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Estrogen Regulation of the Pregnant Baboon Placental 11β-Hydroxysteroid Dehydrogenase (11β-HSD)-Catalyzed Metabolism of Cortisol and Cortisone and Its Effect on the Development of the Fetal Hypothalamic-Pituitary-Adrenal Axis

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ABSTRACT

ESTROGEN REGULATION OF THE PREGNANT BABOON PLACENTAL 11β-HYDROXYSTEROID DEHYDROGENASE (11βHSD)-CATALYZED METABOLISM OF CORTISOL AND CORTISONE AND ITS EFFECT ON THE DEVELOPMENT OF THE FETAL HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

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Pepe and Albrecht previously demonstrated that the estrogen-regulated change in transuteroplacental metabolism of cortisol (F) and cortisone (E) from preferential reduction (E to F) at midgestation to oxidation (F to E) near term results in a decline of bioactive cortisol crossing the placenta and reaching the fetus culminating in activation of the hypothalamic-pituitary adrenal axis of the baboon and the ontogenesis of rate-limiting steroidogenic enzymes culminating in *de novo* F secretion. Protein kinase A (PK-A) activity in the baboon fetal adrenal gland cytosolic fraction was increased 2-fold both at term [day 165 of gestation (term = 184 days)] and following treatment with estrogen at midgestation (day 100), compared to levels at mid-gestation. Protein kinase C (PK-C) activity in both cytosolic and membrane-bound fractions was similar at mid- and late gestation and not altered by treatment with estradiol.

Secondly, we determined whether maturation of the fetal adrenal reflects enhanced expression of the mRNA for the ACTH precursor proopiomelanocortin (POMC). Pituitary POMC mRNA, was greater in baboon fetuses at term than at mid-gestation and increased on day 100 in estrogen-treated animals. Immunohistochemical studies confirmed that ACTH protein paralleled changes in POMC mRNA. Next, we determined

whether the ontogenetic increase in POMC/ACTH in fetal baboon pituitaries reflected an increase in hypothalamic corticotropin-releasing hormone (CRH). CRH protein and CRH mRNA at mid-gestation were similar to concentrations in fetal baboons of late gestation and were not altered in fetuses in which the mother was treated with estradiol. In conclusion, the onset of fetal adrenal steroidogenic maturation normally near term and prematurely at mid-gestation following estrogen administration reflects increased expression of fetal pituitary POMC mRNA / ACTH protein and are not associated with a concomitant increase in hypothalamic CRH peptide or CRH mRNA.

Thirdly, since the 11β-hydroxysteroid dehydrogenase (11β-HSD)-catalyzed metabolism of maternal cortisol and cortisone by the placenta is an important component in the sequence of events regulating the function of the baboon fetal pituitary-adrenocortical axis, both baboon 11β-HSD-1 and –2 gene promoters were isolated and sequenced and shown to exhibit extensive homology to their respective human sequences. Both genes were subcloned into luciferase reporter vectors and transiently-transfected into human placental JEG-3 cells. In the presence of 17β-estradiol and estrogen receptor α, basal promoter activities of both 11β-HSD-1 and -2 increased 8-fold. Finally, RT-PCR analysis demonstrated a significant reduction of both 11β-HSD-1 and –2 mRNAs in baboon placental syncytiotrophoblast-enriched fractions following reduction of maternal serum estrogen *in vivo* by a highly-specific P450-aromatase enzyme inhibitor, CGS 20267.

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CHAPTER I

BACKGROUND AND INTRODUCTION

A better understanding of the biology of pregnancy maintenance and fetal development is essential for formulating strategies for reducing the relatively high incidence of neonatal morbidity and mortality associated with prematurity and labor-associated fetal stress. In addition, recent studies have proposed the "fetal origins of adult disease" (FOAD) hypothesis stating that several of the major diseases of adulthood, including coronary heart disease, hypertension, and type 2 diabetes, originate in impaired intrauterine growth and development (Barker and Godfrey, 2000). A major factor regulating fetal growth and development is the adrenal gland hormone cortisol. It is believed that estrogen, produced by the placenta to levels almost 100-fold higher than levels seen in the menstrual cycle, plays a central integrative role in both human and baboon pregnancies (Figure 1; Pepe and Albrecht, 1998). For ethical reasons, our understanding of the regulation of feto-placental development in humans has been limited to hormone concentrations in the circulation, analyses of hormone formation *in vitro*, results from abnormal pregnancies and limited endocrine manipulations during elective caesarean section (Pepe and Albrecht, 1998). Although transgenic approaches in rodents have resulted in major advances, there are significant differences between rodents and primates in placentation, hormone biosynthesis and the endocrine interactions of the mother, fetus and placenta. Therefore, a non-human primate model, in which invasive experiments can be conducted, is required to study *in vivo*, the development of the feto-placental unit and its impact on function in adulthood.

Since the 1970's, Pepe and Albrecht have utilized the baboon as a non-human

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The journal model for this dissertation is *Human Reproduction Update*.

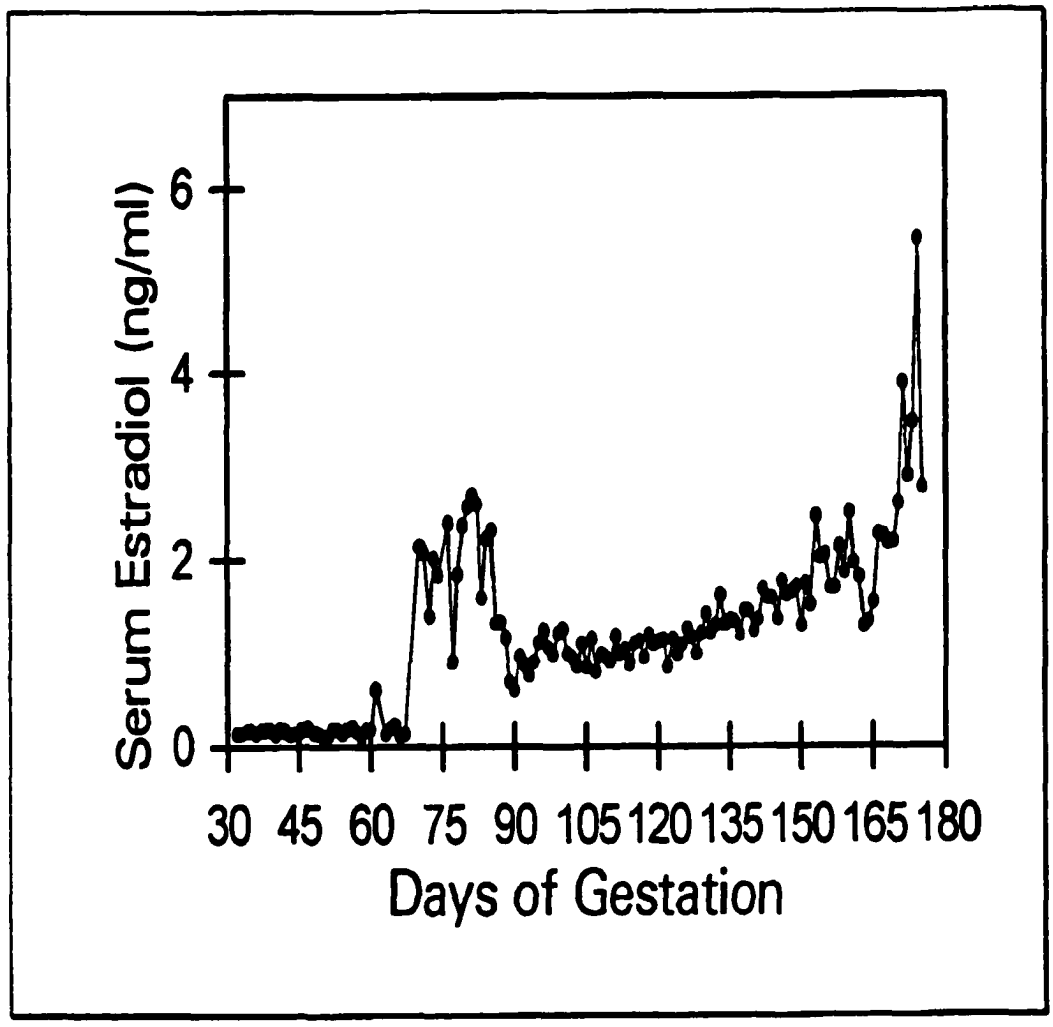

Figure 1. Mean maternal serum estradiol concentrations during baboon pregnancy (term =184 days).

primate model for the study of the endocrinology of pregnancy and have demonstrated that estrogen, the production of which increases during gestation (Figure 1), has three major roles in the development and maintenance of pregnancy and fetal growth. First, estrogen regulates the production of progesterone within the syncytiotrophoblasts via the receptor-mediated uptake of low-density lipoprotein-cholesterol (LDL) and the P450 cholesterol side-chain-cleavage enzyme (P450scc). Second, estrogen acts directly on the fetal adrenal gland to modulate the production of androgen precursors that are subsequently aromatized to estrogen by syncytiotrophoblast P450 aromatase enzyme system. Third, estrogen regulates the syncytiotrophoblast 11β- hydroxysteroid dehydrogenase (11β-HSD-1 and –2) enzymes controlling placental cortisol-cortisone metabolism and thus indirectly regulates the development of the fetal hypothalamic-pituitary-adrenal axis (HPAA). Research endeavors outlined in this proposal have focused on this third area of estrogen-regulated processes.

The fetal milieu depends on a functioning placenta, which develops in parallel with the fertilized ovum. By 6 to 7 days after conception, the blastocyst consists of an outer layer of trophoblast cells and an inner cell mass destined to become the embryo (Williams *et al.*, 1998). Implantation commences as the outer trophoblasts invade the endometrium and two layers of developing placenta can now be demonstrated. Invading trophoblasts form columns to anchor the placenta to the endometrium. Other precursor cytotrophoblasts differentiate into syncytiotrophoblasts and are in direct contact with the maternal circulation. As the placenta develops, the chorionic villi containing the fetal capillaries extend into the maternal lakes of blood within the maternal decidua. The syncytiotrophoblast is the major source of hormone production as well as the major site

of diffusion between the maternal and fetal blood. Some of the more important hormones produced by the placenta include the estrogens (estradiol and estrone), progesterone and the protein hormones chorionic gonadotropin and placental lactogen.

PLACENTAL CORTISOL-CORTISONE METABOLISM

Cortisol is a 21-carbon, lipid-soluble, cholesterol-derived, readily membrane-diffusible steroid hormone. It is measurable in the fetal circulation throughout pregnancy in the human (Murphy, 1982) and non-human primates (Pepe and Albrecht, 1984; Jaffe *et al.*, 1978). Cortisol is biologically active and thus binds to the glucocorticoid receptor. In contrast, cortisone does not bind to this receptor and thus is inactive. In adult serum, the amount of cortisol is 5 – 10 times greater than that of cortisone, while in fetal serum, cortisone levels exceed those of cortisol (Murphy, 1981). Studies in the human (Beitins *et al.*, 1973; Migeon *et al.*, 1957), the rhesus monkey (Kittinger, 1974, Mitchell *et al.*, 1982), and the baboon (Pepe and Albrecht, 1984) indicate that there is extensive movement of cortisol across the placenta, between the fetal and maternal circulation throughout gestation. However, it has generally been considered that the major role of the primate placenta was to catabolize cortisol to cortisone and thus protect the fetus from the relatively high concentrations of cortisol in the maternal circulation (Pepe and Albrecht, 1990). Indeed, based exclusively on experiments performed in late gestation in humans and non-human primates (Pepe and Albrecht, 1995), the placenta actively converts biologically-active cortisol to its inactive metabolite cortisone. The latter mechanism is considered an essential component of the FOAD hypothesis.

Thus, in baboons (Pepe and Albrecht, 1984), as well as in humans (Beitins *et al.*, 1973), the transfer constant (i.e. percent conversion) for the oxidation of cortisol to

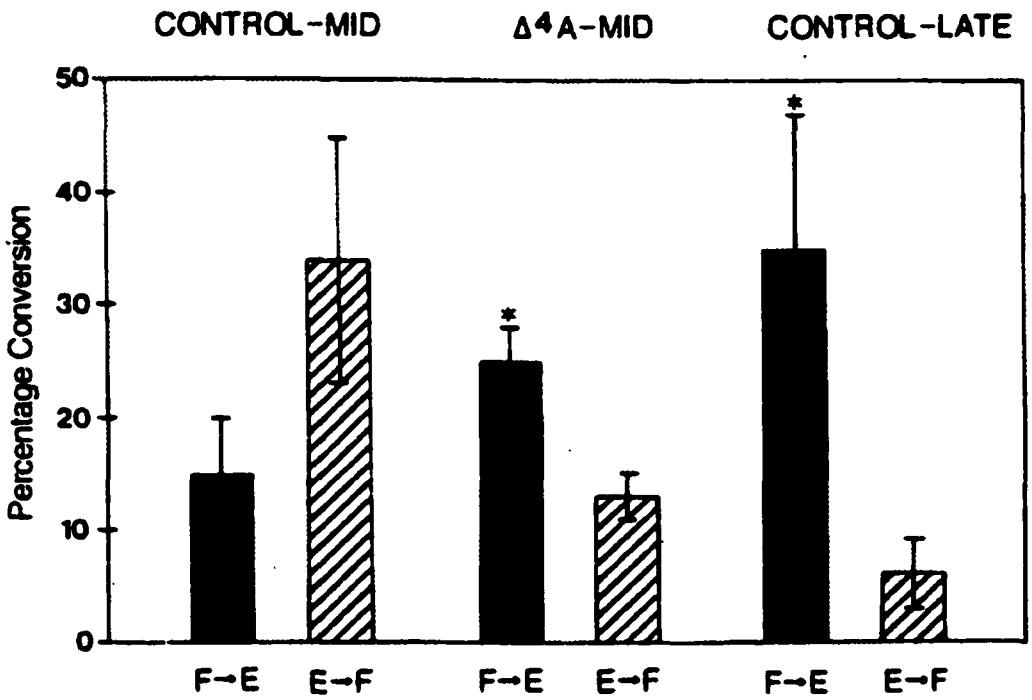

Figure 2. Transuteroplacental interconversion of cortisol (F) and cortisone (E) in untreated baboons on days 100 (mid; n = 7) and 165 (near term; n = 4) of gestation and in nine baboons treated at midgestation with androstenedione which increased placental estrogen production. Values (mean ± SE) represent corticosteroid metabolism by the uterus and placenta corrected for fetal contributions. The asterisk indicates that the percentage F to E is greater ($P<0.05$) than the percentage E to F (by *t*-test for dependent observations).

cortisone across the placenta near term (36%) was 6-fold greater than that for the reduction of cortisone to cortisol, which was minimal (6%). However, when similar *in vivo* isotopic steady-state infusion studies (Figure 2) were performed in baboons at mid-gestation (Pepe *et al.*, 1990), it was observed that the reduction of cortisone to cortisol was quantitatively substantial (35%), and significantly greater than the transfer constant for the oxidation of cortisol to cortisone (15%). This same pattern of cortisol metabolism (i.e. preferential reduction early in pregnancy to preferential oxidation late in pregnancy) may also occur in humans since estimates of cortisol metabolism early in pregnancy showed extensive conversion of cortisone to cortisol (Pasqualini *et al.*, 1970). In contrast, at both mid- and late gestation, almost all fetal tissues (except the placenta) actively convert cortisol to cortisone and thus protect the fetus from abnormally high levels of cortisol (Murphy, 1981; Pepe and Albrecht, 1995).

Using the same *in vivo* experimental paradigms, it was demonstrated that the increase in placental oxidation of cortisol to cortisone near term is regulated by estrogen (Pepe and Albrecht, 1998). Thus, treatment of intact baboons at mid-gestation with estradiol or with androstenedione, which increased placental estrogen production to values normally seen late in gestation, significantly increased the placental transfer constant for the oxidation of cortisol to cortisone and induced a pattern of placental cortisol-cortisone metabolism similar to that seen at late gestation (Figure 2). Further support for the regulatory role of estrogen in this process was realized when the elimination of estrogen production in the later half of gestation via fetectomy or antagonism of the estrogen receptor by ethamoxytriphetol (MER-25) resulted in a pattern of metabolism similar to that seen at mid-gestation (Pepe and Albrecht, 1987).

Recent studies have now confirmed that the interconversion of cortisol and cortisone is catalyzed by two different 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes (Stewart *et al.*, 1994a; Stewart *et al.*, 1994b; Pepe and Albrecht, 1995; Stewart *et al.*, 1995a; Stewart *et al.*, 1995b): 11β-HSD-1, an NADP(H)-dependent dehydrogenase/oxo-reductase with low affinity for substrate cortisol (1,000 nM); and 11β-HSD-2, a high affinity (10-100 nM), NAD^+-dependent dehydrogenase only oxidizing substrate cortisol to cortisone (Albiston *et al.*, 1994; Stewart and Mason, 1995; Whorwood *et al.*, 1995). Given the nanomolar K_m levels for 11β-HSD-2, this form is more likely to play an important physiological role and appears to be the major form expressed in the primate placenta (Burton and Waddell, 1999). In fact, a well-documented role for 11β-HSD-2 exists in the distal nephron of the kidney, where the enzyme ensures aldosterone-selective access *in vivo* to otherwise nonspecific mineralocorticoid receptors (MR or type I versus glucocorticoid receptor or type II) by rapidly inactivating glucocorticoids (i.e. cortisol), serum levels of which are 100- to 1000-fold greater than those of aldosterone. Inhibition of this enzyme by competitive inhibitors found in licorice (glycyrrhizic acid, glycyrrhetinic acid or its derivative carbenoxolone) allows illicit occupation of MR (which *in vitro* has identical affinity for cortisol and aldosterone) in the distal nephron by glucocorticoids, producing sodium retention, hypokalemia and hypertension (Agarwal *et al.*, 1995). This syndrome in the congenital form is called apparent mineralocorticoid excess (AME). Classic enzyme kinetics (Pepe and Albrecht, 1985a; Baggia *et al.*, 1990a; Baggia *et al.*, 1990b) demonstrated that baboon placental NAD^+-dependent 11β-HSD-2 oxidase activity at mid-gestation was increased 4-fold in animals in which placental estrogen production was increased by treatment of the mother with aromatizable

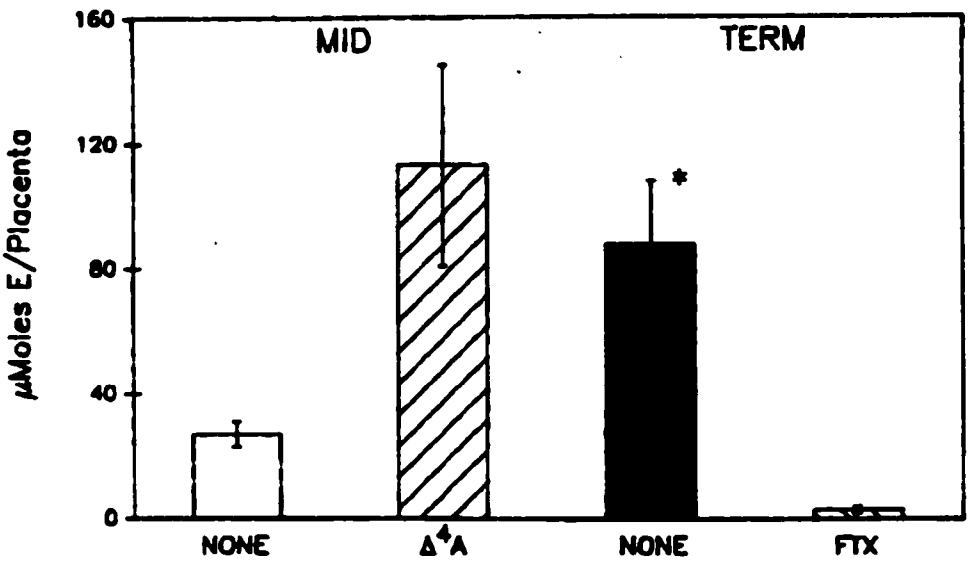

Figure 3. Total NAD-dependent 11β-hydroxysteroid dehydrogenase activity (micromoles of cortisone (E) formed per minute/placenta) in baboons at midgestation treated with androstenedione and near term after fetectomy (FTX). Asterisked values (mean ± SE) in control baboons are greater at term than at midgestation ($P<0.01$, by unpaired *t*-test), and the value after FTX is lower than that in untreated animals at term ($P<0.01$, by unpaired *t*-test).

androstenedione or estradiol and decreased at term in baboons in which placental estrogen production was depleted by fetectomy (Figure 3). *In vitro* studies (Pepe *et al.*, 1996b) demonstrated that the baboon placenta expressed the mRNA for 11β-HSD-1 at mid-gestation favoring the formation of cortisone to cortisol, presumably catalyzed by the 11β-HSD-1 enzyme protein. Although 11β-HSD-1 mRNA or protein has not been consistently detected in human placenta (Lakshmi *et al.*, 1993; Stewart *et al.*, 1994b; Whorwood *et al.*, 1995; Sun *et al.*, 1996a), the mRNA and protein for 11β-HSD-2 have always been detected (Stewart *et al.*, 1994b; Krozowski *et al.*, 1995; Stewart *et al.*, 1995a; Brown *et al.*, 1996a). Recently, both 11β-HSD-1 and -2 enzyme protein and mRNA have been demonstrated in baboon and human placenta (Pepe *et al.*, 1996a; Pepe *et al.*, 1999a). Moreover, levels of mRNA and protein increased for both enzymes with advancing gestation. Up-regulation of 11β-HSD-1 seems to contradict previous results where transplacental conversion of cortisone to cortisol *in utero* declined with advancing gestation. To address these conflicting results, it has been recently demonstrated that changes in transplacental corticosteroid metabolism results from an estrogen-dependent change in the compartmentalization of 11β-HSD-1 and -2 within the syncytiotrophoblast (Pepe *et al.*, 2001). Thus, 11β-HSD-1 was localized extensively to the microvillus membranes (MVM) juxta the maternal circulation, whereas 11β-HSD-2 was located throughout the remainder of the syncytiotrophoblast, including the basal membrane (BM_m) facing the fetal blood compartment. Moreover, there was a developmental increase in the ratio of 11β-HSD-2 to11β-HSD-1 in the syncytiotrophoblast membranes juxta the fetal blood compartment, which we propose is consistent with and perhaps the subcellular mechanism responsible for the previously demonstrated switch in trans-

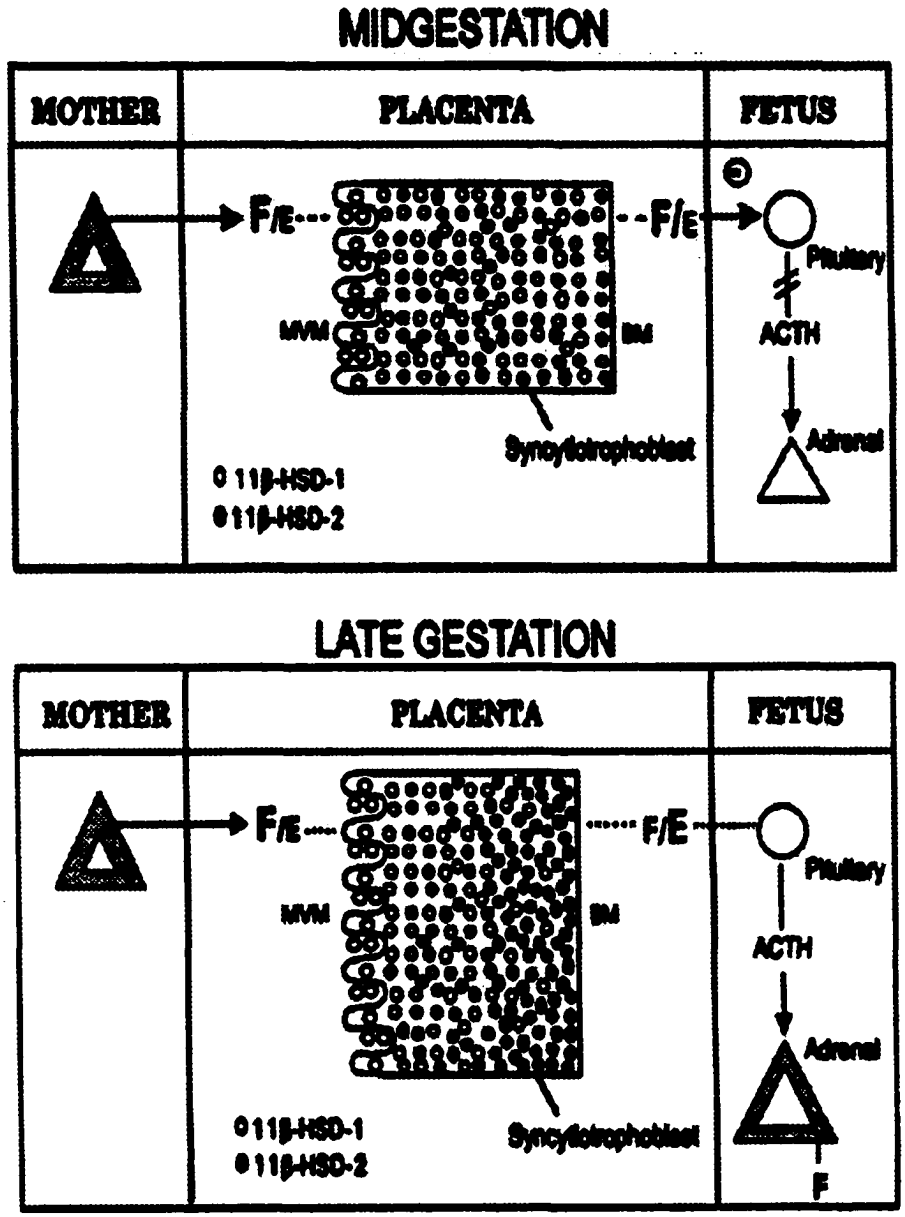

Figure 4. Subcellular localization of 11β-HSD-1 and -2 in the baboon placental syncytiotrophoblast at mid- and late gestation; control of cortisol (F) and cortisone (E) secretion into the fetus and regulation of the fetal pituitary-adrenal axis.

placental glucocorticoid metabolism from reduction at mid-gestation to oxidation late in gestation (Figure 4; Pepe *et al.*, 2001).

