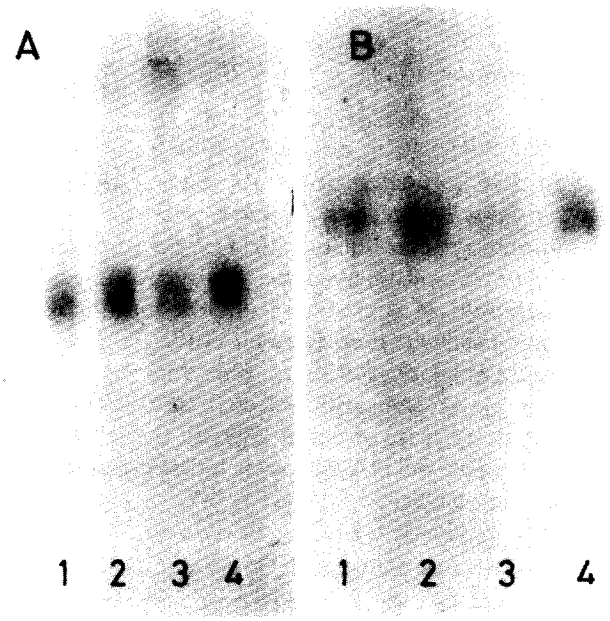

Fig. 2. Effect of addition of E_2 and or AI or TMX to first trimester placental minces on α- and β-hCG specific mRNA: Northern analysis, representative autofluorogram. Placental minces were incubated with: (A) α-hCG: 15 μM AI (Lane 1); 5 μM TMX (Lane 2); no addition (Lane 3); 15 μM AI + 10 nM E_2 (Lane 4). (B) β-hCG: no addition (Lane 1); 10 nM E_2 (Lane 2); 15 μM AI (Lane 3); 15 μM AI + 10 nM E_2 (Lane 4), for 4 h at 37°C under 95% O_2 and 5% CO_2 after which tissue was processed for isolation of total RNA. 20 μg of RNA was resolved by denatured formaldehyde agarose gel electrophoresis and Northern analysis was carried out.

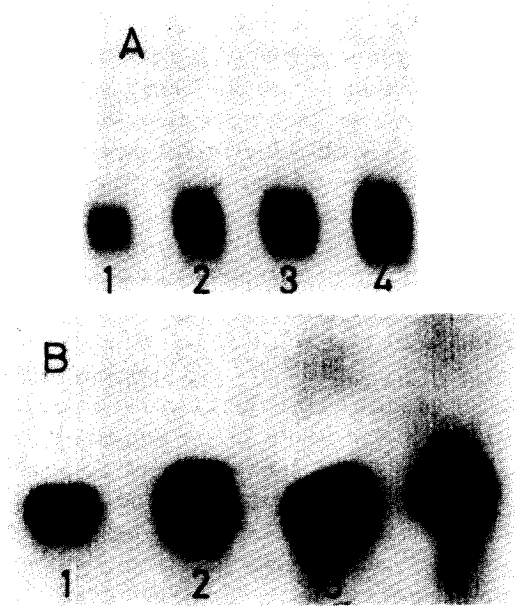

Fig. 3. Effect of addition of P_4 and or RU486 on α- and β-hCG mRNA levels Northern analysis; representative flurogram. For experimental details see Fig. 2 legend. (A) α-hCG mRNA; (B) β-hCG mRNA. (Lane 1) No addition; (Lane 2) RU486 0.1 μM; (Lane 3) P_4 63 μM; (Lane 4) RU486 0.1 μM + P_4 63 μM.

and the products were processed for SDS–PAGE analysis followed by autofluorography. An increase in the synthesis of the α-hCG subunit was seen [Fig. 5(A), Lanes 2 and 3] with RU486 and P_4 and this could not be reversed by simultaneous addition of P_4 along with RU486. In the case of β-hCG, an increase with AI [Fig. 5(B), Lane 4] and decrease with E_2 [Fig. 5(B), Lane 3] and TMX [Fig. 5(B), Lane 4] was seen.