IMPACT OF PLACENTAL CORTISOL METABOLISM ON THE FETUS

Due to the absence of key rate-limiting steroidogenic enzymes (Pepe and Albrecht, 1990; Mesiano and Jaffe, 1997), specifically 3β-hydroxysteroid dehydrogenase (3β-HSD) which converts pregnenolone to progesterone, the fetal adrenal gland in humans (Lanman, 1953; Neville and O'Hare, 1982) and non-human primates (Kerr *et al.*, 1969; Pepe *et al.*, 1977; McNulty *et al.*, 1981) does not develop the enzymatic capacity to synthesize cortisol *de novo* (i.e., from endogenous cholesterol) until very late in gestation (i.e., the last 10%). The anatomical correlates of brain function (Scott and Pepe, 1987) and the pituitary's ability to secrete adrenocorticotrophic hormone (ACTH) in response to corticotropin releasing hormone (CRH) *in vivo* (Berghorn *et al.*, 1991) indicate that the fetal baboon hypothalamus and pituitary gland develop relatively early in gestation as previously shown in the human (Ackland et al., 1986; Blumenfeld and Jaffe, 1986). The developmental maturation of the fetal adrenal gland seems to be "out-of-synchrony" with that of the hypothalamus and pituitary which are supposed to be integrated as the HPAA. It has been demonstrated that at mid-gestation, almost 100% of the fetal baboon serum cortisol is of maternal origin whereas at term less than 50% originates from the maternal compartment (Figure 5). Based on these observations, it was proposed that the placenta, via metabolism of cortisol-cortisone, determines the qualitative and quantitative patterns of these corticosteroids reaching the fetal pituitary gland at mid- and term gestation. Consequently, this unique process regulates the fetal HPAA and the timely onset of *de novo* cortisol production by the fetal adrenal gland. Thus, preferential conversion of

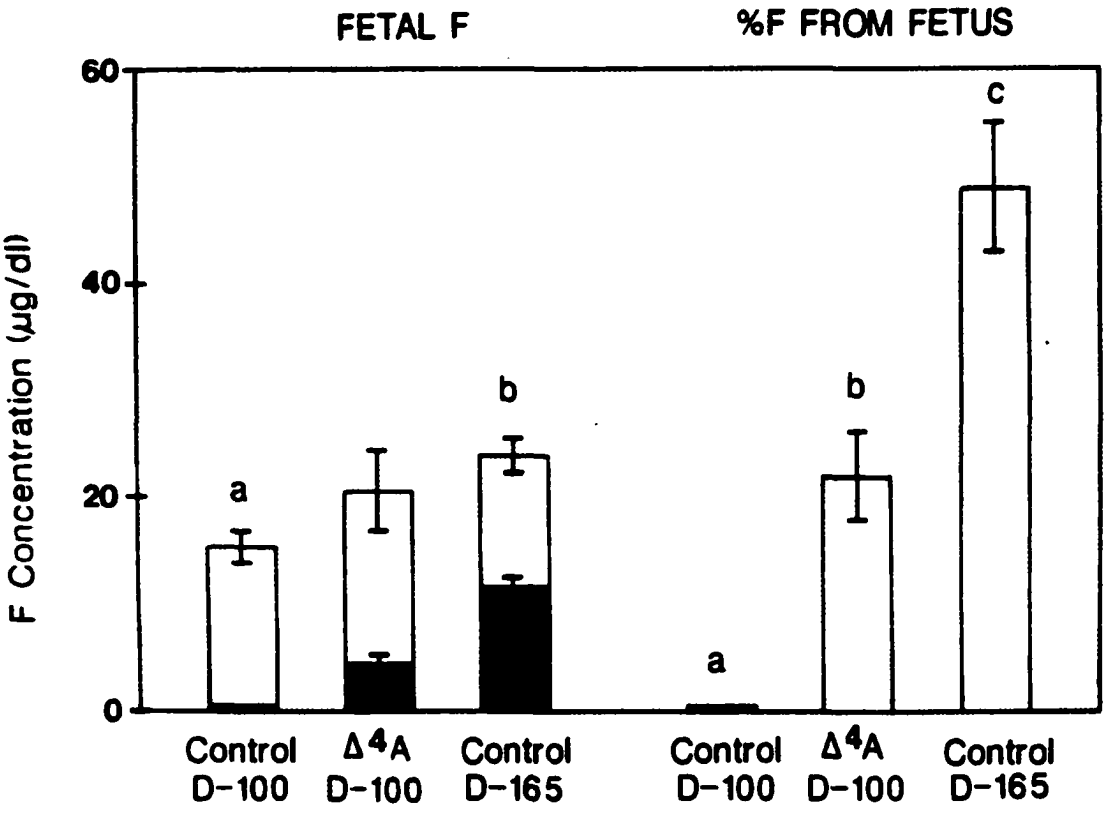

Figure 5. Fetal serum cortisol (F) concentrations (left panel) and percentage of fetal F derived from production in the fetus (right panel) by the fetal adrenal gland in untreated baboons on day 100 (mid) and 165 (late) of gestation and in baboons at midgestation following maternal treatment with androstenedione (Δ^4A). The proportion of fetal F produced within the fetus (right panel) and present within fetal serum (darkened area of bars of left panel were determined by specific activities of serum F in maternal and umbilical veins. Values (mean ± SE) with different superscripts differ from each other at $P<0.05$ (ANOVA with multiple comparison of means by Least Significant Difference statistic).

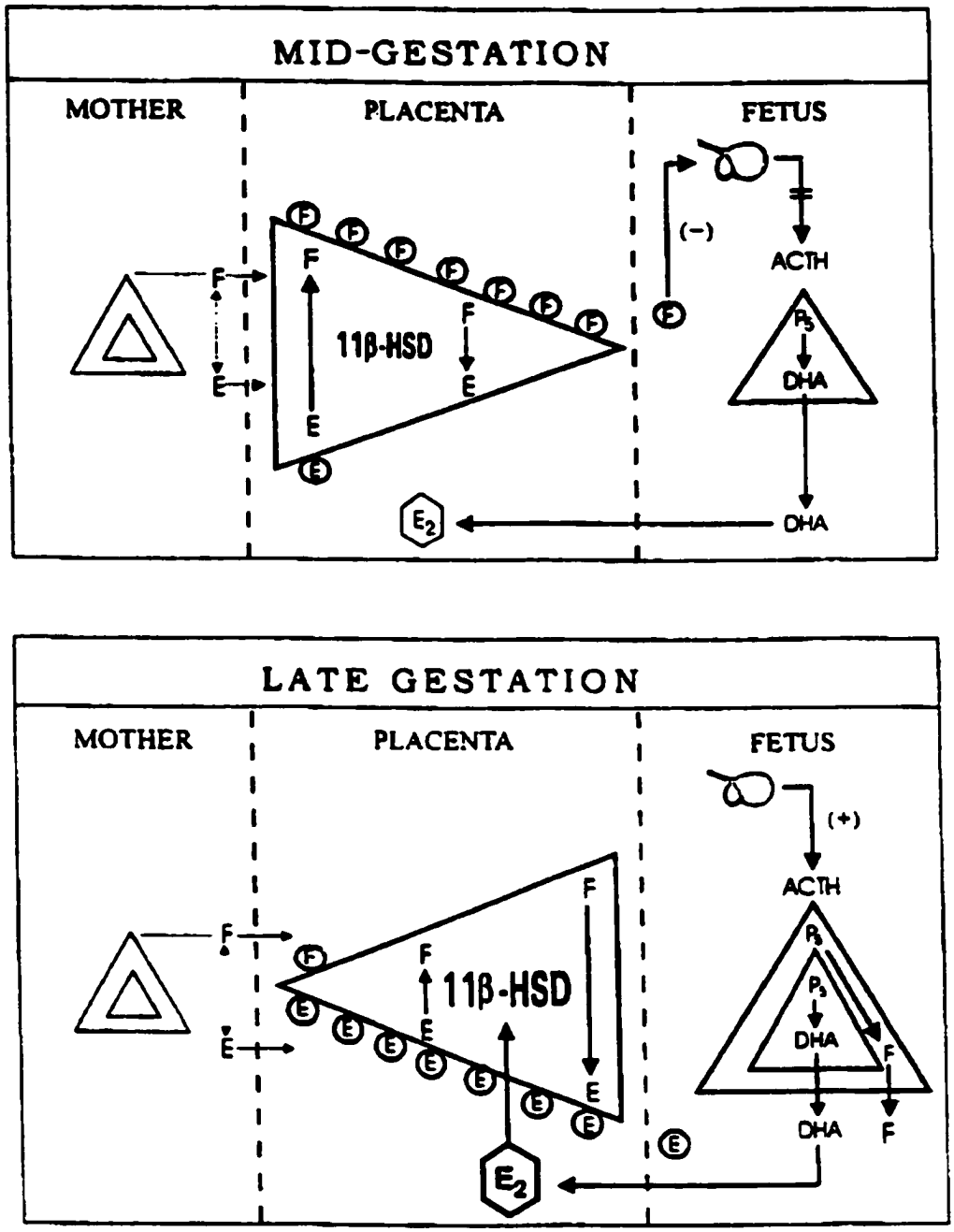

Figure 6. The role of estrogen (E_2) on placental 11β-hydroxysteroid dehydrogenase (11β -HSD)-catalyzed metabolism of cortisol (F)-cortisone (E) and regulation of the fetal pituitary-adrenocortical axis at mid- and late gestation in the baboon. With advancing gestation, the estrogen-induced increase in placental 11β-HSD-catalyzed oxidation of maternal cortisol to cortisone decreases the concentration of maternal cortisol in the fetus and thus disinhibits the fetal pituitary leading to increased adrenocorticotrophic hormone (ACTH) production and maturation of and *de novo* cortisol production by the fetal adrenal gland. DHA = dehydroepiandrosterone; P_5 = pregnenolone.

maternal cortisone to biologically active cortisol by the placenta occurs at mid-gestation when fetal production of cortisol is low (Figure 6). This has a negative feedback effect on fetal pituitary ACTH secretion, thereby limiting fetal adrenal definitive zone cell growth and the ontogenesis of 3β-HSD activity as well as *de novo* cortisol synthesis throughout most of pregnancy. In fact, the activity of 3β-HSD, the gene and protein for which are apparently restricted to the definitive zone of the fetal adrenal gland, is minimal during most of gestation in the fetal adrenal glands of humans, baboons, rhesus monkeys and sheep (Pepe and Albrecht, 1995). The production and secretion of C_{19} androgens, dehydroepiandrosterone (DHA) and DHA sulfate (DHAS), primarily from the fetal adrenal fetal zone cells, is critical for the continued high estrogen levels throughout pregnancy. This androgen elaboration would not be compromised because factors of placental and/or fetal origin (Brown *et al.*, 1979; Pepe and Albrecht, 1985a; Pepe and Albrecht, 1985b; Pepe *et al.*, 1988) have been shown to modulate adrenal androgen production. By term-gestation, however, the estrogen-induced increase in oxidation of cortisol to biologically-inactive cortisone by the placenta, as well as fetal tissue (Waddell *et al.*, 1988b), reduces fetal serum cortisol levels, allowing stimulation of fetal pituitary secretion of trophic hormone ACTH and the timely onset of *de novo* cortisol production by the fetal adrenal gland. In support of this hypothesis, it has been demonstrated that the ontogenesis of *de novo* fetal adrenal cortisol production (Figure 5) was markedly increased with advancing gestation and enhanced prematurely at mid-gestation following induction of placental NAD^+-dependent 11β-HSD-2 oxidase activity by estrogen to concentrations observed normally at term-gestation (Pepe *et al.*, 1990).

FETAL ADRENAL GLAND

The adrenal gland originates embryonically from mesodermal cells attached to the urogenital ridge. The fetal adrenals are recognizable by mid-first trimester when neuroectodermal (chromaffin) cells invade to commence formation of the medulla. Shortly thereafter, the adrenal gland is recognizable as consisting of a thin, outer layer "definitive" zone and a large, inner "fetal" zone.

In most mammalian species, products of the fetal adrenal gland play an important role in regulating maturation of various organ systems in the fetus (Pepe and Albrecht, 1990) as well as responding to stress (Matthew and Challis, 1995). Preceding parturition, fetal organs undergo accelerated maturation that facilitates the transition from intrauterine to extrauterine life. Cortisol, produced from cholesterol in the fetal adrenal glands or converted from inactive cortisone in the placenta, is one of the key chemical messengers involved in stimulating fetal/neonatal lung maturation (Kotas and Avery, 1971; Farrell and Zachman, 1973; Mendelson and Boggaram, 1991; Weaver and Whitsett, 1991; NIH Consensus Conference, 1995). Cortisol works in concert with hormones such as thyroid hormone, prolactin, and insulin to accelerate lung tissue surfactant dipalmitoyl phosphatidylcholine (DPPC) synthesis, lung liquid reabsorption as well as lung structural modifications (Pepe and Albrecht, 1995; Williams *et al.*, 1998). DPPC is the major component of surfactant, which reduces surface tension at the alveolar air-liquid interface. Surfactant is synthesized by type II epithelial cells in the lung alveolus and secreted into the air by exocytosis. Glucocorticoids also stimulate at least four key phosphatidylcholine (PC) biosynthesis enzymes, including fatty acid synthase, choline phosphate cytidylyltransferase, phophatidylphophatase and lysoPC:acyl-CoA

acyltransferase (Pepe and Albrecht, 1995). Approximately 10% of the mass of pulmonary surfactant is made up of specialized proteins, known as surfactant proteins (SP). The largest and most abundant is SP-A, a 35,000 dalton glycoprotein. Two other surfactant-associated hydrophobic proteins are SP-B and SP-C, with molecular weights of 8,000 and 5,000 daltons, respectively. Glucocorticoids, at physiological doses (i.e., 1 mM), also have stimulatory effects on the production of these three proteins.

Cortisol also stimulates gluconeogenesis from amino acids and liver glycogen synthesis/accumulation while decreasing peripheral utilization of glucose (Greengard and Dewey, 1970; Greengard , 1973; Eisen *et al.*, 1973; Seron-Ferre and Jaffe, 1981). Moreover, glucocorticoids promote the differentiation of preadipocytes into mature fat cells and stimulate adipocyte lipoprotein lipase to increase lipid mobilization and triglyceride sequestration (Whorwood *et al.*, 2001). Additionally, cortisol increases methylation of norepinephrine to epinephrine, the hepatic conversion of T_4 to T_3 and the induction of several enzymes in the brain, retina, pancreas and gastrointestinal tract of the fetus (Moscona and Piddington, 1966; Piddington and Moscona, 1967; Moog, 1971; Giannopoulos, 1975). In addition to cortisol's major effects on intermediary metabolism, cortisol suppresses the immune response (promotes lymphocyte lysis) and the inflammatory response (decreases the number and migration of leukocytes and inhibits the production of prostaglandins and leukotrienes). While glucocorticoids play a crucial role in the final maturation of fetal organ systems, excess glucocorticoid exposure retards fetal growth, including pancreas and adrenal glands, and may impair fetal brain development and immune system function (Kay *et al.*, 2000). Because glucocorticoids provide key signals in cellular differentiation, many of their effects are long-lasting, and

excess glucocorticoid exposure *in utero* has been directly linked to the subsequent development of hypertension and hyperglycemia in adult life (Burton and Waddell, 1999) and perhaps delayed puberty (Smith and Waddell, 2000).

In most species investigated to date, late pregnancy is characterized by rising concentrations of cortisol in the fetal circulation (Liggins, 1994) and the maternal circulation (Burton and Waddell, 1999). In sheep, cortisol concentrations rise sharply prepartum and induce parturition, presumably by increasing the placental enzyme, P450c17αhydroxylase (Liggins *et al.*, 1973; Seron-Ferre and Jaffe, 1981) which results in decreased placental progesterone secretion, increased placental estrogen secretion and consequently increased uterine prostaglandin production. In human and non-human primates, fetal cortisol levels increase more slowly and the role of the cortisol surge in the initiation of labor is less convincing (Muller-Huebach *et al.*, 1972; Liggins, 1976; Liggins *et al.*, 1977; Casey and MacDonald, 1988). Nevertheless, fetal adrenal gland *de novo* cortisol production appears to be a factor in determining the timing of parturition (Sucheston and Cannon, 1969; Turnbull and Anderson, 1984).

In addition to these roles for fetal adrenal cortisol, it is well established that the fetal adrenal gland in primates, as well as humans, is important to the synthesis and secretion of androgen precursors essential to the production of estrogen by the P-450-placental aromatase enzyme complex (Diczfalusy, 1964; Siiteri and MacDonald, 1966; Oakey, 1970; Diczfalusy, 1974). The principal secretory androgen of the human fetal adrenal gland throughout gestation is DHAS and to a lesser extent its nonconjugated form DHA (Seron-Ferre and Jaffe, 1981). The production of adrenal androgens from pregnenolone requires 17α-hydroxylation, thus the major source of DHAS in the fetus is the adrenal

fetal zone. In the fetal rhesus monkey, the serum levels and daily production rates of DHAS increase steadily during gestation and parallel fetal cortical growth (Seron-Ferre *et al.*, 1983). DHAS is extensively metabolized to 16α-OH DHA by the fetal liver in the human but not in the baboon. Both DHAS and 16α-OH DHAS can be hydrolyzed by the human placental steroid sulfatase to the non-conjugated form (Bolte *et al.*, 1964), and then converted by placental 3β-HSD to androstenedione (A_4). A_4 can then be converted to either estrone (E_1) directly by P450 aromatase or to testosterone by 17β-HSD II and then subsequently aromatized by P450 aromatase to estradiol (E_2). 16α-OH DHAS is aromatized by the placenta to estriol (E_3), quantitatively the major estrogen present in pregnant woman, whereas DHA is aromatized to estrone (E_1), and estradiol (Seron-Ferre and Jaffe, 1981). Estriol and estrone are weaker estrogens than estradiol and exhibit activities $1/80^{th}$ and $1/12^{th}$, respectively, of that of estradiol. Thus, fetal adrenal androgens are precursors for placental aromatase production of estrogens in humans (Simmer *et al.*, 1964; Easterling *et al.*, 1966; Chang *et al.*, 1976) and non-human primates (Kling *et al.*, 1972; Townsley, 1976; Walsh *et al.*, 1979; Jaffe *et al.*, 1981; Seron-Ferre *et al.*, 1983; Pepe and Albrecht *et al.*, 1980). The interdependence of fetus, placenta and mother in the formation of estrogens has led to the concept of the "fetoplacentomaternal unit" (Seron-Ferre and Jaffe, 1981; Pepe and Albrecht, 1990). Human and non-human primate placentas lack P450c17α-hydroxylase enzyme and are therefore solely dependent on both the maternal adrenal and especially the fetal adrenal glands to provide necessary androgen precursors in the form of DHA and DHAS for the continually increasing placenta production of estrogen via placental P450-aromatase.

The adult adrenal gland is comprised of an outer cortex and an inner medulla. The outer cortex is richly vascularized and is composed of three zones: an outer zona glomerulosa that lacks P450c17α-hydroxylase and can only produce aldosterone, a zona fasciculata (75% of the cortex) and an inner zona reticularis. Both zonae fasciculata and reticularis are responsive to ACTH and generate cortisol and androgens. The fetal adrenal gland, on the other hand, lacks the typical zonation of the adult adrenal gland. Thus, throughout the majority of gestation, the fetal adrenal gland in human and non-human primates is comprised of two morphologically distinct zones: an inner fetal cortical zone, which comprises about 85% - 95% of the gland, and an outer definitive, that occupies the remaining 5% - 15% of the gland late (Pepe and Albrecht, 1990; Seron-Ferre and Jaffe, 1981). This type of zonation and adrenal development is not present in fetal adrenal glands of non-primates. The fetal zone contains large eosinophilic cells with large pale nuclei and distinct cell outlines that have the ultrastructural characteristics of steroid-secreting cells. The definitive zone consists of small basophilic cells with dark staining nuclei (Seron-Ferre and Jaffe, 1981). Structural and functional evidence have described a third zone, the transitional zone (Sucheston and Cannon, 1968; Mesiano and Jaffe, 1997). This zone, which appears in human and non-human primates late in gestation lies between the definitive and fetal zones and is comprised of large acidophilic cells with indistinct cell boundaries and is believed to be the site of cortisol synthesis. These three zones are thought to form the zona glomerulosa (definitive), fasciculata (transitional) and reticularis (fetal zone) of the adult adrenal.

During the course of gestation in human (Lanman, 1953; Neville and O'Hare, 1982) and non-human primates (Kerr *et al.*, 1969; Pepe *et al.*, 1977; McNulty *et al.*, 1981;

Seron-Ferre *et al.*, 1983; Albrecht and Pepe, 1988), the fetal adrenal gland grows rapidly, with marked growth occurring primarily during the last one-third of intrauterine development. In humans, the weight of both fetal adrenal glands is less than 100 mg at 10 weeks gestation and increases to approximately 2000 mg by week 20 (Swinyard, 1941; Bocian-Sobkowska *et al.*, 1997a; Bocian-Sobkowska *et al.*, 1997b). Between 20 and 30 weeks of gestation, the gland doubles in size, with the ontogenesis of adult-type zonation (Sucheston and Cannon, 1968). By term, the human fetal adrenal weighs approximately 4000-8000 mg (Swinyard, 1941), about twice the weight of adult adrenals, and represents 0.5 percent of total body weight, compared with 0.005 percent in the adult (Miller and Tyrrell, 1995). Similar increases in fetal adrenal weight occur in the baboon (Pepe *et al.*, 1985b) and the rhesus monkey (McClellan and Brenner, 1981). The increase in weight is achieved mainly by an increase in the size of the fetal zone with some growth in the outer zones, but not until late in gestation. Thus, by day 150 of gestation in the rhesus monkey (term = 165 days), a presumptive zona glomerulosa and a zona fasciculata are present but these adult-type zones still only occupy less than 20% of the cortex (McClellan *et al.*, 1981). In the human fetal adrenal gland, the fetal zone apparently undergoes rapid involution during early postnatal life. By the second week after birth, adrenal weight has decreased by one-third. The fetal zone is no longer detectable by age 1 year (Miller and Tyrrell, 1995). A similar process occurs in the baboon and rhesus monkey (McNulty *et al.*, 1981), since distinct adult-type zones are observed in neonates by 6-12 months of age (Pepe and Albrecht, 1985a; Pepe and Albrecht, 1985b; Pepe and Albrecht, 1985c).

The factors regulating the remodeling of the fetal gland into the permanent mature cortex remain to be elucidated. After birth in the non-human primate, proliferation of the definitive zone with absence of necrosis within the fetal zone was observed. This postnatal morphology in rhesus monkey was interpreted to represent the development of the zona fasciculata by transformation of the fetal zone (Holmes, 1968). In baboons (*Papio anubis*), it was suggested that the zona fasciculata develops by proliferation of new cells within its upper margins and not by transformation of preexisting cells in the fetal zone (Ducksay *et al.*, 1991). In contrast, it was originally suggested (Sucheston and Cannon, 1968) that the fetal zone in humans disappears and that it is the fetal definitive cortex which gives rise to the three zones of the adult adrenal gland (i.e. glomerulosa, fasciculata and reticularis) during the first year of life. However, more recent studies have questioned this theory and it has been proposed that each zone develops independently with the definitive zone being analogous to the adult zona glomerulosa, the transitional zone to the adult zona fasciculata and the fetal zone to the adult zona reticularis (Mesiano and Jaffe, 1997).

The principal secretory products of the human fetal adrenal gland are the C_{19}-androgens, DHA and DHAS, and the C_{21}-steroids, aldosterone and cortisol. Based primarily on *in vitro* studies utilizing human fetal adrenal tissues obtained early in gestation, it is apparent that cholesterol, derived from circulating low density lipoprotein (LDL)-cholesterol produced in the fetal liver, is a major substrate for fetal adrenal steroidogenesis (Williams *et al.*, 1998). Thus, the quantity of DHAS and cortisol synthesized by human fetal adrenal cells in culture increased as a function of the concentration of LDL-cholesterol present in the culture media (Mason and Rainey, 1987).

Additionally, using *in situ* perfusion, it was demonstrated that neither circulating acetate nor free cholesterol were efficient precursors for human fetal steroidogenesis (Solomon *et al*, 1967). Similar conclusions have been deduced from *in vivo* studies in baboon (Pepe and Albrecht, 1984) and rhesus monkeys (Ducsay *et al.*, 1985) in which less than 5% of placental progesterone and pregnenolone secreted into the fetus were utilized for cortisol production between mid and late gestation. Pregnenolone does not appear to be a quantitatively significant precursor in the human as well, because the total amount of this precursor estimated to be used for steroidogenesis is less than 1% of the fetal production rate of DHAS (Simpson *et al.*, 1985).

Fetal adrenal uptake of LDL-cholesterol occurs by LDL receptor-mediated endocytosis. Treatment of human fetal tissue fragments with radiolabeled LDL-cholesterol, in the presence or absence of ACTH, indicated that fetal adrenal LDL-cholesterol uptake was saturable, and presumably mediated by a population of high-affinity, low-capacity binding sites (Carr *et al.*, 1980a; Carr *et al.*, 1980b; Carr *et al.*, 1980c). As in the adult adrenal, where less than 10 percent of LDL enters the cell by a receptor-independent mechanism (Brown *et al.*, 1979; Gwynne and Strauss, 1982), LDL-cholesterol binds to fetal adrenal gland LDL receptors that are internalized in coated vesicles. Following this, the LDL-cholesterol is then separated from its receptor, which is recycled back to the plasma membrane and acid proteinases degrade LDL-cholesterol apolipoproteins to amino acids, and acid lipases free cholesterol from esters (Miller and Tyrrell, 1995). ACTH stimulates LDL receptor synthesis, the uptake of LDL-cholesterol (Golos and Strauss, 1988) and degradation of LDL-cholesterol by fetal adrenal tissue fragments (Carr *et al.*, 1980c; Carr and Simpson, 1981). To investigate the nature and

regulation of the receptors involved in the endocytosis of LDL-cholesterol, the binding of LDL-cholesterol to membrane fractions prepared from adrenals of normal and anencephalic human fetuses was assayed (Carr and Simpson, 1981; Ohashi *et al.*, 1981a; Ohashi *et al.*, 1981b). In tissues from both sources, binding was of high affinity. However, compared to values in tissues from anencephalics, LDL-cholesterol binding and degradation were greater in normal fetuses. Only in normal tissue, however, were the latter processes stimulated by ACTH. Although there was substantial binding of high-density lipoproteins (HDL) by human fetal adrenals (Ohashi *et al.*, 1981a; Ohashi *et al.*, 1981b), HDL was not degraded nor regulated by ACTH. Moreover, HDL does not stimulate or sustain human adrenal steroidogenesis (Gwynne and Strauss, 1982; Ohashi *et al.*, 1981a; Strauss and Miller, 1991).