Nuclear run off transcription

Nuclear run off studies using nuclei isolated from placental villi treated *in vitro* with TMX or AI revealed that the effects observed in the mRNA levels are also due to an actual decrease or increase, respectively, in the synthesis of specific messages for both α- and β-hCG. It is evident from the autoflurogram (Fig. 6) that following addition of AI there is an increase in the signal for both α [Fig. 6(A), Lane 3] and β [Fig. 6(B), Lane 4] and a decrease following addition of TMX [Fig. 6(A), Lane 4 and Fig. 6(B), Lane 3]. As can be expected this effect of AI could be partially reversed by the simultaneous addition of E_2. Interestingly, when TMX was added along with AI it had an overriding effect by blocking the increase due to addition of AI [Fig. 6(A), Lane 6]. In addition determination of radioactivity in the hybridization spots revealed that while there was over a 50% decrease in the case of TMX, an increase of over 133% was seen following addition of AI.

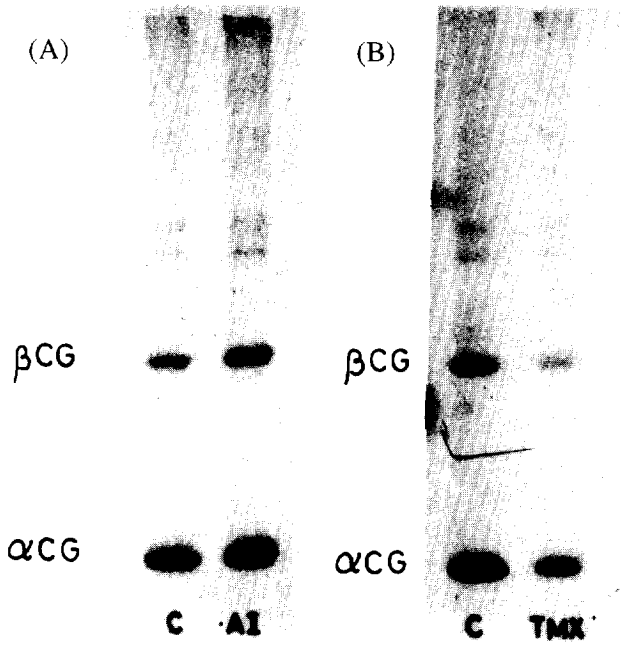

Fig. 4. Effect of addition of E_2 and or AI or TMX on the levels of α- and β-hCG subunit mRNA as judged by *in vitro* translation. Representative autoflurogram of SDS–PAGE analysis of *in vitro* translated and immunoprecipitated products using antiserum to hCG. (A) Effect of AI. (B) Effect of TMX. (Reproduced with permission from the *Journal of Endocrinology*)

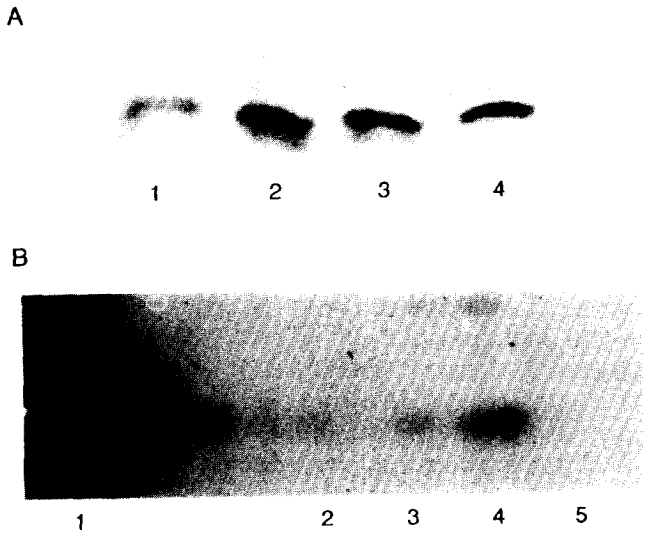

Fig. 5. Effect of addition of P_4 and or RU486 on the levels of α-hCG subunit and E_2 or AI or TMX on beta hCG subunit as judged by *in vitro* biosynthesis. First trimester human placental minces were incubated with 30 μCi of [^{35}S]methionine for 4 h at 37°C in an atmosphere of 95% O_2 and 5% CO_2 after which the tissue was homogenized and processed for immunoprecipitation using specific antiserum to α- (A) and β-hCG (B) antiserum, (A) (Lane 1) No addition; (Lane 2) RU486 0.1 μM; (Lane 3) P_4 63 μM; (Lane 4) RU486 0.1 μM + 63 μM P_4. (B) (Lane 1) [^{125}I] β-hCG subunit; (Lane 2) no addition; (Lane 3) E_2 10 nM; (Lane 4) AI 15 μM; (Lane 5) TMX 5 μM.