Because no more than 20% of cholesterol in the fetus is derived from the maternal compartment (Lins *et al.*, 1977; Simpson *et al.*, 1985), it has been suggested that the majority of circulating LDL-cholesterol arises from *de novo* synthesis within the fetus. In support of this, extensive cholesterol production by human fetal liver, testes and adrenal glands has been demonstrated (Carr and Simpson, 1982). Despite the importance of LDL-cholesterol, it has also been demonstrated (Carr *et al.*, 1981; Carr and Simpson, 1981; Mason and Rainey, 1987) that the adrenal readily synthesizes cholesterol from acetyl-CoA in response to trophic stimulation by ACTH. Moreover, *de novo* cholesterol production catalyzed by the rate limiting enzyme 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, could account for as much as 30% of the daily secretion rate of DHAS and cortisol by the human fetal adrenal. As in the adult, fetal *de novo* cholesterol synthesis was only an important source of substrate in certain situations. Thus, in the

absence of LDL-cholesterol, steroidogenesis by human fetal adrenal cells in culture could be maintained almost entirely by the *de novo* pathway (Mason and Rainey, 1987). Additionally, the principal molecular species of fetal adrenal HMG-CoA reductase in both cultured and fresh adrenal tissue was a 97,000-dalton protein, the appearance of which correlates with *in vitro* measures of enzyme activity. Moreover, because loss of HMG-CoA reductase enzyme protein associated with culture of fetal adrenal cells in the absence of ACTH was prevented by concomitant treatment with ACTH, it appeared that HMG-CoA reductase activity in fresh adrenal tissue was regulated by ACTH. Indeed, *de novo* cholesterol synthesis in adrenals of anencephalics was significantly lower than that in tissue of normal fetuses (Carr *et al.*, 1981). In the adult adrenal, HMG-CoA reductase is inactivated by phosphorylation and is activated by dephosphorylation in response to intracellular increases in cAMP following the binding of ACTH to its ACTH-receptor (Gwynne and Strauss, 1982). In the fetal adrenal however, ACTH may not activate HMG-CoA reductase by stimulating dephosphorylation but rather, as demonstrated in adult bovine adrenals cells, ACTH may promote increased rates of accumulation of HMG-CoA reductase protein (Rainey *et al.*, 1986).

CHEMISTRY OF STEROID HORMONES

Cholesterol (C_{27}), the original precursor molecule for steroid synthesis, is stored mainly within lipid droplets esterified (acyl-CoA:cholesterol acyltransferase {ACAT}) to polyunsaturated fatty acids following *de novo* synthesis from acetate via HMG-CoA reductase and/or acquisition from LDL-cholesterol extracellularly via LDL-receptor uptake. About 80% of the cholesterol used for steroid hormone synthesis comes from dietary sources and is transported to the adrenal in plasma as LDL-cholesterol.

Stimulation of adrenal cells (i.e. ACTH) stimulates cholesteryl ester hydrolase (CEH), inhibits ACAT, and results in the release of free cholesterol. Cholesterol is then transported to the mitochondria via sterol carrier protein 2 (SCP-2) assisted by steroidogenic acute regulator (StAR) protein. StAR is rapidly induced by cAMP. The conversion of cholesterol to pregnenolone in mitochondria by P450 cholesterol side-chain-cleavage (P450scc) is the first and rate-limiting step in the synthesis of steroid hormones from cholesterol. This step involves three distinct chemical reactions: 20-hydroxylation, 22-hydroxylation, and scission of the C_{20}-C_{22} cholesterol side chain to yield pregnenolone (C_{21}) and isocaproic acid (Miller, 1988). Pregnenolone is then returned outside the mitochondria to the cytosol before further steroidogenesis occurs. Pregnenolone may be converted to 17α-hydroxypregnenolone by P450c17α-hydroxylase or to progesterone by $\Delta^5$3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD). 3β-HSD, a single 42,000-dalton microsomal enzyme (Thomas *et al.*, 1989), catalyzes both 3 β-hydroxysteroid dehydrogenation and the isomerization of the double bond from the Δ^5 steroid to the Δ^4 steroid (Δ represents double bond). There are at least two isoforms of human 3β-HSD, encoded by two different genes. The 3β-HSD type I gene is expressed in the placenta, skin, mammary gland, and possibly other peripheral tissues (Lorence *et al.*, 1990; Lachance *et al.*, 1991). A distinct 3β-HSD type II gene is expressed in the adrenals and gonads (Lachance *et al.*, 1991; Rheaum *et al.*, 1991). Both pregnenolone and progesterone may undergo 17α-hydroxylation to yield 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, respectively. These two reactions are mediated by a single enzyme, cytochrome P450c17α-hydroxylase, 17/20-lyase (P450c17). As P450c17 has both 17α-hydroxylase activity and $C_{17,20}$-lyase activity, it is a key branch point in

steroid hormone synthesis, catalyzing production of either glucocorticoids (17α-hydroxylation but not $C_{17,20}$ cleavage) or androgens (both 17α-hydroxylation and $C_{17,20}$ cleavage). These 17α-hydroxylated steroids may then undergo scission of the C_{17} / C_{20} carbon to yield the 19-carbon steroids, DHA and androstenedione (Δ^4A). Subsequent conversions of progesterone and 17α-hydroxyprogesterone to 11-deoxycorticosterone and 11-deoxycortisol respectively occur in the smooth endoplasmic reticulum by P450c21-hydroxylase. The nature of the 21-hydroxylating step has been of great clinical interest because congenital adrenal hyperplasia most frequently results from 21-hydroxylase deficiency. In human fetuses affected with this genetic disorder, the dysfunctional adrenal can produce enough androgen to virilize a female fetus to a male phenotype (New *et al.*, 1981; Saenger, 1984). 11-deoxycortisol is then further hydroxylated within the mitochondria by the P450c11β1-hydroxylase enzyme to form cortisol. This enzyme is inhibited by metyrapone and therefore interrupts the negative feedback on the HPAA. 11-deoxycorticosterone undergoes three conversions all catalyzed by a single mitochondrial enzyme, P450c11β2-hydroxylase enzyme (aldosterone synthase) to yield the mineralocorticoid aldosterone. A summary of steroid biosynthesis in the adrenal gland with the major secretory products and enzymes is presented (Figure 7).

REGULATION OF FETAL ADRENAL STEROIDOGENESIS

Steroidogenesis by the fetal adrenal is regulated at least partly by ACTH. The orphan nuclear receptor steroidogenic factor 1 (SF-1) is critical for development and function of the fetal adrenal gland. Many cell transfection studies have highlighted important roles for SF-1 in the transcriptional activation of the genes that encode

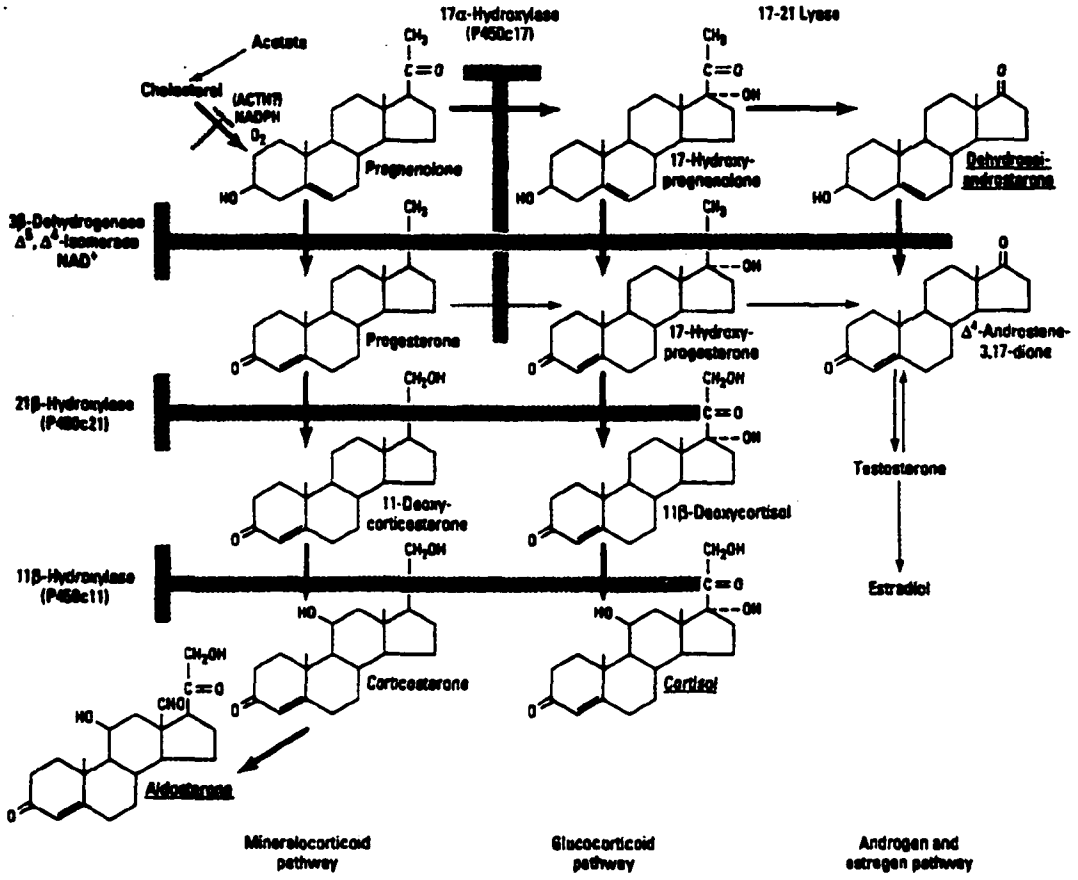

Figure 7. Outline of major pathways in adrenocortical hormone biosynthesis. The major secretory products are underlined. Pregnenolone is the major precursor of corticosterone and aldosterone, and 17-hydroxypregnenolone is the major precursor of cortisol. The enzymes and cofactors for the reactions progressing down each column are shown on the left and from the first to the second column at the top of the figure. When a particular enzyme is deficient, hormone production is blocked at the points indicated by the shaded bars.

steroidogenic enzymes, while targeted disruption of the mouse gene encoding SF-1 clearly defined the essential role for SF-1 in adrenal, as well as gonadal, development (Hanley *et al.*, 2001). It is well established that the expression and activity of the enzyme 3β-HSD, responsible for the synthesis of $\Delta^4$3-ketosteroids (i.e. cortisol) from pregnenolone (P_5) is minimal during the majority of intrauterine development in the fetal adrenal of human (Lanman, 1953; Neville and O'Hare, 1982) and non-human primates (Pepe *et al.*, 1977; Kerr *et al.*, 1969; McNulty *et al.*, 1981; Simonian and Gill, 1981; Seron-Ferre *et al.*, 1983; Albrecht and Pepe, 1988; Mesiano and Jaffe, 1997; Coulter *et al.*, 1996). When, however, human fetal definitive zone explants from the first and second trimester are cultured in the presence or absence of ACTH and other agonists of the protein kinase A pathway, the capacity for cortisol secretion is significantly induced as is the activity of 3β-HSD (Bolte *et al.*, 1964; Beitins *et al.*, 1972; Beitins *et al.*, 1973; Branchaud *et al.*, 1978; Doody *et al.*, 1990). Consequently, adrenals of fetuses early in gestation are competent to respond to ACTH but may not have been exposed to sufficient ACTH *in vivo* during this developmental period.

Recent immunohistochemical evaluation of the cellular localization and ontogeny of steroidogenic enzymes has provided additional insight into the regulation of steroidogenesis *in utero*. P450scc is expressed in all cells of the adrenal cortex in both the mid-gestation and the term-gestation rhesus monkey fetus (Coulter *et al.*, 1996). In contrast, 3β-HSD was localized primarily in the definitive zone cells from mid-gestation fetuses, whereas at term-gestation it was localized in both the definitive and transitional zone cells. P450c17 expression was detected in the transitional and fetal zones. ACTH only increased secretion of cortisol by fetal transitional zone cells, indicating that 3β-

HSD is not present in fetal zone cells (Doody *et al.*, 1990). Western blot confirmed the absence of 3β-HSD protein in the fetal zone and its exclusive presence in the definitive/transitional zone (Doody *et al.*, 1990). The absence of 3β-HSD in the fetal zone explained the low ACTH-stimulable cortisol secretion from these cells *in vitro* relative to a 5-fold increase in ACTH-stimulable cortisol secretion from similarly cultured definitive/transitional zone cells. Coexpression of 3β-HSD and P450c17 enzymes in the transitional zone by term-gestation in the primate fetal adrenal gland indicates the capacity of these cells to synthesize *de novo* cortisol. These findings are also supported by *in vivo* observations where a rise in the serum concentrations of cortisol in the baboon fetus (Pepe and Albrecht, 1984) and in the umbilical artery in humans (Murphy and Diez d'Aux, 1972; Smith and Shearman, 1974; Murphy, 1982) and rhesus monkeys (Mitchell *et al.*, 1982) occurs late in gestation. Studies in the baboon (Pepe *et al.*, 1977; Pepe and Albrecht, 1984; Waddell *et al.*, 1988a; Waddell *et al.*, 1988b) have confirmed that the increase in cortisol levels reflects production by the fetal adrenal gland and is not the result of an increase in placental transfer of maternal cortisol nor an alteration in fetal cortisol metabolic clearance (Figure 5).

In contrast to expression late in gestation of 3β-HSD, the activities, mRNA and proteins for the enzymes catalyzing conversion of cholesterol to pregnenolone (P450scc) and hydroxylations (P450c17, 21,11) at steroid carbons 17, 21, and 11 respectively appear to be present earlier in gestation (Smith and Shearman, 1974; Pepe *et al.*, 1977; Sholl, 1981; Sholl, 1982; Sholl, 1983; Voutilainen and Miller, 1986; Di Blasio *et al.*, 1987; Jaffe *et al.*, 1988). However, despite the apparent abundant levels of C-17, -21, and -11 hydroxylases throughout gestation, the primate fetal adrenal gland (Pepe *et al.*,

1988; Pepe and Albrecht, 1990) appears to have a very limited capacity to utilize exogenous progesterone (Pepe *et al.*, 1979; Winkel *et al.*, 1980; Ducsay *et al.*, 1985) or pregnenolone (Pepe, 1977; Pepe and Albrecht, 1980) as substrate for cortisol. Thus, although it was originally suggested that the fetal adrenal utilizes placental progesterone for corticosteroid synthesis (Solomon *et al.*, 1967; Voutilainen *et al.*, 1979; Branchaud *et al.*, 1985), it has been shown in rhesus monkeys (Ducsay *et al.*, 1985) and baboons (Pepe and Albrecht, 1980) that less than 6% of placental progesterone and pregnenolone secreted into the fetus prior to term is used to synthesize cortisol. Although the steroidogenic pattern as well as the growth and differentiation of the definitive and fetal cortical zones of the human and non-human primate fetal adrenal gland are clear features of intrauterine development, our understanding of the factors regulating these processes is incomplete.

FETAL PITUITARY GLAND

The pituitary gland is an oval, bean-shaped, symmetrical, brownish red organ. It weighs about 100 mg in a term human fetus, 25 mg in a term baboon fetus and 600 mg in the adult human. The pituitary is located under the brain in a bony cavity called the sella turcica and is attached to the median eminence of the hypothalamus by a stalk through which axons (about 100,000) and neurosecretion-containing blood pass. The pituitary gland develops embryologically as a fusion between an up-growth of ectodermal cells from the pharynx (Rathke's pouch) to form the anterior lobe (adenohypophysis), and a down-growth of neural tissue from the hypothalamus to form the posterior lobe (neurohypophysis). Between both lobes is found a small partition or intermediate lobe. The adenohypophysis constitutes 80% of the gland. The pituitary is evident early in the

first trimester and near the end of the first trimester is recognizable as an adult pituitary, only smaller. Both growth hormone and ACTH are evident in the adenohypophysis by this time (Williams *et al.*, 1998). Luteinizing, follicle-stimulating and thyroid-stimulating hormones as well as prolactin are evident in the adenohypophysis shortly thereafter. The neurohypophysis contains unmyelinated, hypothalamus-originating nerve fibers that contain and release oxytocin and arginine vasopressin (AVP).

The adenohypophysis is the most richly vascularized of all mammalian tissues, receiving 0.8ml blood / g / min from a unique capillary-vein-capillary network called the hypothalamo-hypophysial portal system. This portal circulation connects the median eminence of the basal hypothalamus and the adenohypophysis and plays an important role in the regulation of adenohypophysial hormone production and secretion.

During the past decades, it has generally been accepted that ACTH is the principal trophic hormone regulating the fetal adrenal gland (Gulyas *et al.*, 1977; Carr and Simpson, 1981; Jacobs *et al.*, 1994). ACTH, whose biological half-life is approximately 8 minutes, is a 39-amino-acid (4,500-dalton) peptide synthesized as part of a large, 241-amino-acid (28,500-dalton) precursor molecule, pro-opiomelanocortin (POMC). POMC is translated from a single mRNA molecule (chromosome 2) and undergoes extensive post-translational processing, including glycosylation, enzymatic cleavage and phosphorylation, NH_2–terminal acetylation, and COOH-terminal amidation of certain cleaved peptides. Enzymatic cleavage of POMC protein occurs by prohormone convertases (PC), a family of seven subtilisin-like endoproteases. PCs are responsible for the processing of neuropeptides, adhesion molecules, receptors, growth factors, cell surface glycoproteins and enzymes. PC1 and PC2 are the best-known and most studied

PCs that accomplish neuroendocrine-specific processing of protein precursors directed to the regulated secretory pathway (Muller and Lindberg, 1999). PC1 and PC2 share more than 50% homology in their catalytic domains, but exhibit differences in substrate specificity and susceptibility to inhibitors (Apletalina *et al.*, 2000).

POMC protein is cleaved at dibasic amino acids by PC1 and/or PC2 into (a) ACTH, which can give rise to melanocyte-stimulating hormone (α-MSH) and corticotropin-like intermediate lobe peptide (CLIP): (b) β-lipotropin (β-LPH), which can yield γ-LPH, β-MSH, and β-endorphin (and thus α- and γ-endorphins); and (c) a large NH_2–terminal peptide, which generates γ-MSH. In the human adenohypophysis however, $ACTH_{1-39}$ is not cleaved any further. Both immunocytochemical and biochemical studies have shown that ACTH is present in the human pituitary gland after ten weeks of gestation (Baker and Jaffe, 1975; Silman *et al.*, 1976; Begeot *et al.*, 1977). Corticotrophs (ACTH-containing cells) appear basophilic (vice acidophilic) upon hematoxylin and eosin staining and represent about 15 – 20% of all adenohypophysial cells. Mean plasma ACTH concentrations of 240 pg/ml have been found in cord blood samples obtained at 14-34 weeks in human fetuses delivered by hysterectomy. A decrease in plasma ACTH concentrations to 160 pg/ml occurs after 34 weeks. However, these single point determinations of fetal ACTH may not accurately reflect hypothalamic-pituitary function *in vivo* because of the multiple sites of secretion, stress and potential pulsatile nature of pituitary ACTH release (Pepe *et al.*, 1994). In sheep, immunoreactive ACTH becomes detectable in fetal blood on about day 100 of gestation and steadily increases at a rate of approximately 5 pg/ml between days 100 to 140 of gestation (Jones *et al.*, 1977; Jones and Roebuck, 1980; Saez *et al.*, 1984; Challis and Brooks, 1989). Progressive gestational

increases in fetal pituitary POMC mRNA and ACTH output by the human (Siler-Khodr, 1974) have also been described. In anencephalic human (Johannsson, 1979; Bocian-Sobkowska *et al.*, 1997a; Bocian-Sobkowska *et al.*, 1997b) and rhesus monkey (Novy *et al.*, 1977) fetuses, fetal adrenal gland size and weight are considerably reduced compared with normal. In human anencephalic fetuses, plasma levels of ACTH are low (Seron-Ferre and Jaffe, 1981), and the fetal adrenal gland develops normally up to 15 weeks of gestation (Williams *et al.*, 1998). Fetal adrenal adenylate cyclase activity was not detectable in cultured human fetal adrenal cells from anencephalics, although enzyme activity was very high in both the definitive and cortical zones of glands obtained from normal fetuses. When the HPAA is suppressed in normal fetuses treated with glucocorticoids, the fetal adrenal gland in humans (Easterling *et al.*, 1966; Simmer *et al.*, 1974; Gray and Abramovich, 1980; Ballard *et al.*, 1980), rhesus monkeys (Challis *et al.*, 1974) and baboons (Townsley, 1976; Leavitt *et al.*, 1997; Aberdeen *et al.*, 1998; Leavitt *et al.*, 1999) atrophies and exhibits reduced steroidogenesis. Similar observations have been made in the lamb in which the maturation of immature fetal cells was prevented by removal of the fetal pituitary (Liggins *et al.*,1973; Robinson *et al.*,1983).

Recent studies using *in vitro* and *in vivo* experimental paradigms to study the relative importance of fetal pituitary ACTH upon fetal adrenal growth, differentiation, and steroidogenesis have yielded equivocal results. For example, it has been shown in cultures of human fetal adrenal cells that ACTH inhibits (Simonian and Gill, 1981), activates (Diblasio *et al.*, 1990), partially activates (Jaffe *et al.*, 1977) or has no effect (Simonian and Gill, 1981) on cell proliferation and/or steroidogenesis. A stimulatory effect of ACTH on proliferation of the adult-type definitive zone cells was reported after

culture of collagenase-dispersed human fetal adrenal cells obtained early in gestation. However, ACTH caused involution and subsequent disappearance of the large fetal cortical zone cells under the same experimental conditions (Kahri *et al.*, 1976; Fujieda *et al.*, 1982). Jaffe *et al* (1977) demonstrated ACTH binding and cortisol secretion in the definitive/transitional zone cells of human fetal adrenals in early gestation but ACTH induced inconsistent secretion of DHAS from the fetal zone cells. In different culture conditions, addition of ACTH increased the rate of growth and the cytoplasmic size (i.e. hypertrophy) of the definitive cells over an 8-day period but had no apparent effect on fetal zone cells (Kahri and Halinen, 1974; Kahri *et al.*, 1976). These results contrast with studies demonstrating that ACTH induced proliferation and enhanced steroidogenesis of cultures of fetal zone cells obtained from fetuses at 18-20 of weeks of gestation (Diblasio *et al.*, 1990).

Moreover, 3β-HSD expression, used as a marker to assess adrenal corticosteroid-producing cell proliferation, was increased in response to ACTH in human definitive and fetal zone cells *in vitro* (Mesiano and Jaffe, 1997; Mason *et al.*, 1993). Additionally, stimulation of rhesus monkey fetal pituitary ACTH release resulted in increased 3β-HSD protein and mRNA expression in both the definitive and transitional zones (Coulter *et al.*, 1996). The ACTH-induced increase in P450c17α-hydroxylase mRNA in the transitional zone exceeded that in the fetal zone. In contrast, the relative abundance of P450scc detected in all three zones was not altered by increased ACTH secretion. It has also been shown that ACTH inhibits mitosis of adult human and bovine adrenocortical cells in culture (Hornsby and Gill, 1977; Hornsby and Gill, 1978) as well as human fetal definitive and cortical cells cultured for more than 6 days (Simonian and Gill, 1981).

Additionally, ACTH appears to be important to the increase in P450c17α-hydroxylase measured late in gestation, but ACTH may not be required for the initial expression of the P-450 hydroxylases (c11β1-, c11β2- and 21-) in sheep fetal adrenal glands (John *et al.*, 1987). Similar conclusions have been obtained using bovine fetal adrenal cells (Lund *et al.*, 1988). An important limitation of primary cultures of fetal adrenal cortical cells is that they undergo "maturation" and other phenotypic changes *in vitro* including the capacity to synthesize cortisol in the presence of added ACTH (Kahri and Halinen, 1974). Simonian and Gill (1981) demonstrated that initially, fetal and definitive zone cells in culture increased their secretion of their characteristic steroids, DHAS and cortisol, respectively, however, after 3-4 days of exposure to ACTH the pattern of steroid secretion by the fetal zone cells shifted to cortisol production, indicating induction of 3β-HSD activity in these cells. Therefore, primary cell culture of fetal adrenal gland may not be the best model to evaluate the role of ACTH on fetal and definitive zone function and development *in vivo*.

Collectively, these findings suggest that factors of fetal pituitary origin (i.e. ACTH) are essential to fetal adrenal growth and maturation, at least during the last two-thirds of intrauterine development. Since adrenal glands develop normally up to 15 weeks of gestation in anencephalic fetuses, extrapituitary factors, perhaps of placental and/or maternal origin, may also be important to adrenal maturation, at least early in gestation. Some of these factors may be placental ACTH, placental CRH, fetal adrenal/hepatic IGF-II (Aberdeen *et al.*, 1999), placental estradiol and placental chorionic gonadotropin (HCG). Both activin and inhibin appear to inhibit fetal zone proliferation and ACTH-stimulable shift of steroid production from DHAS to cortisol (Williams *et al.*, 1998).

Therefore, the role of ACTH *per se* on growth (hyperplasia and/or hypertrophy), maturation and steroidogenic capacity of the fetal adrenal gland is only partially understood.

In the adult adrenal, ACTH stimulates the synthesis and release of steroids by binding with high-affinity to its cell-surface ACTH receptor (ACTH-R), stimulating adenylate cyclase via G-protein coupled activation and the production of cAMP (Nicholson *et al.*, 1978; Kojima *et al.*, 1985; Jefcoate *et al.*, 1986). Classically, cAMP then binds to the regulatory subunits of cAMP-dependent protein kinase A (PKA) which releases the catalytic subunits. PKA catalytic subunits are now capable of phosphorylating other cytosolic and/or nuclear proteins at their serine and/or threonine amino acids. One of the proteins phosphorylated is cholesteryl ester hydrolase (CEH), increasing its activity. Consequently, more free cholesterol is released from cholesterol esters and converted to pregnenolone in the mitochondria (Greenspan and Strewler, 1997). Two other roles for cAMP include activation of cAMP-gated channels and binding to and activating cAMP-Guanine Nucleotide Exchange Factors (cAMP-GEFs). cAMP is inactivated by hydrolysis by any one of twenty-one mammalian phosphodiesterases (Conti, 2000).