DISCUSSION

Negative and positive regulation of pituitary gonadotropins in both male and female mammals by gonadal steroids namely E_2 and P_4 and testosterone is well documented in literature [5, 6]. As mentioned in the introduction, considering the fact that placenta also functions as a transient hypothalamo–pituitary–gonadal axis by its ability to produce protein, peptide and steroid hormones, it is only justified in assuming that similar regulatory mechanisms may be operative in placenta also. Based on this hypothesis, studies were initiated to examine the role of E_2 and P_4 in regulation of hCG in the human placenta. However, unlike the whole animal model, where the effect of added E_2 or P_4 can be studied in relatively complete absence of influence of endogenous steroids by gonadectomy, such an approach is not possible in the case of placenta as the syncytiotrophoblast is the site of production of both protein, peptide and steroid hormones. Thus, the effect of endogenous steroid hormone cannot be completely eliminated. In the present study we have attempted to examine the effect of deprival of E_2 or P_4 by blocking their synthesis or their action, on CG levels in the human placenta.

The result of the present study using AI indicates that the synthesis of both α- and β-hCG subunits is under the negative control of E_2. This conclusion is based on the results obtained after monitoring the effects of added AI and TMX on the levels of (a) immunoreactive hCG; (b) α- and β-hCG specific mRNA; and (c) *in vitro* translation of poly A^+ RNA as well as nuclear run off experiments to monitor the synthesis of specific messages. In contrast to these results the results obtained following addition of RU486 and P_4 indicate that the synthesis of α- and β-hCG is positively modulated by P_4. These studies on the role of P_4 were initiated with the assumption that it may also exert an inhibitory effect like E_2 as it is produced in large quantities. In fact, based on the increase in immunoreactive hCG levels following addition of RU486, it was initially assumed that it was also exerting a negative control like E_2. However, it was interesting to note that the effect of RU486 could not be reversed by simultaneous addition of P_4 and addition of P_4 also resulted in an increase in hCG levels. These effects on the increase in immunoreactive hCG were reflected at the mRNA level as well as in the synthesis of α- and β-hCG subunits. Preliminary studies suggest that addition of aminoglutathamide, which blocks the activity of the cholesterol side chain cleave enzyme which results in a decrease in the level of P_4, also resulted in a decrease in the P_4-induced increase in the hCG message, thus providing supportive evidence to the above conclusion. The effect of E_2 or P_4 on the level of house keeping genes like actin was not monitored as reports indicate that its mRNA varies with E_2 treatment [14, 15]. In addition, in the case of studies involving Northern blot analysis of RNA following addition of P_4

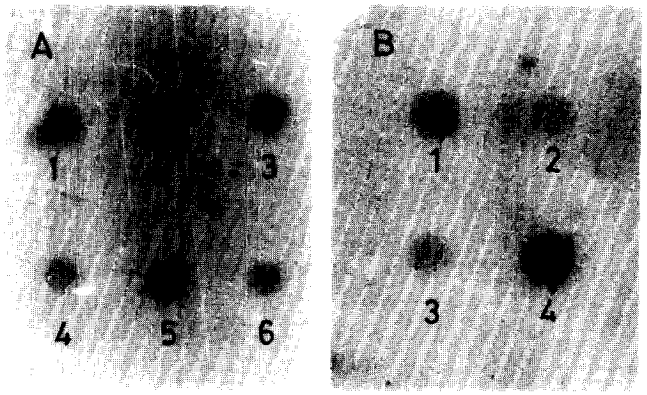

Fig. 6. Nuclear run off transcription of α- and β-hCG genes. Effect of addition of E_2 and or AI or TMX. First trimester human placental minces (0.5–1 g) wet weight were incubated with E_2 or AI or TMX for 4 h at 37°C in an atmosphere of 95% O_2 and 5% CO_2 for 4 h following which active nuclei were isolated from each sample. 200 μg DNA equivalent of nuclei were once again incubated with E_2 or AI or TMX in a total volume of 150 μl at 26°C along with 100 μCi [^{32}P]UTP (3000 Ci/mmol) for 30 min after which radioactive RNA was isolated and processed for hybridization with unlabelled α-hCG and β-hCG cDNA probes. (A) α-gene transcription: (Lane 1) control; (Lane 2) E_2 10 nM; (Lane 3) AI 15 μM; (Lane 4) TMX 5 μM; (Lane 5) AI 15 μM + E_2 10 nM; (Lane 6) AI 15 μM + TMX 5 μM. (B) β-gene transcription: (Lane 1) control; (Lane 2) E_2 10 nM; (Lane 3) TMX 5 μM; (Lane 4) AI 15 μM.

or RU486, the filters were probed with β-hCG cDNA after probing them first with α-hCG cDNA and stripping the probe. However, it should be noted that at this stage the possibility that the observed increase may also be due to stabilization of the message cannot be completely ruled out.

One important and intriguing result of the present study is the agonistic effect seen with TMX and RU486. Initially the objective in using TMX or RU486 was to block the action of E_2 or P_4 at the receptor level. However, consistently we have only observed the stimulatory effect with TMX, which suggested that it is exerting an agonistic effect. Although traditionally TMX is used as an estrogen receptor antagonist it is known to behave both as agonist or antagonist depending on the dose and type of tissue used and the gene in question [16]. It is possible that in the present situation TMX may be acting as an agonist and thus exerting a negative influence on the synthesis of hCG.

With regard to the agonistic action of RU486, although traditionally it is used as a P_4 receptor antagonist [17], it is also known to exert an agonistic effect. In human endometrial cells a significant increase in prolactin and IGF-βP mRNA was observed following RU486 treatment [18]. Growth stimulation by RU486 in human breast cancer cells (T47D wild type) has also been reported [19]. It has been suggested that these discrepancies could be due to P_4 receptor content as well as differences in P_4 receptor in different cell lines and tissues. Recently Das [20] reported that the syncytiotrophoblast contains receptors for P_4 which have higher affinity for RU486 than for P_4 itself and they observed that a 10-fold higher concentration of P_4 was required to overcome the effect of RU486. Our results also indicate that the concentration of P_4 required is relatively much higher than the concentration of RU486 required to observe a stimulatory effect.

While our results permit us to conclude that hCG is modulated positively by P_4 and negatively by E_2, earlier studies [21] reported that addition of either P_4 or E_2 to human placental tissue had no effect on hCG secretion. It has also been reported that there was a decrease in the α- and β-hCG subunits mRNA, following the addition of P_4, while no effect was seen following the addition of E_2 [22]. However, it should be noted that in these studies no attempt was made to minimize the influence of endogenous steroids. It has been suggested that as the placenta is already exposed to large quantities of E_2, the effect of added steroids seen are over and above those that are due to endogenous hormone [23]. In this connection it is pertinent to mention that glucocorticoids also stimulate CG secretion [24] and RU486 also binds to glucocorticoid receptors [25]. Considering this, the possibility that RU486 is exerting its action by binding to both the P_4 receptor and glucocorticoid receptor cannot be excluded and this perhaps can partly account for the discrepancy between the present and earlier studies. In fact preliminary studies have indicated that the addition of RU486 and dexamethasone together has an additive effect on α- and β-hCG mRNA levels and α-hCG mRNA is differentially regulated in the first trimester and term placenta. Finally, it should be noted that the results obtained with P_4 on hCG regulation are not totally inconsistent with physiological requirements during pregnancy. It is very well known that during early pregnancy when hCG is indispensable, the main function of hCG is to stimulate the synthesis of P_4 which is also indispensable for successful maintenance of pregnancy. Considering this fact, it is only reasonable to assume that during early pregnancy P_4 should also exert a stimulatory effect on the synthesis of hCG, as an inhibitory effect will have a deleterious effect on the course of pregnancy. It is pertinent to note in this connection that it has been reported that the secretion of hCG was increased 3–4-fold by addition of P_4, DHEA and cortisol and a combination of steroids had an additive effect. While the results of the present study permit us to suggest that these two steroids, E_2 and P_4, may have a role in the regulation of hCG, it should be pointed out that these could be only two of the several factors which are reported to be involved in the regulation of hCG in the human placenta.

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