The cAMP response element binding (CREB) and cAMP response element modulator (CREM) proteins bind to cAMP response elements (CRE) in genes regulated by cAMP. Both CREB and CREM are members of a family of DNA-binding phosphoproteins which contain glutamine-rich transactivation domains and basic amino acid (leucine zipper) rich regions (bZIP). CREB protein is a cAMP-inducible activator of transcription following PKA phosphorylation and binds as a homodimer to CREs, a palindromic octamer (TGACGTCA) that usually lie upstream of the basal promoter of a

significant number of genes. CREB is therefore responsible for regulation of gene transcription in response to hormonal stimulation of the cAMP pathway (Meyer and Habener, 1993). CREM protein, on the other hand, acts as a transcriptional repressor in most tissues (Daniel *et al.*, 2000). cAMP also autoregulates its own expression by modulating three cAMP response elements responsible for cAMP induction of transcription (Meyer *et al.*, 1993; Walker *et al.*, 1996). Extracellular calcium, which enters through T-type calcium channels, independent of adenylate cyclase activation, is required for optimal ACTH binding and ACTH-induced steroidogenesis (Williams *et al.*, 1998). All of the specific proteins phosphorylated in response to ACTH to activate steroidogenesis are not known. Increases in expression of immediate early genes *fos-B*, *jun-B*, c-*fos* and c-*jun* have been implicated (Williams *et al.*, 1998). However, accumulation of cAMP does precede steroidogenesis, and binding of cAMP to the regulatory subunit of protein kinase correlates well with stimulation of steroidogenesis (Hayashi *et al.*, 1979). It has been proposed that cAMP also acts directly on exocytotic machinery independent of PKA to stimulate hormonal release (Ozaki *et al.*, 2000).

The effects of ACTH on steroidogenesis are two-fold. Acute effects occur within a few minutes and involve an increase in adrenal blood flow and stimulation of cholesterol uptake (via LDL-cholesterol receptors), availability and transport to the inner mitochondrial membrane and conversion to pregnenolone by mitochondrial P450scc (Williams *et al.*, 1998). Steroidogenic acute regulatory protein (StAR) is an ACTH- and SF-1-inducible protein and is a key modulator of cholesterol transport into mitochondria. A second protein involved in this process is the peripheral-type benzodiazepine receptor (PBR), which completes the final step of cholesterol delivery to P450scc for

transformation into pregnenolone (Lacroix *et al.*, 2001). ACTH also up-regulates the immediate early genes c-*fos* and c-*jun* via the PKA pathway as well as its own receptor, the ACTH-R. Only small amounts of cortisol are stored in the adrenal. The long-term or chronic specific responses to ACTH require hours or days and are manifested as increased synthesis in DNA and mRNA for most of the steroidogenic enzymes (3β-HSD II, P450c17, P450 c21 and P450c11) and also cellular growth (hyper-vascularization, cellular hypertrophy, and cellular hyperplasia). This long-term regulation is complex, as no clear correlation exists between mRNA and protein levels of steroidogenic enzymes *in vivo* (Lacroix *et al.*, 2001).

The ACTH-R is the smallest member (297 amino acids) of the super family of G protein-coupled receptors with seven hydrophobic transmembrane domains (Mountjoy *et al.*, 1992). The ACTH-R belongs to the melanocortin receptor subfamily which is characterized by short NH_2–terminal extracellular domains (which contain two putative N-glycosylation sites), short COOH-terminal intracellular domains and unusually short fourth and fifth transmembrane-spanning domains (Allolio and Reincke, 1997). As in the adult adrenal, *in vitro* studies of human adrenal cortical cells from mid-gestation fetuses has shown that these cells do contain ACTH receptor mRNA and that ACTH receptor expression is upregulated by its own ligand and also by angiotensin II. ACTH specifically increases the transcription rate of the ACTH-R and the ACTH-R mRNA half-life. This upregulation is apparently mediated in the human (Lebrethon *et al.*, 1994a; Lebrethon *et al.*, 1994b; Mesiano *et al.*, 1996) and baboon (Davies *et al.*, 1993) fetal adrenal by a PKA (i.e. cAMP mediated), not a PKC (i.e. calcium and/or diacylglycerol), mechanism. Recently, our laboratory (Albrecht *et al*, 1996) demonstrated that adrenal

ACTH-R mRNA levels in the baboon fetus increase 13-fold from early to mid gestation and then unexpectedly decline by 70% in term adrenal. This biphasic, monomodal developmental expression contrasts with previous data demonstrating a progressive increase in adrenal weight (Albrecht *et al.*, 1996), an ontogenic increase in activity of steroidogenic enzymes (Pepe and Albrecht, 1991) and an increase in *de novo* cortisol production near term (Pepe *et al.*, 1990). These three increases are consistent with action of ACTH in both fetal and definitive zone cells.

Additionally, it has recently been demonstrated that in contrast to the developmental increases in growth and function of the definitive zone, there is a decline in the output per cell of fetal zone-specific DHA, between mid- and late gestation (Berghorn *et al.*, 1995). Although the secretion of DHAS increased with advancing gestation, the absolute levels of DHA and DHAS were lower compared to cortisol levels near-term. The increase in size of the fetal adrenals, apparently accounts for the progressive increase in total adrenal C_{19}-steroid output. These findings suggest that there is an uncoupling of development between the fetal zone and the definitive zone in the second half of pregnancy, again indicating a multifactorial regulation of fetal adrenal maturation.

FETAL HYPOTHALAMUS

The hypothalamus and pituitary form a unit which is recognizable and mature by mid-gestation and able to exert control over adrenal function (Ackland *et al.*, 1986; Blumenfeld and Jaffe, 1986; Scott and Pepe, 1987; Berghorn *et al.*, 1991). The median eminence at the base of the third ventricle of the hypothalamus is the site at which adenohypophysis-regulating neurons release their secretions into the capillaries of the hypothalamo-hypophyseal portal system. The median eminence, which is evident by the

9th gestational week, contains special features including densely packed nerve endings, fenestrated (windowed) capillaries and supporting glial and ependymal (tanycytes) cells which allow feedback effects of pituitary and adrenal gland hormones (Williams *et al.*, 1998). Some of the nerve endings in the median eminence are the terminals of corticotropin releasing hormone (CRH) neurons, which originate in the paraventricular nucleus of the mediobasal hypothalamus. CRH, a 41 amino acid peptide, is synthesized as a 196 amino acid pro-hormone and undergoes enzymatic cleavage at basic amino acid pairs prior to release. CRH belongs to a family of peptides that includes sauvagine from frog skin and urotensin I from teleost fish, both of which have ACTH-releasing and hypotensive activities like those of CRH (Williams *et al.*, 1998). CRH acts by binding to its specific CRH receptor type I (CRH-R1) on adenohypophysial corticotrophs and activating adenylate cyclase, thereby increasing intracellular cAMP levels which summarily activate PKA, increasing the influx of extracellular calcium through L-type calcium channels and increasing the production of lipoxygenase metabolites of arachidonic acid. A prompt release of ACTH ensues as well as an increase in the POMC mRNA transcription rate (Williams *et al.*, 1998). About 50% of CRH-containing parvocellular neurons contain arginine vasopressin (AVP) which can be concomitantly released with CRH to synergistically stimulate ACTH release from the adenohypophysis. AVP exerts its effect by binding to vasopressin (V_3) receptor, thereby activating phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol 1,4,5-trisphosphate (IP_3), which mobilizes intracellular calcium, and diacylglycerol, which activates phospholipid- and calcium-dependent protein kinase C (PKC). CRH and AVP are secreted into the hypophyseal-portal circulation in a pulsatile

fashion. These rhythms are responsible for pulsatile release (about 15 – 18 pulses per 24 hour day) of ACTH and contribute to peak levels of ACTH and cortisol in the early morning. Important hypothalamic excitatory inputs which increase CRH and AVP release come from the suprachiasmatic nucleus (the regulator of circadian rhythms), the amygdala (a way station of the limbic system, i.e. "emotional" brain), the raphe nuclei of the brain stem (site of origin of the serotonergic system), and the locus ceruleus of the brain stem (site of origin of ascending noradrenergic fibers). Vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide (PACAP), angiotensin II (Ang-II), neuropeptide Y (NPY), cholecystokinin (CCK), gastrin-releasing peptide (GRP), interleukin-I (IL-1), tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), serotonin, histamine and acetylcholine mildly enhance CRH and AVP secretion (Lacroix *et al.*, 2001). Inhibitory inputs on CRH secretion arise in the hippocampus and in the locus ceruleus of the midbrain (Williams *et al.*, 1998). Atrial natriuretic peptide (ANP), substance P, somatostatin and nitric oxide (NO) suppress CRH release. Psychological and physical stress can activate the HPAA with an increase in both ACTH and cortisol secretion. In addition to adrenal cortisol release, the ACTH released by CRH stimulates the secretion of other adrenal steroids, including aldosterone and DHA. Cortisol inhibits secretion of CRH, AVP and ACTH and the synthesis of their respective mRNAs. The set point of plasma cortisol feedback inhibition is determined by the central nervous system.

In humans, as well as non-human primates, CRH is produced by the placenta (Pepe and Albrecht, 1999). Placental CRH is identical in immunoreactivity and bioactivity to hypothalamic CRH. During human pregnancy, maternal plasma CRH concentrations rise exponentially in the second and third trimesters and fall precipitously after delivery. The

absolute CRH levels, and perhaps the rate of increase, correlate with the timing of parturition (Reis *et al.*, 1999). Apparently, part of this parturition mechanism involves CRH binding to its type-II receptor in the myometrium and modulating labor (Stevens *et al.*, 1998). In baboons, plasma CRH concentrations peak in the first half of pregnancy, then decline to lower levels for the remainder of gestation. In both species, placental CRH is secreted into the fetus but at concentrations 100-fold lower than those in the mother. CRH stimulates placental ACTH production *in vitro* suggesting a possible paracrine role for placental CRH (Williams *et al.*, 1998). However, cortisol has no inhibitory effect on CRH or POMC release as in the hypothalamo-pituitary unit. CRH type-I receptors have also been localized to human fetal adrenal gland fetal zone cells. Moreover, CRH, via the phospholipase C-inositol phosphate PKC second messenger system vice the classical adenylate cyclase-cAMP-PKA pathway seen in pituitary corticotrophs, increased the mRNA for P450scc and P450c17α-hydroxylase, but not 3β-HSD (Chakravorty *et al.*, 1999). Additionally, CRH was as effective as ACTH in stimulating DHAS production and release in cultured human fetal adrenal fetal zone cells, but not cortisol release (Smith *et al.*, 1998). Finally, among the hypophyseotropic hormones, CRH is the only one for which a specific binding protein (in addition to the receptor) exists in tissue or blood. (Williams *et al.*, 1998). The placenta and livers are the principal sources of pregnancy-related CRH-binding protein, which complexes, and renders inactive, up to 80% of plasma CRH (Pepe and Albrecht, 1999). The functional significance of the CRH-binding protein is not fully understood although its presence in corticotrophs may act to sequester and/or terminate the action of membrane-bound CRH.

A second role for the CRH system is in energy homeostasis via direct central actions independent of HPAA control. A CRH-related peptide, urocortin, has a much higher affinity for another CRH receptor, (CRH-R2), which is expressed in the heart, muscle, GI tract and brain. Both CRH and urocortin, when administered centrally, inhibit feeding, decrease appetite and stimulate metabolism, in opposition to the role of hypothalamic CRH in stimulating cortisol release and enhancing energy storage (Cullen *et al.*, 2001).

STEROID HORMONE ACTION

Estrogens exert a wide variety of effects on growth, development and differentiation, including important regulatory functions within the reproductive, central nervous and hypothalamo-pituitary axis systems. Estrogens mediate these activities through binding to specific, high-affinity, intranuclear receptor proteins, the estrogen receptor alpha (ERα), and the estrogen receptor beta (ERβ), encoded by two separate genes located on human chromosomes 6 and 14, respectively. Both receptors function as signal transducers and transcription factors to modulate expression of target genes (Klinge, 2000). Both receptors are sequestered in an "inactivated" form in the nucleus and, upon binding of ligand, undergo conformational changes, presumably by dissociation of heat shock proteins (hsp90 and hsp70) to form an "activated" receptor. The estrogen receptors can then dimerize (αα, ββ or αβ) and bind to specific DNA sequences, estrogen response elements (EREs), and stimulate estrogen-target gene transcription. The minimal consensus ERE is a palindromic inverted repeat (IR): 5'-GGTCAnnnTGACC-3', where n = any nucleotide. Most estrogen-regulated genes contain imperfect, non-palindromic EREs (Klinge, 2000). Secondly, ER can enhance gene expression without direct DNA

binding. One mechanism accounting for this effect is interaction between ER and other transcription factors that may stabilize the DNA binding of that transcription factor (Webb *et al.*, 1999). Examples of this DNA-binding-independent mechanism include ER α interaction with SP1 and ERα/ ERβ interaction with AP-1 (Klinge, 2000). Thirdly, in addition to the classic ligand-mediated activation pathways, it has been shown that ER, in the absence of ligand, can be activated following phosphorylation by growth factors (EGF) or kinases (PKA, MAPK or CDK2; Hall and McDonnell, 1999). Finally, the fourth possible mechanism for estrogen action is the putative existence of a cytoplasmic membrane, G-protein coupled ER (Razandi *et al.*, 1999), with downstream effects on calcium flux and cAMP generation.

ERα and ERβ are members of the steroid hormone/nuclear receptor (NR) superfamily (about 150 members) of transcription/enhancer proteins. The superfamily includes the class I NR, the steroid receptors, e.g. glucocorticoid, mineralocorticoid, progesterone, and androgen receptors and the class II NR, e.g. retinoic acid receptor, retinoid X receptor, vitamin D receptor, thyroid receptor and peroxisome proliferator activated receptor. Additionally, the NR superfamily includes about 50 "orphan receptors" which are putative NRs that have no identifiable ligand. Molecular cloning of the cDNA of the NRs has led to rapid advances in our knowledge of these proteins including the fact that they all share common functional domains (A, B, C, D, E and F) for DNA binding (DBD), ligand binding (LBD) and activation function (AF). The N-terminal domain, termed the A/B domain, is of variable length and has the lowest degree of sequence similarity. This domain usually harbors an activation function (AF-1) that contributes to transcriptional activity. Adjacent to the A/B domain is the C domain or

DNA-binding domain (DBD). This is the most highly conserved region and consists of two cysteine-rich (8 total cysteine amino acids) Zn^{2+} finger motifs that confer specific DNA-binding capacity. Within the DBD is the P-box which encompasses six amino acids (four cyteines) and the first Zn^{2+} finger and confers DNA-binding specificity (i.e., recognition of target genes). Additionally within the DBD is the D-box, which utilizes the second Zn^{2+} finger to confer dimerization activity and is important for efficient DNA binding at palindromic, direct repeat or inverted repeat hormone response element DNA sequences. Adjacent to the DBD or C domain is the D domain which is poorly conserved and contains the hinge region (40 – 50 amino acids) involved in association with the molecular chaperone heat shock protein 90 (hsp90), nuclear localization, dimerization and cofactor binding. At the C-terminus is the ligand binding region or E/F domain which displays a high degree of homology among the NR superfamily members. This multifunctional region is involved in dimerization, ligand binding (agonists and antagonists), cofactor binding, activation functions (AF-2), nuclear localization and hsp90 interactions.

The human ERα gene is extremely complex. To date, six different mRNAs (± 1,785 bases) transcribed utilizing three different promoters from the same gene have been described (Flouriot *et al.* 1998). These transcripts encode a common protein (595 amino acid, 66,182-daltons) but differ in their 5'-untranslated region. ERα has a rapid turnover rate in cells (3 – 4 hours). The human ERβ gene also appears to be complex (Hall and McDonnell, 1999). Three isoforms of ERβ have been described: ERβ-short (485 amino acids) and ERβ-long (548 and 530 amino acids). The expression patterns of ERα and

ERβ mRNAs are different. ERβ mRNA (7 kb and 9.5 kb) is more widely expressed than ERα and is highest in endometrium, placenta, prostate, ovary, lung, adrenal, testis, kidney and spleen (Albrecht *et al.*, 1999; Brandenburger *et al.*, 1997). The highest expression of ERα mRNA in the human has been found in ovary, testis, pituitary, colon and uterus (Enmark *et al.*, 1997; Mosselman *et al.*, 1996).

The DBDs of the two ERs share approximately 97% sequence similarity. In the LBD, the overall amino acid identity is 55% (Pettersson and Gustafsson, 2001). The major differences in ligand binding between ERα and ERβ lie in the affinities for various compounds, and the transcriptional response a given compound is able to elicit. Binding of an agonist to the LBD induces a conformational change that is associated with the transition to a transcriptionally active complex. With regard to the ability to activate transcription with ERE-containing constructs, ERβ is weaker (20–60%) than ERα in most cell systems tested. This is at least partly attributable to the fact that ERβ does not contain a strong AF-1 within its amino-terminus A domain, but rather contains a repressor domain that when removed, increases the overall transcriptional activity of the ERβ (Hall and McDonnell, 1999). Finally, phosphorylation plays a major role in the multistep process of ER activation. ER phosphorylation occurs as part of both ligand-induced activity and ligand-independent transcriptional activation (Pettersson and Gustafsson, 2001). Phosphorylation of both ER's tyrosine in the LBD, serines 118 and 236 in ERα and serines 106 and 124 in ERβ enhances dimerization and/or transcriptional activity *in vitro*.

However, significant gaps still exist in our understanding of the mechanism by which receptors stimulate or repress gene transcription. A more recent advance in the

understanding of NR gene regulation considers a new class of proteins called coregulators. These proteins have the faculty to repress (corepressors) or to enhance (coactivators) the activity of genes regulated by nuclear hormone receptors in a ligand-dependent fashion (Robyr *et al.*, 2000). Approximately 19 different ERα coactivator proteins have been identified. Three of the most studied include SRC-1, SRC-2 and CBP/p300 which form complexes with ER to increase the rate of hormone-responsive gene transcription. These coactivators contain unique, conserved leucine-rich amino acid repeats (LXXLL), also called NR boxes, which form hydrophobic surfaces and are responsible for binding to/interacting with ERs. Some of these coactivators, including SRC-1 and -3 (steroid receptor coactivator) have histone acetyltransferase (HAT) activity. HATs acetylate lysine residues on the N-terminal tails of histones H3 and H4 in chromatin, resulting in weaker association of the histones with DNA. This disruption of higher-order chromatin structure facilitates access of transcription factors (i.e., RNA polymerase II) to promoter elements and is associated with increased transcriptional activity (Pettersson and Gustafsson, 2001). Corepressors, on the other hand, recruit proteins that have histone deacetylase (HDAC) activity that is believed to repress gene expression by maintaining chromatin in a more condensed state (Klinge, 2000). Corepressors may also interact with ERs and obstruct recruitment of coactivators. Two such corepressors are NcoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptors). Recent studies suggest that it is the repertoire of transcription factors (including coactivators and corepressors) present within a cell in addition to the selective use of a specific promoter that determines transcriptional activity of ERα and ERβ (Donaghue *et al.*, 1999).

Cortisol can bind to two intracellular receptors, type I (the mineralocorticoid receptor, so named because it binds aldosterone and glucocorticoids with high affinity) and type II (glucocorticoid receptor, which has low affinity for mineralocorticoids). Glucocorticoid action involves binding of the steroid-receptor complex to regulator sequences in the genome and/or binding to coregulator proteins. Type I receptors are saturated by basal levels of glucocorticoids, whereas type II receptors are not saturated under basal conditions but approach saturation during peak phases of the circadian rhythm (i.e. early AM) and during stress (Williams *et al.*, 1998). The majority of cortisol effects occur via the type II GR, which exists in two isoforms, GRα and GRβ. The GRα and GRβ transcripts are a result of alternative gene splicing (chromosome 5) with identical NH_2-terminal amino acid sequences through amino acid 727. Thereafter GRα has an additional 50 amino acids whereas GRβ has an additional, non-homologous 15 amino acids. In contrast to GRα, GRβ does not bind glucocorticoid agonists or antagonists but apparently exerts inhibitory actions by heterodimerizing with ligand-bound GRα and/or repressing GRα actions on glucocorticoid responsive promoters (Dahia *et al.*, 1997; Bamberger *et al.*, 1995; Oakley *et al.*, 1996). In addition to the hypothalamo-pituitary unit mentioned above, the placenta is also a glucocorticoid target organ with placental growth and endocrine function known to be affected by glucocorticoids (Burton and Waddell, 1999). Glucocorticoid binding activity has been demonstrated in placental tissue of rodents, rabbits and humans (Burton and Waddell, 1999). Rat placental GR mRNA has been detected and placental 11β-HSD enzymes may locally regulate access of glucocorticoids to their receptors within the placenta (Burton

and Waddell, 1999). This phenomenon has been demonstrated in rat testes Leydig cells (Monder *et al*, 1994a; Monder *et al*, 1994b).

Circulating steroid hormones (i.e. cortisol and estradiol) are largely bound to plasma proteins. The major binding proteins are corticosteroid-binding globulin (CBG or transcortin), sex hormone-binding globulin (SHBG) and albumin. The binding globulins have high affinity (CBG for cortisol and SHBG for estradiol) and low capacity for steroid binding, whereas albumin has low affinity and high capacity. Under physiological conditions, cortisol (90-97%) is protein bound, most to CBG (383 amino acids; 59,000-dalton glycosylated protein) and the rest to albumin. About 90% of DHA and DHAS are bound to albumin, as are estrogens. Cortisol and other steroids can also associate with erythrocytes. The physiological role of plasma protein binding of steroid hormones is not fully understood.

PLACENTAL P450-AROMATASE ENZYME AND ITS INHIBITION

The rate-limiting step in the biosynthesis of estrogens is the aromatization of androgens androstenedione (A_4) and testosterone (T_4) to estrone (E_1) and estradiol (E_2), respectively, by aromatase enzyme (estrogen synthetase). As mentioned previously, A_4 can be converted to T_4, and E_1 can be converted to E_2 via 17β-HSD. Aromatase exists in a membrane-bound enzyme complex consisting of a cytochrome P-450 hemoprotein (aromatase, $P\text{-}450_{arom}$, a 55-kDa protein of 503 amino acids), and a flavoprotein (reduced NADPH-cytochrome P-450 reductase). Conversion of the C_{19} androgens is thought to occur by three sequential reactions at C-19, each requiring 1 mole of NADPH and 1 mole of molecular oxygen, culminating in the elimination of the C-19 atom as formic acid and the aromatization of ring A of the steroid molecule (Brodie and Njar, 2000).

The aromatase mRNA is composed of 10 exons and is transcribed from a 75 kb gene which contains five major promoter regions in it's 5'-untranslated end. The enzyme is expressed in the brain, in testis Leydig cells, muscle, adipose tissue (including that in the breast), ovarian granulosa cells and the placental syncytiotrophoblast cells, where fetal/maternal adrenal androgens are converted to estrogens. The presence of aromatase enzyme proximal to or within breast tumors which are estrogen-dependent/sensitive has stimulated the development of effective strategies to inhibit this rate-limiting enzyme (Brodie and Njar, 2000).

Aromatase inhibitors fall into two major categories; steroidal inhibitors and nonsteroidal inhibitors. Steroidal inhibitors compete with androstenedione for the active site and include 4-hydroxyandrostenedione (4-OHA), exemestane, atemestane and 10-propagyl-androstenedione. The nonsteroidal inhibitors possess a nitrogen-containing heterocyclic moiety which binds with the iron atom of hemoprotein P-450_{arom} and excludes substrate androstenedione from the active site. The two major nonsteroidal aromatase inhibitors are triazole compounds; anastrozole (arimidex, ZN1033) and letrozole (femara, CGS20267). Clinical trials with CGS 20267 have demonstrated no clinically relevant changes in blood chemistry and hematology tests (Trunet *et al.*, 1993; Iveson *et al.*, 1993). Serum cortisol and aldosterone levels were not affected. As expected, there were dose-dependent increases in T_4, LH and FSH. Both inhibitors (Albrecht et al., 2000) are highly potent, competitive and reversible and have been recently approved as treatments for postmenopausal breast cancer (Brodie and Njar, 2000).

PHYSICAL AND ANATOMICAL CONSIDERATIONS OF THE BABOON

The baboon (*Papio anubis*) is a considerably large animal with the adult females averaging about 22-33 pounds. Yet, the animal is docile, easy to handle and extremely adaptable. Because of its size, blood and other tissue samples can be collected repeatedly during gestation and surgical procedures. As in humans, the baboon possesses a hemochorial and monodiscoid placenta, which becomes the major source of estrogen and progesterone around 30 days post ovulation (Albrecht and Pepe, 1988). Fetal adrenal androgen precursors are used for placental estrogen synthesis (Albrecht *et al.*, 1980). The growth and maturation of both the human and baboon fetal adrenal glands exhibit similar developmental patterns (Pepe *et al.*, 1977; Pepe and Albrecht, 1990). As in women, the baboon placenta syncytiotrophoblast cells synthesize and secrete unique proteins including chorionic somatomammotropin (Musicki *et al.*, 1997), pregnancy-associated plasma protein A (Pepe *et al.*, 1994a) and insulin-like growth factors (Putney *et al.*, 1990, 1991).

ENDOCRINOLOGICAL CONSIDERATIONS OF THE BABOON

The maternal pattern of placental steroid hormone production exhibited in pregnant baboons is similar in many important aspects to that in pregnant women. The rate of progesterone (P_4) production (Albrecht and Townsley, 1976b) and serum progesterone concentrations (Albrecht and Townsley, 1976a) are elevated during pregnancy in baboons as in women (Lin *et al.*, 1972), although the absolute amounts of progesterone are less in baboons (about 12 ng/ml the last two-thirds of gestation in baboons and 145 ng/ml in women). Additionally, placental progesterone production in both baboons and women is dependent upon low density lipoprotein (LDL) cholesterol, not *de novo* synthesis (Albrecht

and Pepe, 1990). Recent studies in the baboon have demonstrated an estrogen-dependent developmental increase in the receptor-mediated uptake of LDL cholesterol substrate as well as estrogen-induced increases in the mRNA for the LDL receptor and P450scc in baboon placental syncytiotrophoblasts. Finally, the metabolism of progesterone is also similar in these two species during pregnancy, pregnanediol being the major metabolite (Waddell *et al.*,1996).

The concentrations and secretory pattern (Albrecht and Townsley, 1978) of estradiol (E_2) and estrone (E_1) in the maternal and fetal circulation of pregnant baboons are similar to those in pregnant women. Estrone is secreted preferentially into the fetus whereas estradiol is secreted primarily into the maternal circulation (Waddell *et al.*, 1992). Moreover, the absolute amounts of E_2 present in the peripheral circulation at term in baboons are in the same order of magnitude as those in women (i.e., 5-10 ng/ml). In the baboon however, the major urinary estrogen is E_1 and in women, estriol (E_3). Maternal serum estradiol levels during baboon pregnancy have been demonstrated (Figure 1).

The metabolism of cortisol and cortisone in the adult female baboon is also very similar to that in women. In both species, adrenals of adult animals secrete only cortisol and 99% of serum cortisone levels are derived from secreted cortisol (Pepe and Townsley, 1978). During pregnancy, the pattern of cortisol-cortisone metabolism is altered in both species (Pepe and Townsley, 1975) and an increase in corticosteroid binding globulin is observed, without a significant increase in the production rate of cortisol (Oakey, 1975; Pepe *et al.*, 1976). The metabolic clearance rate of cortisol exceeds that of cortisone and the type and degree of conjugation and formation of tetrahydrocortisol and tetrahydrocortisone

are very similar in baboons and women. Examination of urinary metabolites of cortisol in baboon neonates revealed a pattern very similar to that in human neonates; a low cortisol production rate, reduced glucuronidation and an increase in production of highly polar compounds such as 6β-hydroxycortisol (Pepe and Townsley, 1976). Development, function and morphological zonation of the fetal adrenal in the human and the baboon are similar and distinct from those in other species.

BABOON HUSBANDRY

A colony of baboons (*Papio anubis*) was established in 1978 at the Eastern Virginia Medical School in the Central Animal Facility. Female baboons weighing 13-16 kg, are housed individually in aluminum-stainless-steel primate cages (32.5" x 36" x 56", LC-1106 baboon cage, Research Equipment Co, Bryan, TX) in air-conditioned rooms with 12 hours on/12 hours off light/dark lighting schedule. Baboons receive Purina monkey chow (Ralston Purina Co., St. Louis, MO) twice daily, fresh fruit and vitamins once a day and water *ad libitum*. Baboons are cared for and used strictly in accordance with USDA regulations and the NIH Guide for the Care and Use of Laboratory Animals (Publication 85-23, 1985). The experimental protocols employed in this study were approved by the Institutional Animal Care and Use Committee of the Eastern Virginia Medical School.

BABOON MENSTRUAL CYCLE

The menstrual cycle of the baboon resembles that of the human in all major phases. The pattern of expression and levels of E_2 (range 20 – 280 pg/ml) and P_4 (range 1 – 13 ng/ml), as well as, FSH and LH are very similar to those in women. In our colony the average length of the menstrual cycle, as determined by the first day of bleeding of one cycle up to the onset of bleeding in the following cycle is 29 days. Changes in the perineum

(sex skin) are used to determine various stages of the menstrual cycle. They are the proliferative, ovulatory, luteal, and involution phases. These stages of the cycle are correlated with changes in the perineum area and offers a visual indication of hormonal activity and sexual receptivity.

The changes in the sex skin can be described as: a) perineal turgescence b) maximum turgescence c) perineal deturgescence, and d) perineal rest (Hendrickx, 1967). During the perineal turgescence stage, the perineum begins to swell and the color deepens from a dull pinkish red to a brighter red. The turgescence of the perineum continues through the postmenstrual and preovulatory phases. This period lasts from 4 to 8 days. During the maximum turgescence period, which is associated with maximum estrogen production, the perineum becomes fully distended and reaches its deepest and most intense color, a bright red. The entire perineum is devoid of wrinkles and has a smooth, shiny appearance. The period of maximum turgescence encompasses the latter part of the preovulatory phase, the entire ovulatory phase, the transition period and in most instances the postovulatory phase. Usually there is a brief period of near-maximum turgescence followed by another brief period of about five days when the perineum reaches its maximum size. Ovulation seems to occur during the last half of the maximum-turgescence period. The entire period lasts between 5 and 15 days. During the perineal deturgescence stage, the sex skin area shrinks rapidly and occurs because of a decrease in ovarian estrogen production and a concomitant increase in progesterone production by the newly formed corpus luteum. This menstrual cycle period coincides with the postovulatory and luteal phases. The onset of deturgescence is indicated by the beginning of wrinkles on the outer border of the sex skin and by a change in color from a

bright red to a pinkish red. The duration of this period is between 4 and 14 days. Following this, the perineum is at rest. This coincides with the latter part of the luteal phase and the onset of a new menstrual cycle, marked with endometrial shedding and subsequent bleeding. The epithelial surface of the perineum is dull compared to the bright and shiny surface during maximum turgescence. This period lasts from 5 to 15 days. In the pregnant baboon, the perineum remains at rest throughout the gestation period although the color changes to a magenta red (Hendrickx, 1967). Females are paired with male baboons (25-40 kg) for five days at the anticipated time of ovulation. Pregnancy is confirmed 30 days post ovulation by determining the presence of chorionic gonadotropin in urine using the nonhuman primate pregnancy test kit supplied by Gabe Bialy and the National Institute of Health and/or by measuring serum E_2 levels.

CHAPTER II

DEVELOPMENTAL REGULATION OF PROTEIN KINASE-A AND –C ACTIVITIES IN THE BABOON FETAL ADRENAL GLAND

INTRODUCTION

Pepe and Albrecht have previously demonstrated that the estrogen-regulated change in transuteroplacental metabolism of cortisol (F) and cortisone (E) from preferential reduction (E to F) at midgestation to oxidation (F to E) near term results in activation of the hypothalamic-pituitary adrenal axis of the baboon and the ontogenesis of rate-limiting steroidogenic enzymes culminating in *de novo* F secretion (Pepe and Albrecht, 1984; Pepe and Albrecht, 1990; Pepe *et al.*, 1990; Pepe and Albrecht, 1991). Our present investigations have been directed at elucidating the subcellular mechanisms in the hypothalamic-pituitary-adrenal axis that have been activated by estrogen-induced alterations in placental corticosteroid metabolism. With regard to adrenal function, it is well established (Simpson and Waterman, 1988) that translation of messages activated by peptide-mediated binding to membrane receptors can occur via cyclic AMP (cAMP) dependent protein kinase A (PK-A) and/or phospholipid/calcium dependent protein kinase C (PK-C). Our present understanding of the respective roles of these kinases in fetal adrenal function, however, is incomplete. Recently, it has been demonstrated in human fetal adrenal cells in culture, that activation of PK-C by phorbol esters blocked increases in 17α-hydroxylase-C_{20} lyase (17α-OHase) activity (McAllister and Hornsby, 1988) and mRNA (Ilvesmaki and Voutilainen, 1991) elicited by activators of cyclic AMP including ACTH and forskolin. In contrast, activation of PK-C increased the activity of Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD) elicited by forskolin (McAllister and Hornsby, 1988) and also activated the mRNA for this enzyme following long-term treatment alone (Voutilainen *et al.*, 1991). Therefore, it appears that the pattern of fetal adrenal steroidogenesis can be modulated by the relative abundance and subsequent activation of PK-A and PK-C. Although PK-C has been measured in the human fetal

adrenal at 12-18 weeks of gestation and protein content decreased substantially in tissue of near term human anencephalics (Rainey *et al.*, 1988), studies of the ontogenetic development and regulation of PK-C and PK-A in the human and nonhuman primate fetal adrenal have not been performed. The present study was designed therefore, to determine whether the basal levels and/ or ratio of PK-A and PK-C in the fetal adrenal change with advancing baboon gestation and whether basal activities of PK-A/PK-C are regulated by estrogen-induced alterations in placental corticosteroid metabolism and thus could provide the mechanism(s) by which the increase in fetal adrenal 17α-OHase and 3β -HSD is mediated with advancing gestation and maturation of the fetus.

MATERIALS AND METHODS

Blood sampling

Between day 70 - 100 or day 130 - 170 of gestation blood samples were obtained from pregnant baboons at 1300 - 1430 h every alternate day. Animals were briefly sedated by intramuscular administration of 100 mg Ketamine HCL (Parke-Davis, Detroit, MI) and 5 ml blood samples (3 - 7 ml) were obtained from the maternal saphenous or antecubital vein via a 21-gauge scalp vein needle. Serum was stored at -20 °C until assayed and serum E_2, P_4, DHA, DHAS and F concentrations determined by solid phase ^{125}I radioimmunoassay (Coat-A-Count, Diagnostic Products Corp, Los Angeles, CA) or by means of an automated chemiluminescent immunoassay system (Immulite; Diagnostic Products Corp, Los Angeles, CA). Blood samples were also collected at the time of the cesarean section and stored at -20 °C until assayed.

Operative procedure: anesthesia and general surgical method

The endocrine system is sensitive to the potential effects of various anesthetics. Therefore, it was essential to use anesthetic agents that produce the fewest possible

confounding effects. Various anesthetics, including ketamine and halothane, and their effect on serum concentrations of various hormones secreted by the hypothalamus, pituitary and adrenals of female baboons has been investigated (Walker *et al.*, 1987). Administration of ketamine (Ketalar, Parke-Davis, Morris Plains, NJ) was found to maintain anesthesia and had no effect on serum levels of PRL, F and DHA. DHAS levels rose slightly, but only after 75 min of ketamine administration. Halothane had no effect on hormones of the adrenocortical system but acetylpromazine increased PRL secretion and also induced a marked rise in androgen production (Walker *et al.*, 1987).

Accordingly, ketamine was used as an intravenous anesthetic in this study. Ketamine alone, however, is not considered an effective agent in attaining analgesia to visceral pain (Walker *et al.*, 1987). Halothane, an inhalant anesthetic agent, depresses the secondary afferent system as well as the subcortical structures that regulate somatic and viscerai functions. Therefore, in the present study ketamine was used for restraint and halothane/nitrous oxide for induction of anesthesia and sedation.

Baboons were restrained by intramuscular injection of 200 mg ketamine. Twenty minutes after the onset of ketamine, animals were placed in a supine position and intubated with an endotracheal tube through which a mixture of gases (nitrous oxide; N_2O_2, 25%, 1 liter/min; and O_2, 75%, 3 liters/min) were passed. Between 0 and 30 min 1.5% halothane (Fluothane, Ayerst Laboratories, NY) and N_2O_2 were administered. From 30 min until termination of treatment, the concentration of the halothane was reduced to 1.0% in the presence of N_2O_2 carrier. Maternal heart rate was monitored throughout the period of anesthesia and maintained at 90 - 100 beats/min. At the indicated times of pregnancy, animals were restrained and sedated with an intramuscular

injection of ketamine (10 mg/kg body weight). Anesthesia was achieved as indicated above. The uterus was exposed by an abdominal incision, a transverse incision was then made in the uterus and the umbilical cord clamped and the fetus and/or placenta removed. Blood samples were taken at numerous times during the surgery. The mother received approximately 250 ml dextrose-5%-in-water via saphenous canula.

Baboon treatment with estrogen and harvesting of fetal adrenal glands

Female baboons (*Papio anubis*) weighing 10-15 kg, were housed individually in metabolic cages in air-conditioned quarters under standardized conditions (Albrecht, 1980). Females were paired with males for 5 days at the anticipated time of ovulation as estimated by menstrual cycle history and turgescence of the external sex skin. Baboons were used strictly in accordance with USDA regulations and the NIH Guide for the Care and Use of Laboratory Animals (Publication 85-23, 1985). The experimental protocol employed was approved by the Institutional Animal Care and Use Committee of Eastern Virginia Medical School.

Fetal adrenal glands were obtained on day 100 (n=8) and day 165 (n=6) of gestation (term = day 184) from untreated baboons and on day 100 from baboons treated daily with a maximal dose of 0.5 (n=2), 1.0 (n=3), or 2.0 mg (n=5) estradiol benzoate (Sigma Chemical Co., St. Louis, MO) suspended in 0.5 ml sesame oil and injected sc in increasing concentrations (0.25 to 2.0 mg/day, doubling the dose every 5 days between days 70 and 100 of gestation; i.e. 0.25 mg on days 70-74; 0.5 mg on days 75-79; etc). In two additional baboons studied on day 100, pellets of the aromatizable androgen androstenedione (50 mg; Innovative Research Products, Toledo, OH) were administered in increasing concentrations between days 70 and 94 of gestation (i.e., two on day 70;

four on day 78; six on day 86 and eight on day 94) as described previously (11). In an additional study group, 2 baboon fetuses were treated *in utero* with ACTH (Cortrosyn) delivered continuously into the fetal leg muscle between days 70 and 100 via an Alza osmotic pump (0.35 μg ACTH/100 μl saline/day) and adrenal kinase activity determined on day 100. At 2 day intervals between days 80 and 100 or days 145 and 165 of gestation, all baboons were sedated with ketamine-HCl (10 mg/kg BW; Parke Davis, Detroit, MI), and a maternal saphenous blood sample (4-7 ml) collected. Serum was stored at -20°C until assayed for estradiol by a solid phase ^{125}I RIA (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA) as described previously (Pepe *et al.*, 1988).

Adrenals were also available from four adult female baboons euthanized with Beuthanasia-D (Butler Corp., Fredricksburg, VA) at random times during apparently normal menstrual cycles and from three pregnant baboons on day 80 of gestation and treated 48, 24 and 2 h prior to recovery of adrenal tissue with 3 mg betamethasone (Celestone Soluspan; Schering Corp., Kenilworth, NJ). Fetal and adult adrenal tissue were placed in ice-cold saline, trimmed of fat and mesentery, and weighed to the nearest 0.1 mg on a Mettler balance prior to preparation for analysis of PK-A and PK-C activity.

Analysis of PK-A

All procedures were conducted at 4°C unless stated otherwise. After removal of visible chromaffin medullary tissue, approximately 25-40 mg of adrenal cortical tissue was placed into a 7 ml all glass tissue grinder (Wheaton, Milville, NJ) to which had been added 1 ml of 10 mM potassium phosphate (Sigma) buffer (pH 6.8) containing 250 mM sucrose (Sigma), 2 mM ethylenediaminetetraacetic acid (EDTA, Sigma), and 5 μl of a protease inhibitor cocktail composed of chymostatin, leupeptin, pepstatin A (1 mg/ml;

Peninsula Laboratories, Torrence, CA) and antipain (0.05 mg/ml; Peninsula). Adrenal tissue was gently dounced (10-12 strokes) using a large clearance glass pestle (Wheaton), the homogenate transferred to a 1.5 ml eppendorf tube and cell cytosol obtained by centrifugation for 10 min at 12,000 x g using a refrigerated Jouan Microfuge (Jouan Inc., Winchester, VA). After removal of duplicate 10 µl samples for subsequent determination of protein concentration (Lowry *et al.*, 1951), cytosol was diluted 3, 10, 30 and 100-fold with homogenization buffer to which had been added bovine serum albumin (1 mg/ml; Sigma). The activity of PK-A was determined by measuring the incorporation of ^{32}P into the PK-A specific peptide, kemptide (Roskoski, 1983). Briefly, 30 µl of a test mixture composed of 50 mM Tris-HCl (Sigma) pH 7.4, 25 mM magnesium acetate (Sigma), 20 mM potassium fluoride (KF; Sigma), 0.2 mM 3-isobutyl-1-methylxanthine (MIX; Sigma), 0.25 mM adenosine 5'-triphosphate (ATP; Boehringer Mannheim, Indianapolis, IN), 3 µg kemptide (Sigma), and 1 x 10^6 dpm [γ-^{32}P]ATP (SA 6000 Ci/mmol; New England Nuclear Corp., Boston, MA) was placed into 12 x 75 mm glass tubes. After addition of 10 µl distilled water (dH_2O) or 10 µl 0.025 mM cyclic adenosine-3'5'-monophosphoric acid (cAMP; Boehringer Mannheim) in dH2O, tubes were placed in a 30°C shaking water bath and 10 µl of adrenal cytosol dilutions (0.3 to 30 µg protein) added in duplicate. Following incubation for 10 min, 35 µl of reaction mixture was transferred to 1 x 3 cm P81 phosphocellulose strips (Whatman Lab Sales, Hissboro, OR) which were immersed immediately in 10 ml 75 mM phosphoric acid (Mallinckrodt Chemical Co., Paris, KY). Strips were washed 4 times in 75 mM phosphoric acid, rinsed in 10 ml 95% ethanol (Quantum Chemical Corp., Tuscola, IL), air dried, placed into liquid scintillation vials containing 7 ml Beckman Ready-Sol

(Beckman Corp., Fullerton, CA), and radioactivity quantified in a Beckman LS 8000 liquid scintillation spectrometer with automatic external quench correction. Preliminary studies confirmed that less than 10% of total enzyme activity was present in detergent solubilized adrenal membranes (12,000 x g). In addition, reaction velocity was linear with time and with protein concentrations ranging from 0.3 to 10 μg cell cytosol and was not altered by increasing the concentration of kemptide or ATP 2-fold (data not shown).

Analysis of PK-A Subtypes I and II

Analysis of PK-A subtypes in selected adrenal cytosol preparations was determined following diethylaminoethyl-cellulose (DEAE) column chromatography (4°C). Briefly, freshly isolated cytosol (300-800 μg protein) was applied to a 3.1 ml DE-52 anion exchange gel (Whatman) equilibrated in 20 mM Tris-HCl, pH 7.5, 1 mM ethylene glycol bis-(b-aminoethyl ether) N,N,N,N,tetraacetic acid (EGTA, Sigma), 1 mM dithiothreitol (DTT, Sigma; TESH buffer) in a 10 ml Bio-Rad poly-prep column (Bio-Rad Laboratories, Richmond, CA). The column was washed with 10 ml TESH buffer and then eluted with a sodium chloride gradient (25-350 mM NaCl in TESH). Fractions (0.5 ml) collected into glass centrifuge tubes containing 2.5 μl protease inhibitor cocktail and 10 μ l 5% BSA in dH_2O were assayed for PK-A activity as described above and for NaCl content using an IL943 Flame Photometer (Instrumentation Laboratory, Lexington, MA).

Analysis of PK-C

Sections (20-40 mg) of adrenal tissue were dounced (4°C) in 1 ml 20 mM Tris-HCl, pH 7.5, containing 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 5 μl protease inhibitor cocktail. The homogenate was centrifuged for 60 min (4°C) at 105,000 x g and the cytosol diluted 3, 10 and 30-fold in Tris buffer containing 1% BSA. The

microsomal pellet was resuspended in 1 ml Tris buffer containing 0.2% Triton-X (Sigma) and solubilized on ice with agitation for 45 min and then diluted with Tris buffer-1% BSA. PK-C activity was measured (Noland and Dimino, 1986) by determining the rate of incorporation of ^{32}P from [γ-^{32}P]ATP into Histone IIIS (Sigma). Briefly, 20 µl of Tris buffer alone or 10 µl Tris-buffer without EGTA to which had been added 5 mM calcium chloride and 10 µl Tris buffer containing an emulsion of 1.0 µg 1,2-dioleoyl-rac-glycerol (C18:1, cis9; DG; Sigma) and 10 µg L-a-phosphatidyl-L-serine (PS; Sigma) were added to 55 µl of a test mix containing 20 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 0.02 mM ATP, 50 µg Histone IIIS and 1 x 10^6 dpm [γ-^{32}P]ATP (NEN; 6000 Ci/mmol). After addition of 25 µl of buffer or 25 µl of adrenal cytosol or membrane dilutions (0.3-30 µg protein), reaction mixtures were incubated for 5 min in a shaking water bath (30°C) after which 50 µl was removed and transferred to 1 x 3 cm P81 phosphocellulose strips and processed as described for PK-A. Preliminary studies confirmed linearity of product with time. In selected experiments, cytosolic preparations were applied to a 1.0 ml DE-52 anion exchange gel and PK-C activity determined after column chromatography as described above for PK-A.

Mean adrenal weights, serum estradiol concentrations and specific activities of PK-A and PK-C were analyzed by Analysis of Variance with post-hoc comparison of the means (Duncan, 1955).

RESULTS

Maternal serum estradiol concentrations (Table I) were similar in animals treated at midgestation either with androstenedione (1.2 ng/ml) or with estradiol benzoate at daily doses of 0.5 mg (1.3 ng/ml) or 1.0 mg (1.5 ng/ml). The overall mean (± SE) serum

Table I. Maternal serum estradiol (E_2) concentrations and fetal adrenal weight in baboons at mid- and late gestation and at midgestation after treatment with androstenedione or E_2. Baboons delivered on days 100 and 165 (late) of gestation (term = day 184) to mothers untreated or treated sc with increasing numbers of 50-mg implants of androstenedione or with various maximal doses of E_2 benzoate injected sc daily in increasing concentrations between days 70 – 100 of gestation.

Treatment	N	E2 (ng/ml)[a]	Wet Weight (mg/2 adrenals)
Midgestation			
Control	6	0.7 ± 0.1^{b}	104 ± 8^{b}
Androstenedione	2	1.2^{c}	109^{b}
E2 (0.5 mg)	2	1.3^{c}	107^{b}
E2 (1.0 mg)	3	1.5 ± 0.2^{c}	98 ± 14^{b}
E2 (2.0 mg)	5	3.6 ± 0.5^{d}	102 ± 11^{b}
Late Gestation			
Control	5	1.9 ± 0.3^{c}	374 ± 27^{c}

[a] Serum E_2 (mean ± SE) was determined in maternal saphenous venous samples obtained at 1- to 2-day intervals on days 80 – 100 or days 145 – 165 of gestation. [b,c,d] Values with different superscripts differ from each other (by ANOVA and Duncan's multiple range test, P=0.05).

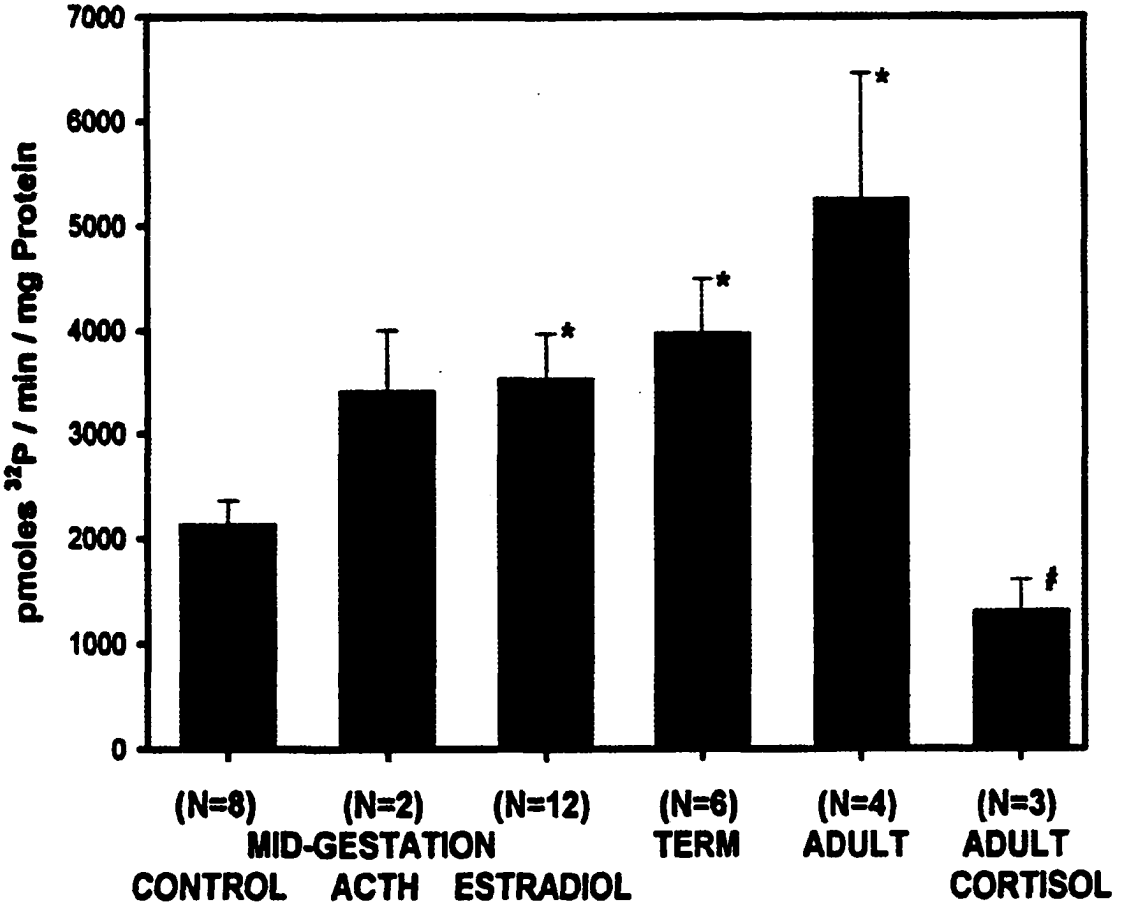

Figure 8. Mean (± SE) PKA activity (picomoles of ^{32}P incorporated into kemptide protein per min/mg protein) in adult adrenal glands, adult adrenal glands treated *in vivo* with betamethasone and fetal adrenal glands obtained at mid (day 100)- and late (day 165) gestation (term = day 184) from untreated baboons and on day 100 from baboons in which estradiol concentrations in the mother were increased by maternal treatment with estradiol or estrogen precursor androstenedione (Mid Estradiol; n = 12; see Table I and Materials and Methods for details). PKA activity was also analyzed in two baboon fetuses (day 100) treated *in utero* with ACTH for 30 days. PKA was determined in the presence of 0.025 mM cAMP. Individual data points for PKA activity determined at the various protein concentrations assayed were similar (± 5%) and, thus, were averaged for each adrenal preparation to yield a representative value for each animal. The *asterisk* indicates that the mean value differs ($P<0.05$) from that in the control at midgestation (by analysis of variance and Duncan's multiple range test, $P=0.05$). The # indicates that the mean value differs ($P<0.05$) from the adult control (by paired *t*-test, $P=0.05$).

estradiol concentration in these baboons (1.3 ± 0.2 ng/ml) was 2-fold greater ($P<0.05$) than that in untreated baboons on day 100, but similar to that of late gestation (Table I). Estradiol concentrations were further increased ($P<0.05$) by administration of 2.0 mg estradiol benzoate to a mean value which was also greater ($P<0.05$) than that on day 165 (Table I). Although fetal adrenal weight was 2-3 fold greater ($P<0.05$) at term than at midgestation, adrenal weight was not changed significantly by any of the treatment regimens (Table I).

Despite apparent dose-dependent increases in serum estradiol following treatment with estradiol-benzoate (Table I), mean (± SE) specific activity of PK-A was similar in adrenals of animals treated with 0.5 mg estradiol-benzoate or androstenedione (3660 ± 892 pmoles/min/mg protein), 1.0 mg estradiol-benzoate (4028 ± 668 pmoles/min/mg protein), or 2.0 mg estradiol-benzoate (3117 ± 666 pmoles/min/mg protein). Therefore, data for these treatment groups were combined and are presented as an overall mean for analysis of the effects of estradiol at mid-gestation (mid-estradiol, Figure 8). PK-A activity at mid-gestation was increased ($P<0.05$) nearly 2-fold by treatment with estrogen to levels which were similar to those measured in animals delivered near term. The data in Table II, when compared with that in Figure 8, indicates that kinase activity measured in the absence of cAMP was always less ($P<0.001$, paired *t*-test) than in the presence of cAMP.

In all groups, the specific activity of PK-C in adrenal cytosol was similar to that measured in the triton-x solubilized membrane fraction and was always 3 to 4-fold lower ($P<0.001$; paired *t*-tests) than that of cytosolic PK-A. In contrast to PK-A, cytosolic PK-C activity was similar at mid- and late gestation and was not influenced by treatment at mid-gestation with estradiol or androstenedione *in vivo* (Figure 9). Similar results were observed for PK-C activity associated with adrenal membranes (Figure 9). Basal kinase activity measured in the absence of calcium and phospholipids was minimal in all treatment groups (Table II). Addition of calcium and phospholipids resulted in a 5 to 12-

Table II. PKA and PKC activities in fetal baboon adrenal cytosols of mid- and late gestation and assayed in the absence of cAMP (PKA – cAMP) or phospholipid/calcium (PKC – lipids). Baboons delivered on day 100 (Mid) and day 165 (Late) of gestation (term = day 184) to mothers untreated or treated with androstenedione or estradiol (E_2) between days 70 – 100 of gestation (see Table I for details). The number of determinations is given in parentheses.

Treatment	PKA – cAMP (pmole ^{32}P/min mg protein)[a]	Ratio +cAMP/ -cAMP	PK-C – lipids (pmole ^{32}P/min mg protein)[b]	Ratio+lipids -lipids
Mid Control	204 ± 35 (n=8)	13.6 ± 3.0	52 ± 48 (n=4)	5.6 ± 2.0
Mid + E2	544 ± 120[c] (n=12)	9.0 ± 1.6	35 ± 22 (n=7)	6.2 ± 1.2
Late Control	358 ± 73 (n=6)	12.4 ± 2.3	27 ± 18 (n=4)	12.9 ± 6.5

[a] PKA assayed in the absence of cAMP. Values are from adrenal cytosols simultaneously assayed for PKA in the presence of cAMP (see Figure 8 and Materials and Methods for details). [b] PKC assayed in adrenal cytosols in the absence of exogenous lipids or Ca^{2+}. [c] Mean (± SE) value different from the control value at midgestation ($P<0.05$, by analysis of variance and Duncan's multiple range test, $P=0.05$).

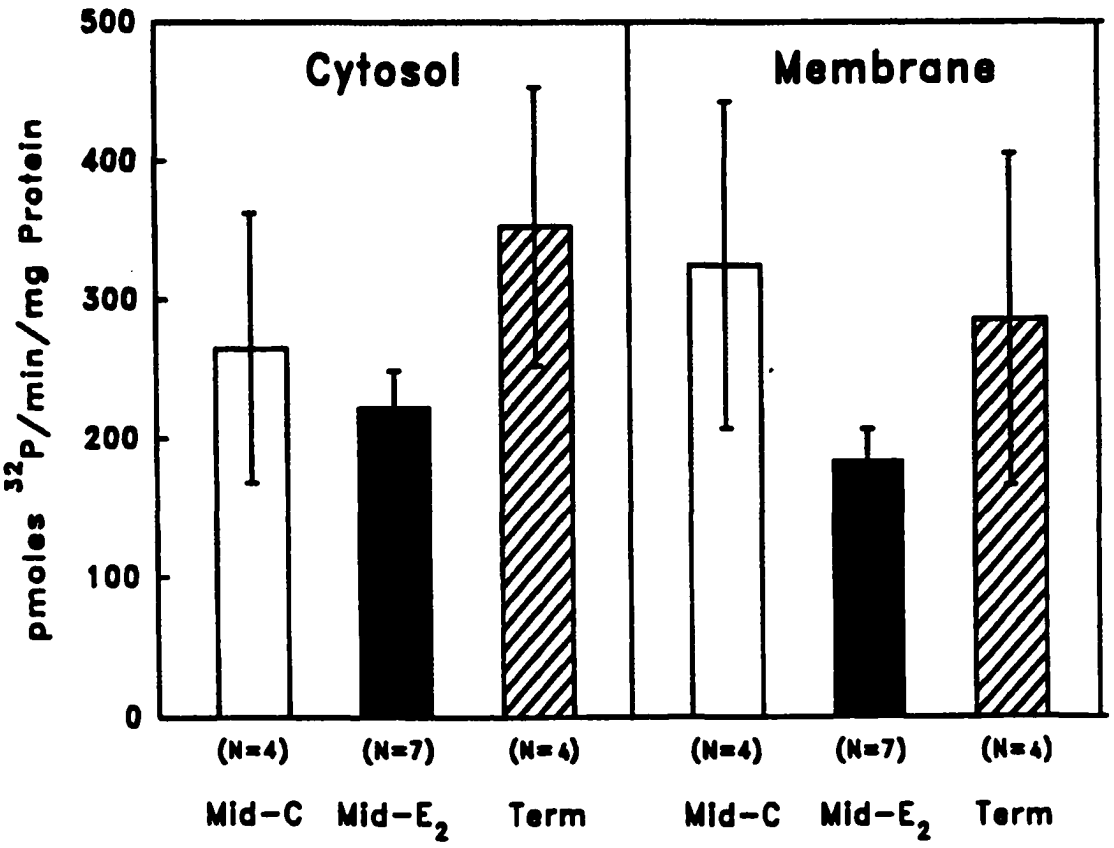

Figure 9. Mean (± SE) PKC activity (picomoles of ^{32}P incorporated into Histone IIIS per min/mg protein) in baboon fetal adrenals obtained at mid (day 100)- and late (day 165) gestation (term = day 184) from untreated baboons and on day 100 from animals treated with estradiol or the estrogen precursor androstenedione (Mid Estradiol; see Table I and Materials and Methods for details). Kinase activity was measured in adrenal cytosolic and membrane fractions (105,000 x g) incubated at two protein concentrations (1-3 μg) in TRIS-HCl buffer containing exogenous lipids and 5 mM Ca^{2+}.

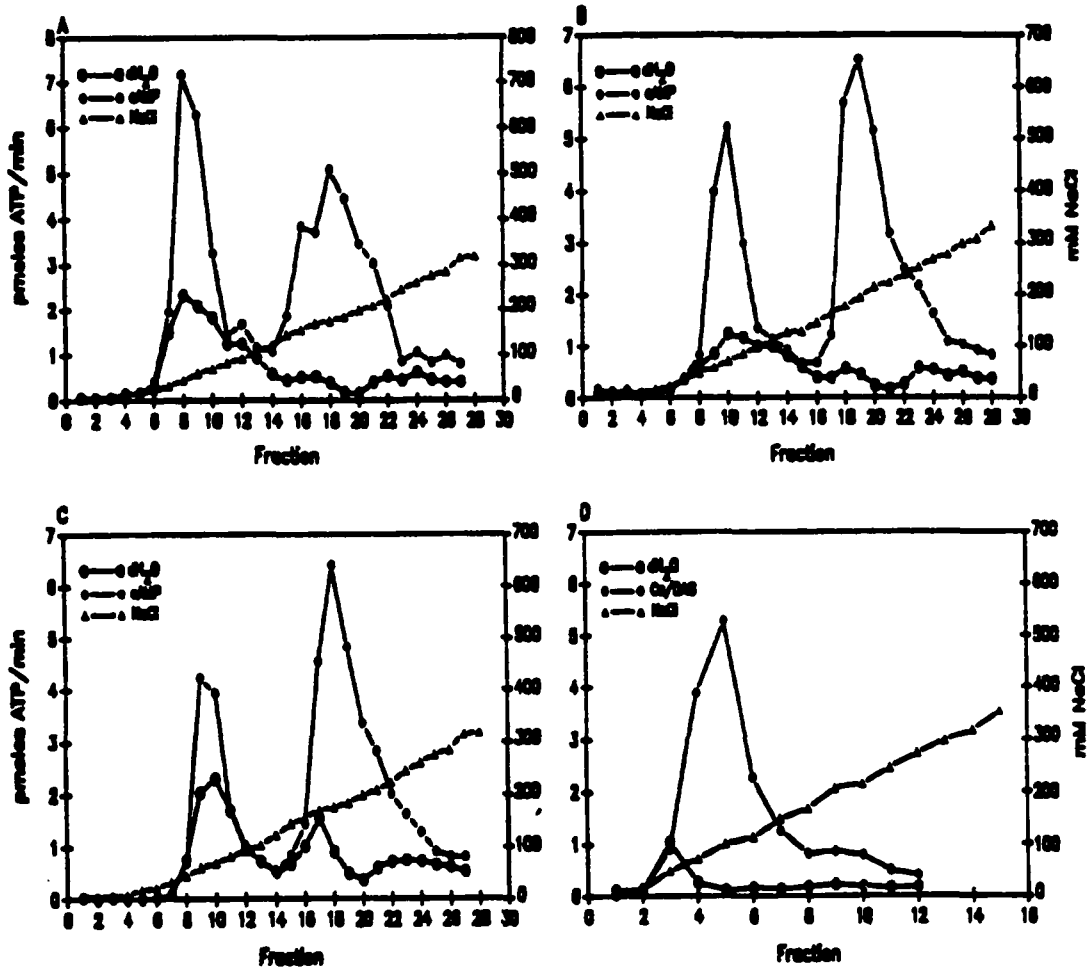

Figure 10. DEAE-cellulose chromatography of baboon fetal adrenal cytosolic PKA (A-C) and PKC (D) activities. Approximately 350–700 µl adrenal cytosol (300-800 µg protein) obtained at mid (day 100;A)- or late (day 165;B) gestation from untreated animals at midgestation from a baboon treated with estradiol (C and D) were applied to DEAE-Sephacel columns (A-C, 3.1 ml; D, 1.0 ml), and protein was eluted with a 0-350 mM NaCl linear gradient. Fractions (500 µl) were collected, and aliquots were assayed for PKA (10 µl) or PKC (25 µl) activity in the presence or absence of cAMP (PKA) or Ca^{2+} and phospholipids (PKC), as outlined in Materials and Methods.

fold mean stimulation of histone IIIS phosphorylation, a degree of activation that was similar in all three treatment groups (Table II).

The data in Figure 10 indicate that there are two types of PK-A in the baboon fetal adrenal, one which is eluted from DEAE cellulose at approximately 50-100 mM NaCl and presumed to be PK-A type I and a second which elutes at a higher salt concentration (175-225 mM NaCl) and presumed to be PK-A type II. Approximately 65% of PK-A activity (area under curve) is associated with PK-A II and 35% with PK-A I. The distribution of PK-A types appears to be similar at mid- and late gestation and unaffected by estradiol treatment at mid-gestation (Figure 10, panels A, B and C). The data in panel D of Figure 10 indicate that PK-C activity is eluted as a single peak from DEAE at a salt concentration of approximately 100-150 mM NaCl, or intermediate between that of PK-A types I and II. This profile of PK-C was also observed at mid- and late gestation and following treatment with estradiol (data not shown).

The specific activity of PK-A in adrenals obtained from adult nonpregnant regularly menstruating female baboons was similar to that measured in adrenals of baboon fetuses delivered late in gestation (Figure 8). In addition, adult adrenal cytosol was comprised of a similar pattern of PK-A type I and type II as determined by DEAE cellulose chromatography (data not shown). Adrenal PK-A activity in adult baboons on day 80 of gestation in which betamethasone was administered daily to the mother 48, 24 and 2 hours prior to removal of the adrenal to block endogenous ACTH production was (1310 ± 270) approximately 20% of that in untreated nonpregnant baboons (Figure 8). In these animals, maternal cortisol concentrations on day 80 (< 5 μg/100ml; data not shown) were 60-80% lower than those (18-30 μg/100 ml) normally observed in our colony of baboons at this time in gestation (Pepe and Albrecht, 1991).

DISCUSSION

The results of the present study indicate that fetal adrenal PK-A activity was greater in late gestation, when estrogen levels are elevated, than at mid-gestation. Moreover,

activity of this kinase was experimentally increased at mid-gestation by prematurely elevating estrogen to levels typically observed late in pregnancy. We previously demonstrated that *de novo* cortisol production by (Pepe *et al.*, 1990) and the specific activities of 3β-HSD and 17α-OHase within (Pepe and Albrecht, 1991) the fetal adrenal were also greater late in gestation than at midgestation and elevated early in pregnancy by the same experimental paradigms utilized in the present study to increase maternal estrogen levels at midgestation. Therefore, based on our previous studies and the results of the present investigation, we suggest that activation of fetal adrenal steroidogenic maturation late in pregnancy and at midgestation after an experimental increase in estrogen is due, in part, to an increase in the activity of the cyclic AMP-dependent second messenger system involving PK-A. Although basal adenylate cyclase activity has been shown to be present in the human fetal adrenal early in gestation and absent in anencephalics near term (Carr, 1986), the present study is the first to demonstrate an ontogenetic increase in fetal adrenal PK-A in the nonhuman primate and to indicate the important role of estrogen in the regulation of the development of this physiologically important cell signaling enzyme system. Our studies also indicate that baboon fetal adrenal protein kinase-A is composed of two subtypes classically identified as type I and type II (Beebe and Corbin, 1986; Corbin *et al.*, 1975). Based on our examination of selected adrenal cytosolic extracts, it would appear that the distribution of type I and II PK-A in the baboon fetal adrenal was not altered during gestation and was comparable to that in the adult adrenal. Thus, we suggest that the estrogen-regulated ontogenetic increase in total protein kinase A activity in the fetal adrenal does not reflect an alteration in kinase subtypes, as appears to occur with follicular maturation and luteal formation in the pig ovary (Dimino *et al.*, 1981) and in various steroidogenic responsive tissues of the male rodent following castration and subsequent treatment with dihydrotestosterone (Fuller *et al.*, 1978).

In preliminary studies recently conducted in our laboratories (Pepe, Davies, and Albrecht, in progress), we demonstrated that daily administration of 0.35 μg ACTH to the fetus between days 75 and 100 of gestation enhanced fetal adrenal PK-A activity and the specific activities of fetal adrenal 3β-HSD and 17α-OHase to levels previously obtained in animals in which the mother was treated with estradiol or androstenedione to increase estrogen production (Pepe and Albrecht, 1991). These findings support the suggestion that the increase in fetal adrenal PK-A, elicited in estrogen-treated baboons of the present study, is the result of an increase in fetal ACTH production which we have proposed (Pepe and Albrecht, 1991) results from the activation of the hypothalamic-pituitary axis by an estrogen-induced increase in placental oxidation of maternal cortisol to its inactive metabolite cortisone prior to entry into the fetus.

Although it remains to be demonstrated that estrogen-induced alterations in placental metabolism activate ACTH production by the fetus, the results of the present study as well as of others, provide indirect evidence consistent with this hypothesis. Thus, in our study, PK-A activity in adrenals of adult pregnant baboons treated with betamethasone to reduce maternal ACTH secretion was only 20% of that measured in adrenals of nonpregnant animals. In human fetal adrenal cells in culture, 17α-hydroxylase activity (McAllister and Hornsby, 1988) and mRNA concentrations (Voutilainen *et al.*, 1991) are increased when cells are cultured in the presence of activators of cAMP, such as forskolin, but not by activators of protein kinase C such as phorbol esters. Moreover, basal adenylate cyclase activity in human fetal adrenals obtained at 10-12 weeks gestation is stimulated *in vitro* by ACTH, but not by several other factors including MSH, prostaglandins and HCG (Carr *et al.*, 1985). Although studies of the human fetal adrenal have focused on the adenylate cyclase component of the PK-A signal transduction pathway, it has been clearly demonstrated that ACTH plays an important role in regulating PK-A activity in adrenals of adult animals and that the effects of ACTH are both acute and chronic. Thus, using immunoreactive gold (Murray

et al., 1987) and fluorescein-coupled immunocytochemical techniques (Murray *et al.*, 1985), Murray and colleagues demonstrated that ACTH treatment for 30 min increased the concentrations of PK-A catalytic subunit in the nucleus and cytoplasm of Y1 adrenal cells in culture. Additionally, it has been demonstrated that short-term treatment with ACTH increased the amount of cyclic AMP bound to the regulatory subunit of PK-A at all concentrations in which ACTH concomitantly elicited an increase in rat adrenal steroidogenesis in vitro (Sala *et al.*, 1979). Finally, it was confirmed that the long term action of ACTH on maintenance of synthesis and activity of key steroidogenic enzymes in cultures of bovine adrenal cells was mimicked by analogs of cyclic AMP (Kramer *et al.*, 1984). Thus ACTH appears to maintain, as well as enhance, steroidogenesis in the adult adrenal by regulating the basal levels and the activation of cAMP-dependent processes including protein kinase A. We propose that this role of ACTH is also apparent in the fetus.

It seems unlikely that the increase in PK-A activity observed in estrogen-treated animals of the present study is the result of a direct effect of the steroid on the fetal adrenal. Thus, at midgestation, estrogen did not stimulate baboon fetal adrenal steroidogenesis in vitro (Albrecht and Pepe, 1987) and inhibited 17α-OHase activity in the human fetal adrenal by acting as a competitive substrate (Couch *et al.*, 1986) and inhibited 3β-HSD activity in human fetal (Fujieda *et al.*, 1982) and neonatal cells (Byrne *et al.*, 1986). Although estradiol has been demonstrated to increase protein kinase C activity in the pituitary gland of the rat (Drouva *et al.*, 1990), fetal adrenal PK-C activity was not affected by treatment with estrogen in our study. Therefore, it is more likely that the increase in PK-A in estrogen-treated animals of the present study is the result of activation of the fetal hypothalamus-pituitary axis and increased ACTH secretion secondary to estrogen-induced alterations in placental F-E metabolism as we have proposed previously (Pepe *et al.*, 1990; Pepe and Albrecht, 1991).

It has been proposed that activation of protein kinase C may play a pivotal role in regulating growth of the fetal adrenal. Thus, it has been demonstrated that the phorbol ester 12-O-tetradecanoyl phorbol acetate (TPA), which activates protein kinase C by substituting for the physiological activator diacylglycerol (Castagna *et al.*, 1982), was a mitogen for human fetal adrenal definitive cells in culture (McAllister and Hornsby, 1987). Moreover, protein kinase C activity can be modulated by IGF I, IGF II, and EGF (McAllister and Hornsby, 1988; Naaman *et al.*, 1989), growth factors which have been demonstrated to enhance fetal adrenal cell growth in culture (Naaman *et al.*, 1989; Simonian and Gill, 1981; Crickard and Jaffe, 1981). Because IGF-I has the capacity to stimulate protein kinase C via increased hydrolysis of glucosyl-phosphatidyl inositols (Low and Saltiel, 1988), factors regulating growth factor production, or activating PK-C directly such as EGF and FGF (McAllister and Hornsby, 1988), in the human fetal adrenal may have profound effects on growth and differentiation. Because fetal adrenal PK-C activity was similar at mid and late gestation and was not altered following estrogen treatment in baboons of the present study, we suggest that the appearance and/or concentrations of growth factors rather than the absolute level of PK-C may play a more significant role in regulating fetal adrenal growth.

In human fetal adrenal cells in culture (McAllister and Hornsby, 1988; Ilvesmaki and Voutilainen, 1991), it has been demonstrated that while activation of PK-A by ACTH or forskolin can induce mRNAs for and activities of key steroidogenic enzymes, these effects can be abolished and/or enhanced by concomitant treatment with factors which activate and/or down regulate PK-C. Therefore, the relative abundance of these two second messenger systems and their respective localization in compartments of the fetal adrenal in conjunction with the appearance of peptide growth factors may play a significant role in orchestrating the steroidogenic and growth pattern of this complex

endocrine gland as has been suggested previously (Pepe and Albrecht, 1990; McAllister and Hornsby, 1988; Voutilainen *et al.*, 1991; McAllister and Hornsby, 1987). The results of the present study are consistent with this suggestion and document that the ratio of PK-A to PK-C in the primate fetal adrenal increases with advancing gestation and that this change occurs concomitant with the ability of the fetal adrenal to synthesize cortisol *de novo*.

In summary, we have demonstrated that fetal adrenal PK-A activity is greater in late gestation when estrogen levels are elevated than at mid-gestation. Moreover, PK-A activity was experimentally increased at mid-gestation by prematurely elevating estrogen to levels typically observed late in pregnancy, treatment paradigms which we previously demonstrated (Pepe and Albrecht, 1991) altered placental cortisol metabolism and activated the fetal hypothalamic-pituitary-adrenal axis resulting in increased 3β-HSD and 17α-OHase activities and the onset of *de novo* cortisol production by the fetus (Pepe *et al.*, 1990). On the basis of our previous and present results, we suggest that activation of fetal adrenal steroidogenic maturation involves an ACTH-induced increase in the cAMP-dependent second messenger system involving PK-A.

CHAPTER III

ACTIVATION OF THE BABOON FETAL PITUITARY-ADRENOCORTICAL AXIS AT MIDGESTATION BY ESTROGEN: ENHANCEMENT OF FETAL PITUITARY PRO-OPIMELANOCORTIN mRNA/PROTEIN EXPRESSION

INTRODUCTION

The steroidogenic maturation of the fetal adrenal gland leading to the onset of *de novo* production of cortisol late in gestation is important to fetal development and adrenocortical self-sufficiency in the perinatal period in human and non-human primates (Liggins, 1976; Jaffe *et al.*, 1988; Pepe and Albrecht, 1990). Using *in vivo* experimental paradigms, we have demonstrated that estrogen, via regulation of placental metabolism of maternal corticosteroids, regulates the timing of fetal adrenal cortisol production presumably via modulation of hypothalamic-pituitary function. Thus, during early to mid-gestation, 11β-hydroxysteroid dehydrogenase (11β-HSD)-catalyzed metabolism of cortisol and cortisone across the baboon placenta favors the formation of cortisol (Pepe and Albrecht, 1984; Pepe *et al.*, 1988) resulting in cortisol being the dominant corticosteroid arriving within the fetus. We have suggested that this cortisol blocks the fetal hypothalamic-pituitary axis and thus ACTH production and/or release thereby preventing *de novo* cortisol synthesis by the fetal adrenal gland. With advancing gestation, the increase in estrogen production (Albrecht and Townsley, 1978) enhances placental NAD^+-dependent 11β-HSD oxidase activity (Baggia *et al.*, 1990a; Baggia *et al.*, 1990b), resulting in increased placental conversion of cortisol to cortisone (Pepe *et al.*, 1988). We further propose that this change in placental corticosteroid metabolism results in a decline in fetal cortisol levels of maternal origin and leads to activation of the fetal hypothalamic-pituitary axis culminating in increased ACTH production required to initiate adrenal steroidogenic maturation. In support of this hypothesis, we (Pepe and Albrecht, 1991) have demonstrated that the ontogenetic increase in the specific activities of the rate-limiting steroidogenic enzymes 17α-hydroxylase/17-20 lyase (17α-OHase)

and Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD), the cell-signaling enzyme protein kinase A (Davies *et al.*, 1993), and the initiation of *de novo* cortisol synthesis near term (Pepe *et al.*, 1990) in the baboon fetal adrenal gland could be induced at mid-gestation in animals in which transplacental oxidation of cortisol was enhanced prematurely following maternal estrogen administration to values normally seen late in pregnancy. Our present studies are directed at determining whether the activation of fetal adrenal maturation following maternal estrogen treatment at mid-gestation and normally at term reflects activation of the fetal hypothalamic-pituitary axis.

Developmental studies conducted in the chronically instrumented sheep fetus have demonstrated that the concentrations of ACTH increase with advancing gestation (Challis and Brooks, 1989). Comparable studies have not been performed in humans or subhuman primates, although single point determinations of fetal (i.e., umbilical) ACTH have been reported in these species at the time of elective cesarean section late in gestation (Pepe and Albrecht, 1990). Because of the multiple sites of secretion and potential pulsatile nature of ACTH, however, the latter may not accurately reflect hypothalamic-pituitary function *in vivo*. It is well established that the ACTH molecule is contained within the large precursor peptide, proopiomelanocortin (POMC) and in fetal sheep (Yang *et al.*, 1991; Myers *et al.*, 1993) and rats (Rundle and Funder, 1988; Scott and Pintar, 1993), measurement of POMC mRNA has been utilized to study ontogenetic maturation of pituitary corticotroph function. Therefore, in the present study we utilized *in situ* hybridization histochemistry to quantify fetal pituitary POMC mRNA levels in order to test our hypothesis that maturation of the baboon fetal adrenal normally near term and prematurely at mid-gestation following estrogen-induced alterations in placental corticosteroid metabolism reflects enhanced fetal pituitary ACTH production.

MATERIALS AND METHODS

Baboon treatment with estrogen and harvesting of fetal pituitary glands

Fetal pituitary glands were obtained on day 100 (n=7) and day 165 (n=5) of gestation (term = day 184) from untreated baboons and on day 100 from baboons (n=6) treated daily with a maximal dose of 1.0 mg estradiol benzoate (Sigma Chemical Co., St. Louis, MO). Estradiol was suspended in 0.5 ml sesame oil and injected sc in increasing concentrations (0.25 to 1.0 mg/day, doubling the dose every 5 days between days 70 and 100 of gestation; i.e. 0.25 mg on days 70-74; 0.5 mg on days 75-79; etc). At 2 day intervals between days 80 and 100 or days 145 and 165 of gestation, all baboons were sedated with ketamine-HCl (10mg/kg BW; Parke Davis, Detroit, MI), and a maternal saphenous blood sample (4-7 ml) collected. Maternal serum and serum from umbilical venous blood samples collected at the time of cesarean section were stored at -20°C until assayed for estradiol by a solid phase ^{125}I RIA (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA) as described previously (Pepe *et al.*, 1988).

Pituitaries were also obtained from 3 adult female baboons euthanized with Beuthanasia-D (Butler Corp., Fredericksburg, VA) at random times during apparently normal menstrual cycles. One of these baboons was treated with 3 mg betamethasone (Celestone Soluspan; Schering Corp., Kenilworth, NJ) 48, 24 and 2 h prior to recovery of pituitary tissue to determine the effect of exogenous corticosteroid on pituitary POMC expression.

POMC *in situ* hybridization

Pituitaries were placed in sterile 15 x 15 x 5 mm Tissue Tex II Cryomolds (Miles Scientific, Elkhart, IN) containing OCT Embedding Medium (Miles), frozen solidly on

dry-ice, wrapped in parafilm and stored at -80°C. Within 2-4 weeks, frozen tissues were positioned in a Leica-Reichart-Jung (Bethesda, MD) precision microtome cryostat (-15°C), sectioned (12μm) and mounted onto Superfrost Plus (Fisher Scientific, Arlington, VA) microscope slides which were sequentially numbered, placed in RNAase-free slide boxes and stored at -80°C until processed for *in situ* hybridization histochemistry.

The procedures for *in situ* hybridization detection and quantification of POMC mRNA expression were adapted for use in our laboratory using previously published methods of others (Young *et al.*, 1986a; Lightman and Young, 1988; Lewis *et al.*, 1978). Purified POMC antisense and sense oligodeoxynucleotide probes complimentary to 30 bases of the human POMC mRNA (Chang *et al.*, 1980) and which encode amino acids 2-11 of the ACTH molecule (Chang *et al.*, 1980; Drouin and Goodman, 1980) were purchased from Oligos Etc. Inc (Wilsonville, OR). Approximately 0.1μM probe was 3' end-labeled with [^{35}S]dATP (SA > 1000 Ci/mmol; Dupont-New England Nuclear, Boston, MA) and terminal deoxynucleotidyl transferase (20 U; Promega, Madison, WI) to a specific activity of approximately 5000 Ci/mmol.

At the time of hybridization, sections of the fetal pituitary (20-40/animal) were selected, equilibrated (10 min) to room temperature, then fixed (10 min) in freshly prepared 4% paraformaldehyde in phosphate buffered saline (pH 7.4). Slides were rinsed in 2X SSC (1X SSC = 0.15M NaCl, 0.015M sodium citrate buffer, pH 7.2), then placed (10 min) in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride-0.9% NaCl (Sigma). Sections were dehydrated through an ethanol series (70%, 1 min; 85%, 1 min; 95%, 1 min; 100%, 1 min), delipidated in chloroform (5 min),

rehydrated in ethanol (100%, 1 min; 95%, 1 min) and then air-dried. Sections were then prehybridized for 3 h (50°C) in 50µl hybridization buffer consisting of 4X SCC, 50% (vol/vol) formamide, 10% (wt/vol) dextran sulfate, 250µg/ml yeast transfer RNA (Gibco BRL), 500µg/ml sheared single stranded salmon sperm DNA (Gibco, BRL), 1X Denhardt's solution (Sigma), 1% $Na_2S_2O_3$, 5mM NaPPi and 50mM freshly prepared dithiothreitol (DTT; Sigma). Slides were rinsed in 2X SCC and then 40µl labeled antisense or sense probe in hybridization buffer added to alternating pituitary sections, and incubated overnight (50°C) under glass cover slips in humidified chambers. Sections were rinsed with 2X SSC and washed twice (2X SCC, 50% formamide) at 60°C (approximately 19C below calculated Tm), then twice in more stringent conditions (0.5X SSC, 60°C). After the last wash, slides were rinsed with 2X SCC (30 min; 22°C), dipped in double distilled water twice, then in ethanol (70% and 95%) and air-dried.

Autoradiography

Slides were placed against Kodak X-Omat film in X-ray film holders and exposed for 5-7 days. After film exposure, representative slides were dipped in fresh Kodak NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with distilled water, placed in light-tight boxes and exposed for 10-20 days. Slides were developed in Kodak D-19 (5 min; 14°C), rinsed in distilled water, fixed (Kodak Fixer; 5 min, 14C), rinsed in running tap water, and counter-stained with 0.7% hematoxylin.

Quantification of POMC mRNA

POMC mRNA expression in the fetal and adult pituitary was determined by densitometric analysis of exposed X ray film using an LKB Bromma Ultroscan XL Enhanced Laser Densitometer (Pharmacia LKB, Piscataway, NY). Areas of film exposed

to pituitary sections labeled with antisense or sense probe were circumscribed and the average density of film determined by computer after subtraction of background which was determined by densitometric analysis of an area of film adjacent to the area of interest. For each animal, an average of 16-22 sections of pituitary were analyzed for POMC antisense expression, while 8-14 sections were concomitantly analyzed for non-specific expression (e.g. sense message). Individual values were combined and an average value for POMC mRNA calculated as the difference between the average antisense and average sense message. Although preliminary studies confirmed that densitometric analysis was being performed in the linear range, to control for potential variance between treatment groups, for each *in situ* analysis pituitary sections from adult, untreated and estradiol-treated mid-gestation and term animals were hybridized concomitantly using the same labeled antisense/sense probes and X ray film exposure time, and concurrently developed and analyzed densitometrically. Finally, to ensure that densitometric analyses of film were not compromised by nonspecific binding not attributable to sense message (i.e., tissue folding), slides of pituitary sections were also examined by light microscopy.

The mean specific (antisense minus sense) number of silver grains/.025mm^2 in fetal pituitary glands from 3 animals in each of the treatment groups was quantified using an Optiphot 2 microscope attached to a Video Based Image-1 Analysis System (Universal Imaging Corp., West Chester, PA). Spatial calibration of the imaging system using a 40x objective was 512 pixels along the horizontal axis and 480 pixels along the vertical axis. For each animal, 4 sections of fetal pituitary and an average of 8 randomly selected areas (140μm x 180 μm)/slide were analyzed.

ACTH immunocytochemistry

To determine whether POMC mRNA cellular expression was associated with concomitant expression of ACTH peptide, localization of ACTH product was determined by peroxidase immunocytochemistry using a rabbit polyclonal antibody to human ACTH and Avidin-Biotin Cytochemicals provided by the manufacturer (Biomeda Corporation, Foster City, CA). Briefly, fetal pituitary sections (n=3-4) adjacent to those in which POMC mRNA was determined were selected from 3-4 animals in each of the experimental groups, brought to room temperature (10 min), fixed for 10 min with freshly prepared 4% paraformaldehyde in phosphate buffered saline (PBS), rinsed twice in PBS (3 min) and treated with pre-immune serum (control) or primary antibody at concentrations recommended by the manufacturer. After incubation at room temperature for 30 min, sections were rinsed twice with PBS, and incubated for 30 min with biotinylated anti-rabbit secondary antibody. After washing with PBS, sections were treated (30 min) with streptavidin peroxidase reagent, washed and incubated (10 min) with chromogen substrate solution. After two washes with distilled water, sections were counterstained with hematoxylin (90 sec), rinsed with distilled water, and, after addition of two drops of crystal mount, baked for 10 min (80°C) for permanent preservation of immunostained slides. Specificity of labeling was verified by antibody neutralization where the primary anti-ACTH antibody was incubated at room temperature for 30 min with 10 mM human ACTH (1-24; Bachem, Inc.) prior to incubation with the pituitary tissue section. Red-staining corticotropes were manually counted microscopically using the Video Based Image-1 Analysis System mentioned above.

Mean serum estradiol concentrations, POMC mRNA concentrations, and number of corticotrophs expressing ACTH peptide were analyzed by analysis of variance with post-

hoc comparison of the means (Duncan, 1955).

RESULTS

The mean (± SE) maternal serum estradiol concentration in baboons treated with estradiol benzoate at midgestation was 3- to 4-fold greater ($P<0.05$) than that in untreated baboons on day 100 but similar to that in late gestation (Table III). In umbilical serum, estradiol concentrations were 5-fold greater ($P<0.05$) at term than at mid-gestation but unlike maternal values, were not significantly increased at mid-gestation following treatment of the mother with estradiol.

Figure 11 depicts representative photomicrographs of sections of fetal pituitary cells hybridized with ^{35}S-labeled POMC sense (Panels A and B) or antisense (Panels C-F) probes. Specificity of POMC labeling was documented by the relative absence of silver grains following incubation of pituitary sections with the sense probe (Figure 11; Panels A and B), the absence of grains in the posterior pituitary of sections hybridized with antisense (Panel C), and the appearance and apparent selective distribution of silver grains over a dispersed population of cells in the anterior pituitary of sections hybridized with antisense, at mid (Panel D) and late (Panel F) gestation and at midgestation following maternal treatment with estradiol (Panel E). In contrast, silver grains were not visible when pituitary sections were pretreated with excess unlabeled antisense probe prior to hybridization with ^{35}S-labeled probe (data not shown).

Densitometric analysis of film exposed to labeled sense probe further confirmed applicability of the methods for POMC mRNA quantification. Thus, average sense message (0.02-0.20 arbitrary absorbance units; AU) was minimal and always 10-20 fold less than that of sections hybridized with labeled antisense probe. Preliminary studies confirmed that densitometric analyses of film exposed to sections hybridized with radiolabeled antisense probe was performed in the linear range. We also confirmed that our analyses were repeatable. Thus, relative intensity of specific labeling (AU antisense

Table III. Maternal and umbilical venous serum estradiol concentrations and fetal pituitary weight in baboons at mid- and late gestation and at mid-gestation after treatment with estradiol.

Treatment	n	Estradiol (ng/ml)[b]		Fetal pituitary weight (mg)
		Maternal	Umbilical vein	
Midgestation				
Control	7	1.0 ± 0.3[c]	0.7 ± 0.2[c]	5.3 ± 0.9[c]
Estradiol (1.0 mg)	6	2.9 ± 0.4[d]	1.1 ± 0.2[c]	4.8 ± 0.5[c]
Late gestation				
Control	5	1.9 ± 0.3[d]	3.7 ± 0.9[d]	20.1 ± 1.1[d]

[a] Baboons delivered on day 100 and day 165 (late) of gestation (term = day 184) to mothers untreated or treated with estradiol benzoate injected daily between days 70-100 of gestation. [b] Serum estradiol (mean ± SE) determined in maternal saphenous venous samples obtained at 1- to 2-day intervals on days 80-100 or days145-165 of gestation. [c,d] Values (mean ± SE) with different superscripts differ from respective values at $P<0.05$ (ANOVA and Duncan's multiple range test at $P=0.05$).

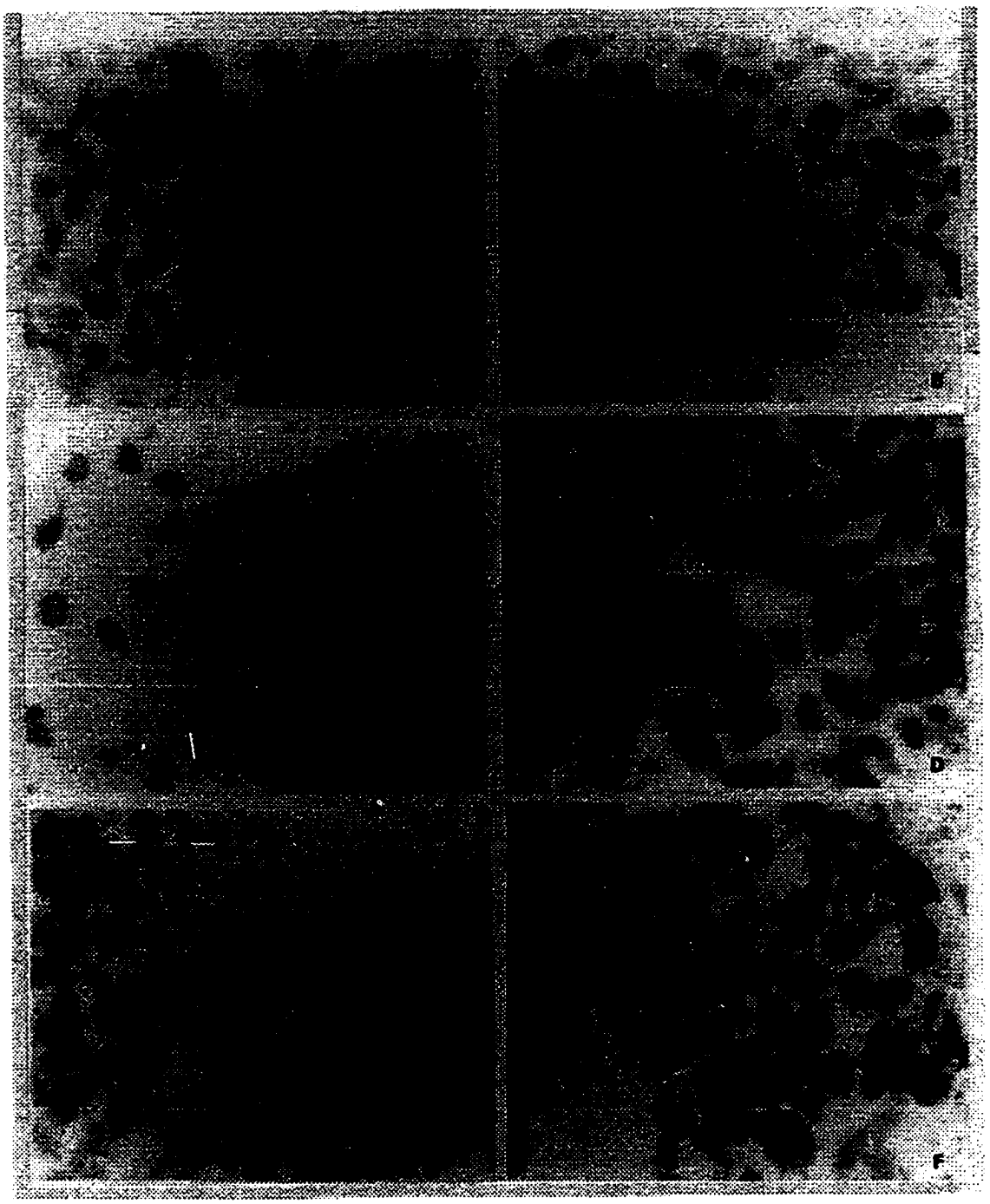

Figure 11. Representative photomicrographs (magnification x 600) of sections of baboon fetal anterior pituitary cells hybridized with ^{35}S-labeled POMC sense (A and B) or antisense (D-F) oligodeoxynucleotide probes. C, Cells of the neural lobe hybridized with ^{35}S-labeled POMC antisense. Pituitaries from untreated animals at mid (day 100; A, C, D) and late gestation (day 165; term day 184; B and F) and at mid-gestation after maternal treatment with estradiol (E).

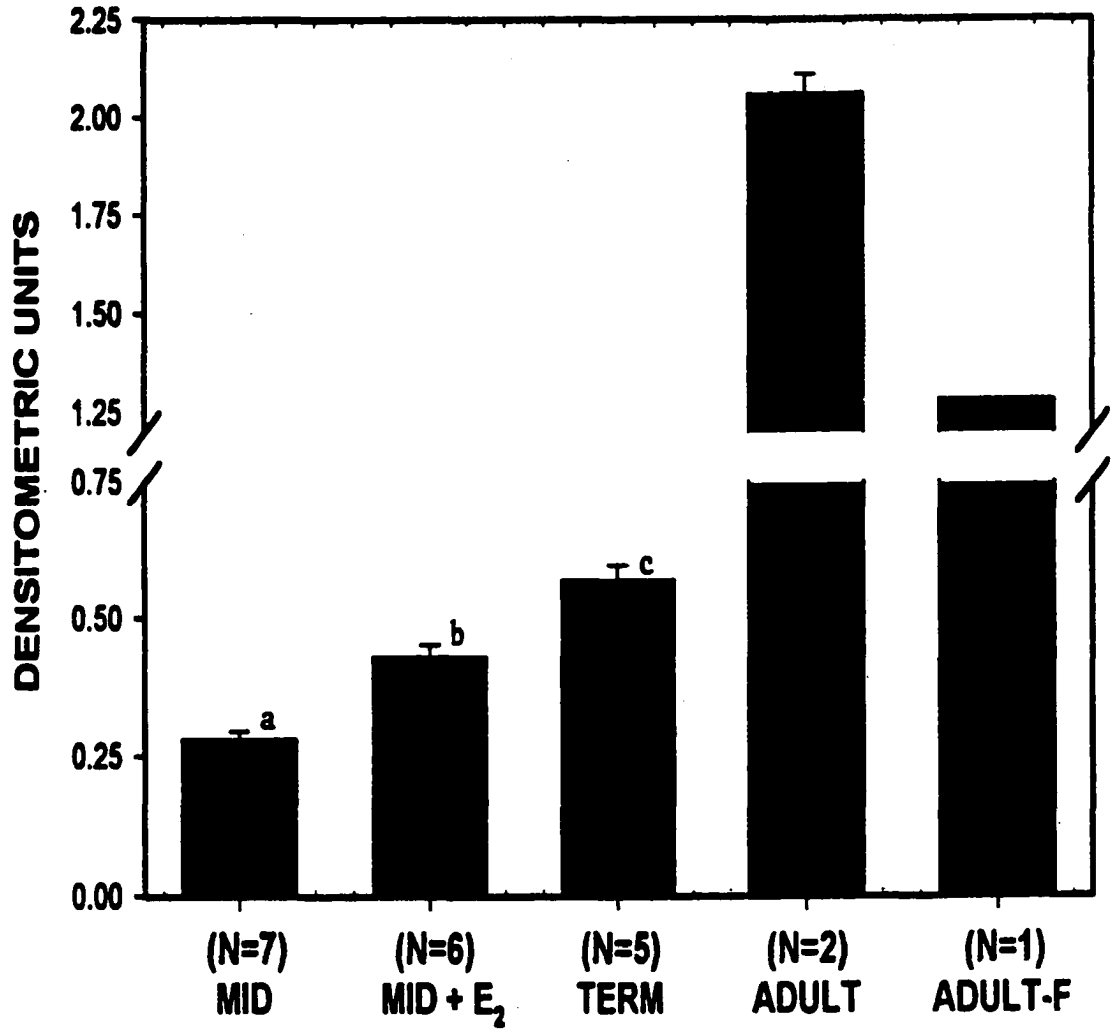

Figure 12. Mean (± SE) POMC mRNA concentrations expressed as specific arbitrary densitometric units (AU antisense minus AU sense) following *in situ* hybridization of fetal pituitaries from untreated baboons at mid- and late gestation and at mid-gestation after maternal treatment with estradiol. POMC mRNA was also determined in pituitaries of adult female baboons (two control and one treated with betamethasone). Values with different letter superscripts differ from each other at P=0.05 (ANOVA; Duncan's multiple range test).

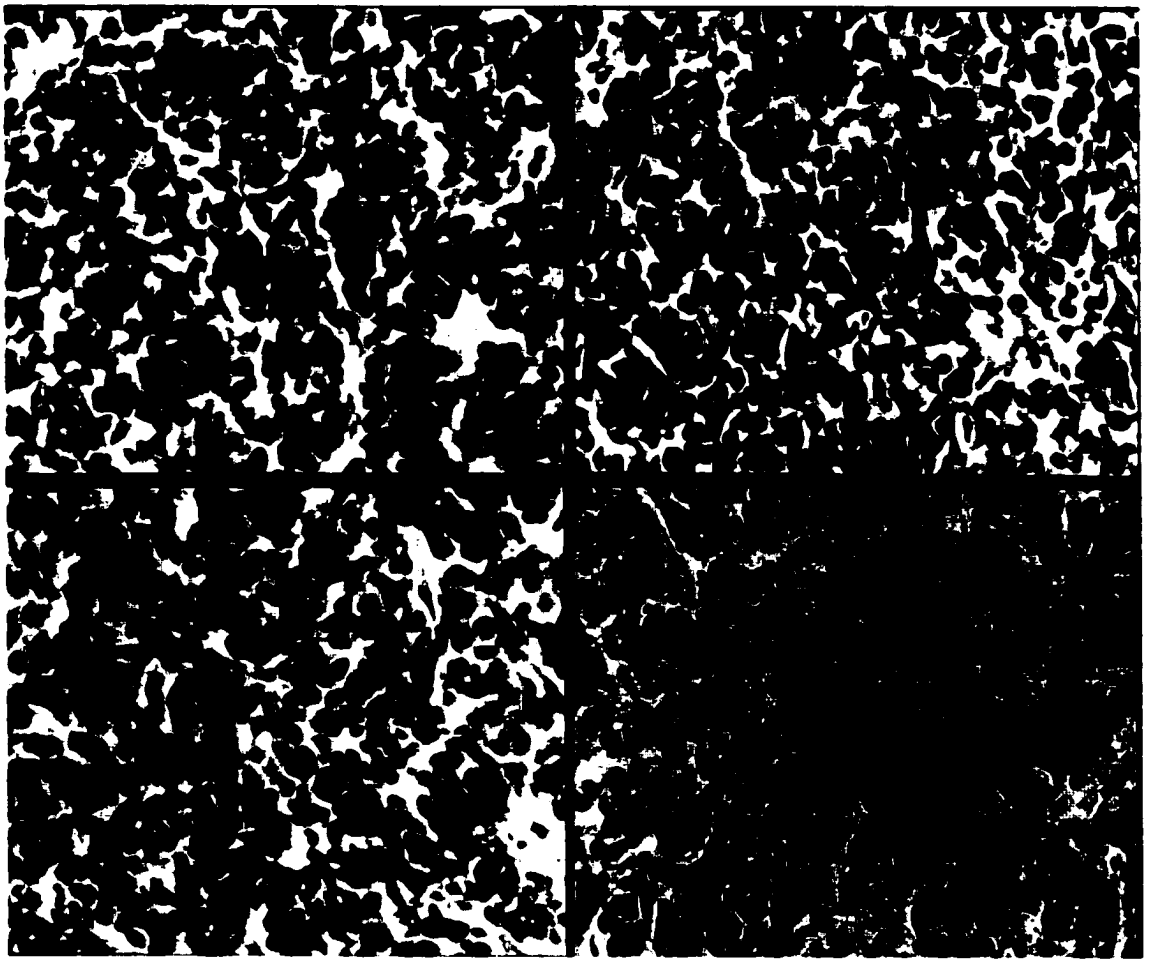

Figure 13. Localization of ACTH peptide in the adenohypophysis of the fetal baboon pituitary gland following immunocytochemistry of frozen sections. **A)** Photomicrograph (200x) of a midterm (day = 100) control pituitary demonstrating the distribution of corticotropes (red chromogen; nuclei counterstained with hematoxylin). **B)** Photomicrograph (200x) of a midterm (day = 100) after maternal treatment with estradiol. **C)** Late gestation (day 165; term day 184) control photomicrograph. **D)** Mid-gestation control adenohypophysis following pre-incubation of the primary antibody with 10nM human ACTH for 30 min prior to ICC.

minus AU sense) was similar in tissue sections analyzed in two separate hybridizations performed at different times (data not shown).

Figure 12 depicts mean POMC mRNA concentrations expressed as arbitrary densitometric units in fetal pituitary glands obtained at mid and late gestation and at midgestation following induction of placental oxidation of cortisol to cortisone by maternal treatment with estradiol. POMC mRNA levels at term (0.57 ± 0.12) were 2-fold greater ($P<0.05$) than at mid-gestation (0.28 ± 0.08) and were significantly ($P<0.05$) increased at mid-gestation in pituitaries from animals in which the mother was treated with estrogen (0.43 ± 0.10). Similar data was obtained when POMC mRNA was quantified as the specific (antisense minus sense) number of silver grains/0.025mm^2 in representative sections of fetal pituitaries from three animals in each of the treatment groups (data not shown). Thus, at mid-gestation in the absence of any change in tissue weight (Table III), the number of silver grains/0.025mm^2 of pituitary tissue from baboons treated with estradiol (401 ± 80) was 3-fold greater ($P<0.05$) than in the untreated controls (101 ± 36). Moreover, despite a 5-fold increase in wet weight of the fetal pituitary late in gestation, the number of silver grains/0.025mm^2 near term (331 ± 64) was still greater ($P<0.05$) than respective values at mid-gestation.

Based on densitometric analysis, POMC mRNA expression in pituitary sections of 2 adult baboons (2.11, 2.07) was 3-4 fold greater than that measured in tissue of the near term fetuses (Figure 12). Although only one animal was studied, it would appear that POMC mRNA expression was lower in the adult animal treated with betamethasone (1.28).

Preliminary studies confirmed the applicability of the methods for quantification of the number of fetal pituitary cells expressing ACTH peptide. Thus, antibody specificity was confirmed by the absence of staining in sections of anterior pituitary cells incubated with pre-immune control serum or with primary antibody that was neutralized prior to

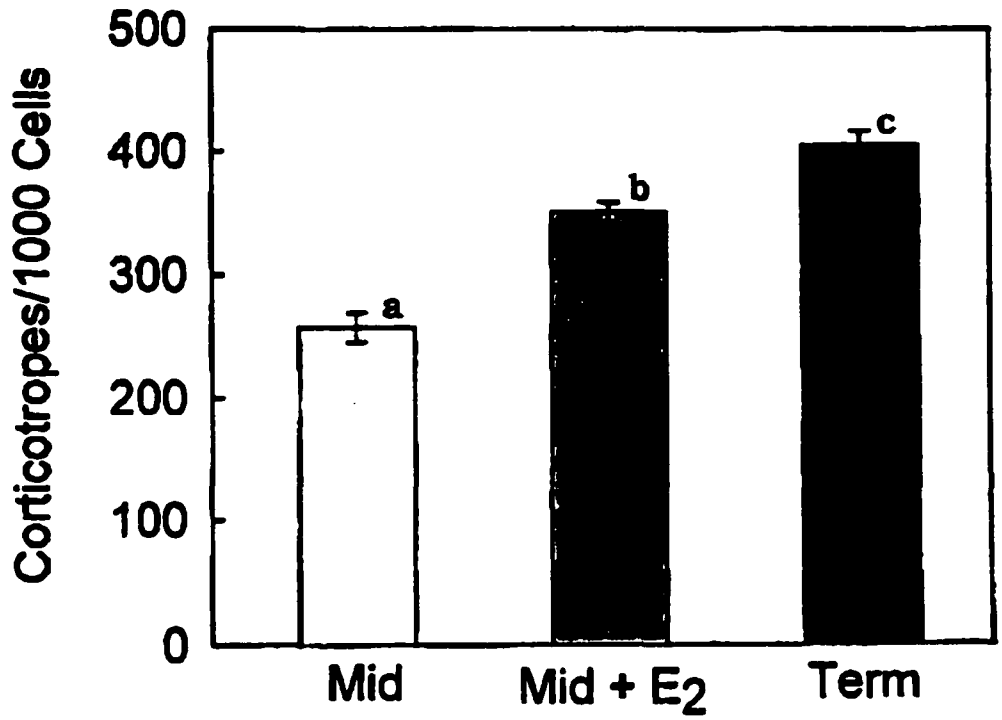

Figure 14. Mean (± SE) number of fetal pituitary cells expressing ACTH peptide following immunohistochemical analysis at mid (n = 4)- and late (n = 3) gestation and at mid-gestation after maternal treatment with estradiol (E_2; n = 4). Values with different letter superscripts differ from each other at P=0.05 (ANOVA; Duncan's multiple range test).

incubation with 10 mM human ACTH (Figure 13D). In contrast, anterior pituitary cells expressing ACTH peptide were clearly visible in sections incubated with primary antibody at mid and late gestation and at mid-gestation following maternal treatment with estradiol (Figure 13A, B, C). As summarized in Figure 14, the mean number of fetal pituitary cells, presumably corticotrophs, expressing ACTH peptide was greater ($P<0.05$) at term (424 ± 21) than at mid-gestation (267 ± 13) and increased ($P<0.05$) at mid-gestation following maternal treatment with estradiol (355 ± 8).

DISCUSSION

The results of the present study indicate that POMC mRNA expression in the baboon fetal pituitary was greater in late gestation when estrogen levels are elevated than at mid-gestation and increased at mid-gestation by prematurely elevating estrogen to levels typically observed late in pregnancy. The increase in POMC mRNA at term and at midgestation following treatment with estradiol was associated with an apparently complementary increase in the number of cells in the fetal pituitaries of these animals expressing ACTH peptide as assessed by immunocytochemistry. Therefore, although we recognize that post-translational processing of POMC is complex and the role of estrogen and advancing gestation on proprotein convertases (Noel and Mains, 1991; Day *et al.*, 1992) such as PC1 and PC2 is unknown at present, we suggest that the estrogen-regulated developmental increase in POMC in animals of the present study was probably accompanied by increased production of ACTH. This suggestion is consistent with previous work on fetal adrenal maturation conducted in our laboratories. Thus, we have shown that the specific activities of fetal adrenal 17α-OHase and protein kinase A were significantly increased with advancing gestation and prematurely increased at mid-gestation by treatment of the mother with estradiol as in the present study (Pepe and Albrecht, 1991; Davies *et al.*, 1993). Moreover, associated with these alterations in

enzyme activity, *de novo* cortisol production which normally is only measurable near term in untreated fetal baboons, was initiated by estrogen treatment early in gestation (Pepe *et al.*, 1990). Finally, in a recent study, we have shown that cortisol secretion by the baboon fetal adrenal in response to ACTH *in vitro* is greater at term than at mid-gestation and increased in animals in which estrogen production was enhanced at mid-gestation (Berghorn *et al.*, 1995), results consistent with exposure of the fetal adrenal to ACTH *in utero*. Since ACTH is known to be a critical regulator of adrenal maturation including maintenance and/or enhancement of the enzymes important to steroidogenesis and cell-signaling (Pepe and Albrecht, 1990), the onset of adrenal maturation with advancing gestation and following estrogen treatment early in pregnancy in the baboon are most likely the result of increased ACTH production and secretion. Therefore, we conclude that the onset of adrenal steroidogenic maturation and responsivity culminating in the onset of *de novo* cortisol production by the fetus normally near term and prematurely at midgestation following estrogen administration is the result of increased expression and subsequent production of ACTH by the fetal pituitary.

The mechanism(s) by which anterior pituitary POMC expression is increased in the baboon fetus with development and prematurely following estrogen treatment remains to be determined. In the fetal sheep, an age-associated increase in fetal corticotroph POMC mRNA expression was dependent upon an intact hypothalamus (Ozolins *et al.*, 1990), suggesting an important role for hypothalamic input presumably via corticotrophin-releasing hormone (CRH). It has been shown that the increase in fetal sheep POMC mRNA with advancing gestation was associated with a concomitant increase in hypothalamic CRH mRNA expression (Myers *et al.*, 1993). Although implantation of a pellet of dexamethasone in the region of the paraventricular nucleus which reduced CRH mRNA, did not have a marked effect on pituitary POMC mRNA expression between days 120 and 125 of gestation in the fetal sheep (Myers *et al.*, 1992), fetal ACTH response to hypotension was curtailed (McDonald *et al.*, 1990). Whether estrogen treat-

ment also alters hypothalamic CRH expression in the fetal baboon is presently under investigation.

Pepe and Albrecht have previously shown (Pepe *et al.*, 1988; Baggia *et al.*, 1990a) that estrogen has a marked direct stimulatory effect on placental 11β-HSD oxidase activity and via this mechanism regulates the amount of maternal cortisol arriving within the fetus. It has been proposed, therefore, that the developmental increase in fetal pituitary POMC mRNA expression observed in the present study is regulated by estrogen indirectly via its action on placental metabolism of maternal cortisol (Pepe *et al.*, 1988). Thus, because maternal cortisol is preferentially secreted into the fetal circulation at midgestation, the relatively low level of fetal pituitary POMC mRNA at this time in gestation presumably reflects the inhibition of fetal hypothalamic CRH and/or fetal pituitary POMC-ACTH expression by maternal cortisol. Indeed, treatment of intact adult animals with synthetic corticosteroids reduces POMC mRNA expression in several species including the rat (Charron and Drouin, 1986; Birnberg *et al.*, 1983; Jingami *et al.*, 1985), sheep (Myers *et al.*, 1992) and baboon (Leavitt *et al.*, 1997). The latter probably also occurs in the fetus since it has been demonstrated that pituitary POMC mRNA expression in fetal sheep can be further increased late in gestation following fetal adrenalectomy (McMillen *et al.*, 1990; Myers *et al.*, 1991). In the fetal rat, it was demonstrated that by embryonic day 15, POMC transcription is responsive to both CRH and dexamethasone and thus functional (Scott and Pintar, 1993). With advancing baboon gestation, we have shown that the rise in estrogen generates placental 11β-HSD oxidase activity (Baggia *et al.*, 1990a) and thus increased catabolism of maternal cortisol to cortisone within the placenta thereby decreasing maternal cortisol transfer to the fetus, which we propose releases inhibition and permits ontogenesis of hypothalamic-pituitary POMC/ACTH expression. While the results of our present and previous studies are consistent with this hypothesis, the experimental paradigm employed in the present study does not exclude the possibility that the increase in pituitary POMC mRNA at term and at

mid-gestation following maternal estrogen treatment reflects a direct action of estradiol on the fetal hypothalamus and/or pituitary. Indeed, estradiol levels in the fetus were greater at term and slightly, but not significantly greater at mid-gestation following maternal treatment with estrogen than at mid-gestation and there is evidence that estrogen can directly modulate hypothalamic/pituitary function. For example, estrogen receptor mRNA has been identified in adult human (Stefaneanu *et al.*, 1994) and rat (Pelletier *et al.*, 1988) pituitary glands and chronic estrogen treatment of ovariectomized rats increases ACTH and corticosterone secretion basally and in response to stress (Burgess and Handa, 1992). Moreover, the human CRH gene contains two perfect half-palindromic estrogen response elements (Vamvakopoulos and Chrousos, 1993) and it has been shown that estrogen enhances the activity of chloramphenicol acetyltransferase (CAT) in CV-1 cells in which CRH-CAT constructs were transiently expressed and supplemented with a co-transfected estrogen receptor cDNA expression plasmid (Vamvakopoulos and Chrousos, 1993). Although the estrogenic enhancement was only 10-20% of that observed with a *Xenopus* vitellogenin CAT construct, the effect of estradiol was specific and reproducible. In contrast to these apparent direct stimulatory effects of estrogen, in studies ongoing in our laboratories, we have shown that the responsivity of the baboon fetal pituitary at mid-gestation to a bolus injection of CRH *in utero* was similar in untreated controls and in animals in which maternal and fetal estrogen production was increased (Berghorn, K.A., Albrecht, E.D., Pepe, G.J., in progress). Therefore, it appears unlikely that the increase in POMC mRNA at term and at mid-gestation following maternal estrogen treatment is the result of a singular direct effect of estrogen on fetal corticotrophs, but rather reflects the effects of estrogen on placental metabolism of maternal cortisol-cortisone. Whether the latter is dependent upon the presence of estrogen in the fetus, however, remains to be ascertained.

Coincident with its action on placental cortisol oxidation, estrogen also appears to modulate cortisol metabolism within the fetus (Albrecht *et al.*, 1981; Waddell *et al.*,

1988a). Thus, the overall conversion of cortisol to cortisone (29%) by the baboon fetus at midgestation (Waddell *et al.*, 1988b) is increased 2-fold (60%) by the time of delivery (Pepe, 1979), increased at midgestation (50%) following maternal estrogen administration (Waddell *et al.*, 1988a) and decreased at term (29%) in neonates delivered to mothers treated with antiestrogen (Albrecht *et al.*, 1981). Moreover, in contrast to the marked ability to catabolize cortisol, the baboon fetus like the rhesus monkey fetus (Mitchell *et al.*, 1982), has limited ability to convert cortisone back to cortisol (Waddell *et al.*, 1988b) and the latter reaction appears to be increased only after birth, presumably as a consequence of metabolic contributions of the lung, a major site of cortisone reduction to cortisol (Murphy, 1978). Whether catabolism of cortisol and cortisone within the neural/pituitary axis is also an important contributor to the onset of POMC mRNA expression remains to be determined, although recent studies in sheep have shown that the mRNA for the 11β-HSD enzyme is expressed in the fetal hypothalamus and pituitary (Yang *et al.*, 1992). Collectively, these observations are consistent with our hypothesis for the importance of the process of oxidative metabolism of cortisol within the placenta and fetus in activating the hypothalamic-pituitary-adrenal axis. Finally, the ability of cortisol to elicit feedback inhibition is dependent upon the presence of receptors for the steroid. Although the latter aspect has not been examined in the human and nonhuman primate fetus, cortisol receptor binding in the fetal sheep hypothalamus and pituitary (Rose *et al.*, 1985) was highest early in gestation, declined with advancing pregnancy only to increase paradoxically near term (Yang *et al.*, 1990). Whether the expression of cortisol receptors in the brain of the primate fetus is coordinated with that for POMC and/or CRH in and also regulated by estrogen remains to be ascertained.

In summary, we have demonstrated that there was a developmental increase in fetal pituitary POMC mRNA expression late in baboon pregnancy, which was reproduced in part by maternal estrogen administration at mid-gestation, presumably via a direct effect of the steroid on placental corticosteroid metabolism. The increase in pituitary POMC

mRNA appeared to be associated with a concomitant increase in pituitary ACTH peptide production, which we propose is essential to fetal adrenal maturation exhibited normally near term and prematurely at mid-gestation in animals in which placental cortisol metabolism is altered by experimental increases in estrogen production. Since estrogen production depends upon androgens of fetal adrenal origin, it would appear that the fetus is ultimately in control of the timing of the maturation of its own hypothalamic-pituitary-adrenal axis, culminating in *de novo* cortisol secretion and adrenocortical self-sufficiency in the perinatal period.

CHAPTER IV

HYPOTHALAMIC CORTICOTROPIN-RELEASING HORMONE (CRH) EXPRESSION IN THE BABOON FETUS AT MID AND LATE GESTATION

INTRODUCTION

Pepe and Albrecht (Pepe *et al.*, 1988; Pepe *et al.*, 1990) have previously demonstrated that estrogen induction of the placental 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes catalyzing oxidation of cortisol to cortisone (Baggia *et al.*, 1990a; Baggia *et al.*, 1990b) at term and at mid-gestation following treatment with estrogen decreased the amount of maternal cortisol arriving at the fetus. We proposed that this change in maternal cortisol secretion to the fetus culminated in activation of the fetal hypothalamus and/or the pituitary leading to increased production of ACTH and steroidogenic maturation of the fetal adrenal gland (Pepe and Albrecht, 1995). In support of this hypothesis we have demonstrated that the increase in expression of the mRNA for the ACTH precursor proopiomelanocortin (POMC) and the number of corticotrophs expressing ACTH peptide in the baboon fetal anterior pituitary (Pepe *et al.*, 1994) and in the specific activity of rate limiting steroidogenic enzymes in the fetal adrenal (Pepe and Albrecht, 1991; Davies *et al.*, 1993) normally observed at term were enhanced at midgestation by maternal estrogen administration. Although these observations confirmed that initiation of fetal adrenal maturation was due to increased fetal pituitary POMC mRNA expression/ACTH secretion, it is not known whether the activation of the fetal pituitary by estrogen-induced changes in placental oxidation of maternal cortisol reflects a concomitant increase in corticotrophin releasing hormone (CRH) mRNA expression and/or peptide production by the fetal hypothalamus. Therefore, the present study was designed, in part, to determine whether the increase in POMC mRNA

previously measured in fetal baboons delivered to mothers treated with estradiol at midgestation reflected a concomitant increase in the expression of hypothalamic CRH.

During human and nonhuman primate pregnancy, the placenta has also been identified as a source of CRH (Shibasaki *et al.*, 1982; Goland *et al.*, 1986; Goland *et al.*, 1986; Sasaki *et al.*, 1987) which is secreted both into the maternal and fetal circulations (Campbell *et al.*, 1987). Although the precise role(s) for placental CRH remains to be elucidated, injection of synthetic CRH into the maternal saphenous vein (Goland *et al.*, 1990) or fetal carotid artery (Berghorn *et al.*, 1991) of pregnant baboons rapidly increased ACTH concentrations in maternal and fetal circulations, respectively. Because our understanding of the role of estrogen on placental CRH production is unclear (Vamvakopoulos and Chrousos, 1994), the present study also determined whether estrogen treatment at mid-gestation enhanced placental CRH production and thus could account, in part, for the increase in POMC mRNA expression previously measured in the baboon fetal pituitary following estrogen administration.

MATERIALS AND METHODS

Baboon treatment with estrogen and harvesting of fetal hypothalami

Fetal hypothalami were obtained on day 100 (n=6) and day 165 (n=5) of gestation (term = day 184) from untreated animals and on day 100 from baboons (n=4) treated daily with a maximal dose of 1.0 mg estradiol benzoate (Sigma Chemical Co., St. Louis, MO) suspended in 0.5 ml sesame oil and injected sc in increasing concentrations (0.25 mg/day, doubling the dose every 5 days between days 70 and 100 of gestation; i.e. 0.25 mg on days 70-74; 0.5 mg on days 75-79; etc). At 2-day intervals between days 80 and 100 or days 140 and 165 of gestation, all baboons were sedated with ketamine-HCl (10

mg/kg BW; Parke-Davis, Detroit, MI) and a maternal saphenous blood sample (4-7 ml) collected. Serum was stored at -20°C until assayed for estradiol by a solid phase ^{125}I RIA (Coat-A-Count, Diagnostic Products Corp, Los Angeles, CA) as described previously (Pepe *et al.*, 1988). On day 100 or 165, animals were sedated with ketamine-HCl then anesthetized with halothane:nitrous oxide (Pepe *et al.*, 1988), a uterine venous blood sample collected, and the fetus delivered. Samples of maternal and uterine venous blood were also obtained from a contemporaneously studied group of baboons at mid (n=3) and late (n=3) gestation and at mid-gestation following maternal administration of estradiol (n=4) as described above. Following injection of an overdose of ketamine to the fetus, the fetal brain was quickly removed and the hypothalamus 2 mm rostral to the optic chiasm extending caudally to the level of the mamillary bodies and 6 mm on either side of the tuber cinereum and 12 mm dorsal to include the paraventricular nucleus (PVN) isolated. Tissue was snap frozen in liquid nitrogen for subsequent analysis of CRH peptide or placed in sterile cryomolds containing OCT Embedding Medium (Miles Scientific, Elkhart, IN) frozen solidly on dry ice, wrapped in parafilm and stored at -80°C for subsequent determination of CRH mRNA.

CRH *in situ* hybridization

Hypothalamic blocks from 3 mid control and 3 term baboons were positioned in a Leica-Reichart-Jung (Bethesda, MD) precision microtome cryostat (-15°C), sectioned (18 μm) in the coronal plane and mounted onto Superfrost Plus (Fisher Scientific, Arlington, VA) microscope slides which were sequentially numbered and stored at -80°C until processed for *in situ* hybridization histochemistry. For each individual hypothalamus, approximately 15% of the sections collected through the entire PVN (determined by Nissl

staining) were utilized for analysis of CRH mRNA expression.

The procedures for *in situ* hybridization detection and quantification of CRH mRNA expression were adapted for use in our laboratory using previously published methods of others (Young *et al.*, 1986a; Young *et al.*, 1986b; Lightman and Young, 1987). A 48 base synthetic oligodeoxynucleotide probe complimentary to the coding region for amino acids 22-37 of rat CRH (21) and exhibiting 94% base sequence homology with the corresponding human sequence (22) was purchased from Oligos Etc. Inc (Wilsonville, OR). Approximately 0.1μM probe was 3' end-labeled with [^{35}S]dATP (SA > 1,000 Ci/mmol; Dupont-New England Nuclear, Boston, MA) and terminal deoxynucleotidyl transferase (20 U; Promega, Madison, WI) to a specific activity of approximately 8,000 Ci/mmol (Pepe *et al.*, 1994).

At the time of hybridization, sections of the fetal hypothalamus through the PVN were selected, equilibrated (10 min) to room temperature, then fixed (10 min) in freshly prepared 4% paraformaldehyde in phosphate buffered saline (pH 7.4). Slides were rinsed in 2 X SSC (1 X SSC = 0.15M NaCl, 0.015 M sodium citrate buffer, pH 7.2), then placed (10 min) in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride-0.9% NaCl (Sigma). Sections were dehydrated through an ethanol series, delipidated in chloroform (5 min), rehydrated in ethanol (100%, 1 min; 95%, 1 min) and air-dried. Sections were prehybridized for 3 h (37°C) in 50 μl hybridization buffer consisting of 4 X SSC, 50% (vol/vol) formamide, 10% (wt/vol) dextran sulfate, 250 μ g/ml yeast transfer RNA (Gibco BRL), 500 μg/ml sheared single stranded salmon sperm DNA (Gibco, BRL), 1 X Denhardt's solution (Sigma), 1% $Na_2S_2O_3$, 5 mM NaPPi and 50 mM freshly prepared dithiothreitol (DTT; Sigma). Slides were rinsed in 2 X SSC and

after addition of 40 µl labeled probe in hybridization buffer, incubated overnight (37°C) under glass cover slips in humidified chambers. Sections were rinsed with 2 X SSC and washed three times (2 X SSC, 50% formamide) at 40°C (approximately 19°C below calculated Tm), then once in 1 X SSC (40°C). After the last wash, slides were rinsed with 2 X SSC (30 min; 22°C), dipped in double distilled water twice, then in ethanol (70% and 95%) and air-dried. Slides were placed against Kodak X-Omat film and exposed for 5-7 days or dipped in fresh Kodak NTB-2 nuclear track emulsion diluted 1:1 with distilled water, placed in light tight boxes and exposed for 21-28 days. Slides were developed in Kodak D-19 (4 min; 15°C), rinsed in distilled water, fixed (Kodak Fixer; 5 min, 15°C), rinsed and lightly counterstained with cresyl violet.

Quantification of CRH mRNA

Specific localization of CRH mRNA expression in the PVN of the baboon fetal hypothalamus was confirmed by light microscopic examination of X-ray film (Figure 15A) and dark-field illumination of hypothalamic sections (Figure 15B-D). Quantification of fetal hypothalamic CRH mRNA expression was determined by counting the specific number of silver grains/nucleus (i.e., per cell) and per 0.025 mm^2 PVN in three control animals at mid- and late gestation using an Optiphot 2 microscope attached to a Video Based Image-1 Analysis System (Universal Imaging Corp., West Chester, PA). Spatial calibration of the imaging system using a 40x objective was 512 pixels along the horizontal axis and 480 pixels along the vertical axis. For each animal, an average of 25 different areas (140 µm x 180 µm) of the PVN each comprised of approximately 85-120 total nuclei were examined. After establishing a gray level, cells with a grain density 10-fold greater than background were considered CRH positive.

Using this criteria, approximately 15-45% of cells/0.025 mm^2 PVN were CRH positive. All grain counts, per cell and per region (number/0.025 mm^2) were averaged and a single value for each animal determined and utilized to calculate overall group mean values.

Tissue extraction/Sephadex chromatography/Purification of CRH protein

Frozen fetal hypothalami from baboons of mid- (n=3) and late gestation (n=2) and from mid-gestation baboons in which the mother was treated with estradiol benzoate (n=4) were homogenized in 2 ml 0.1M hydrochloric acid containing 0.1% (v/v) Triton X-100 (Sigma) and 3,000 cpm [^{125}I]Tyr^{O}-rat/human CRH (2,200 Ci/mmol; Dupont, NEN, Boston, MA) and centrifuged at 6,000 x g, 4°C, for 30 min (Chatelaine *et al.*, 1988). The supernatant was then extracted twice with 2 volumes of ice-cold methanol (Ruckert *et al.*, 1990) and evaporated to dryness using a vacuum centrifuge (Savant Instruments, New York, NY). Extract from one of the two late gestation tissues examined was aliquoted into 3 fractions and each of these, as well as all other hypothalamic extracts, were applied to 35 x 0.9 cm chromatographic columns containing Sephadex G-75 Fine (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.1M HCl (25). After passage of void volume, aliquots were collected at a flow rate of approximately 4 ml/h and ^{125}I-Tyr^{O}-CRH content quantified. Fractions 24-28 (Figure 16A) were pooled, extracted with methanol which was evaporated to dryness, resuspended in 1.0 ml assay buffer (0.05 M sodium phosphate, 0.1M sodium chloride, 0.025M EDTA, 0.1% sodium azide, 0.1% bovine serum albumin and 0.05% Triton X-100; pH 7.3) and stored at -20°C. Duplicate 0.25 ml uterine venous serum samples to which ^{125}I-Tyr^{O}-CRH was added were extracted with methanol, dried, resuspended in 250 µl buffer and stored at -20°C until assayed.

RIA of purified CRH protein

The assay was adapted from that originally described (Vale *et al.*, 1983). Briefly, standard doses (5-250 pg/100 µl assay buffer) of rat/human CRH (Bachem Inc, Torrance, CA) or duplicate aliquots of extracts of hypothalamus (25, 50, 100 µl) or uterine venous serum (50 µl) were added to 12 x 75 mm polypropylene tubes immersed in ice-water. After addition of 300 µl rabbit anti-human/rat CRH antiserum (1:600,000 in assay buffer) generously supplied by Wylie Vale, tubes were vortexed and incubated overnight at 4°C. Tubes were incubated (4°C) for an additional 18-24h following addition of ^{125}I-Tyr^{O}-CRH (20,000 cpm/100 µl assay buffer) and 100 µl normal rabbit carrier (Linco Research Inc., St. Louis, MO). To separate bound/free CRH, 100 µl goat anti-rabbit gamma globulin (Linco) and 500 µl 10% polyethylene glycol (Sigma) in assay buffer were added to reaction mixtures which were vortexed, incubated for 45 min and then centrifuged at 3,000 rpm for 30 min. Assay of serum samples to which 100 or 250 pg authentic CRH were added yielded CRH levels which were 95-105% of those anticipated. The antibody exhibited minimal (<0.5%) cross-reactivity with ovine-CRH, oxytocin, vasopressin, endorphins and sauvagine. All hypothalamic extracts, which exhibited parallelism with CRH standards (Fig. 16B) were evaluated in the same assay. The coefficient of variation for mean CRH value for the 3 aliquots of term fetal hypothalamus was 8.6%.

Mean serum estradiol and CRH concentrations and hypothalamic CRH mRNA and peptide levels were analyzed by Student *t*-tests or by analysis of variance (ANOVA) with post-hoc comparison of the means using the Student-Newman-Keuls test (Snedecor and Cochran, 1980).

RESULTS

The mean (± SE) maternal serum estradiol concentration in baboons treated with estradiol at mid-gestation was greater ($P<0.05$) than that in untreated baboons on day 100, but similar to that in late gestation (Table IV). Fetal hypothalamic wet weight was 3-fold greater ($P<0.05$) at term than at mid-gestation and was not altered at mid-gestation by treatment of the mother with estradiol.

The steady-state concentration of CRH in the baboon fetal hypothalamus (Figure 17) at mid-gestation (15.8 ± 6.0 ng/g tissue) was not altered in fetuses in which the mother was treated with estradiol (17.6 ± 0.9 ng/g). Because the apparent wet weight of the hypothalamus at mid-gestation (0.51 ± 0.05 g) was not altered by maternal estradiol treatment (0.32 ± 0.07 g), the content of CRH in the hypothalamus also was similar in fetuses of control (7.5 ± 2.2 ng/gland) and estrogen-treated (5.5 ± 1.1 ng/gland) animals. Although only two fetuses were studied late in gestation, hypothalamic CRH concentrations in these baboons (20.4 -21.0 ng/g) were similar to mean CRH values measured at mid-gestation. In contrast, due to the marked increase in weight of the fetal hypothalamus with advancing pregnancy, the content of hypothalamic CRH in late gestation (29.6 and 28.1 ng/structure) exceeded ($P<0.01$; ANOVA) the mean value (7.5 ± 2.2) and the 95% confidence interval (16.8 ng/ml) for CRH content in hypothalamic extracts from control baboons of mid-gestation.

Examination of X-ray film after 7 days exposure revealed a high density of labeling of CRH mRNA in the PVN of the fetal baboon hypothalamus (Figure 15A). At this level of exposure, no other areas of the section of neural tissue examined exhibited labeling. Specificity of labeling to the PVN was confirmed when sections of slides placed in emulsion for 28 days were examined by dark-field microscopy (Figure 15C and D). The hybridization signals obtained in the PVN were specific since hybridization of adjacent

Table IV. Maternal serum estradiol concentrations and fetal hypothalamic weight in baboons at mid- and late gestation and at mid-gestation after treatment with estradiol[a].

Treatment[a]	Estradiol (ng/ml)[b]	Fetal Hypothalamus Weight (g)
Midgestation		
Control	1.0 ± 0.2[c] (n = 9)	0.51 ± 0.05[c] (n = 3)
Estradiol (1.0 mg)	2.4 ± 0.4[d] (n = 8)	0.32 ± 0.07[c] (n = 4)
Late Gestation		
Control	2.0 ± 0.2[d] (n = 9)	1.40[d] (n = 2)

[a]Baboons delivered on day 100 and day 165 (late) of gestation (term = day 184) to mothers untreated or treated with estradiol benzoate injected daily between days 70-100 of gestation. [b] Serum estradiol (mean ± SE) determined in maternal saphenous venous samples obtained at 1- to 2-day intervals on days 80-100 or days 145-165 of gestation. [c,d] Values (mean ± SE) with different superscripts differ from respective values at $P<0.05$ (ANOVA and Student-Newman-Keuls test).

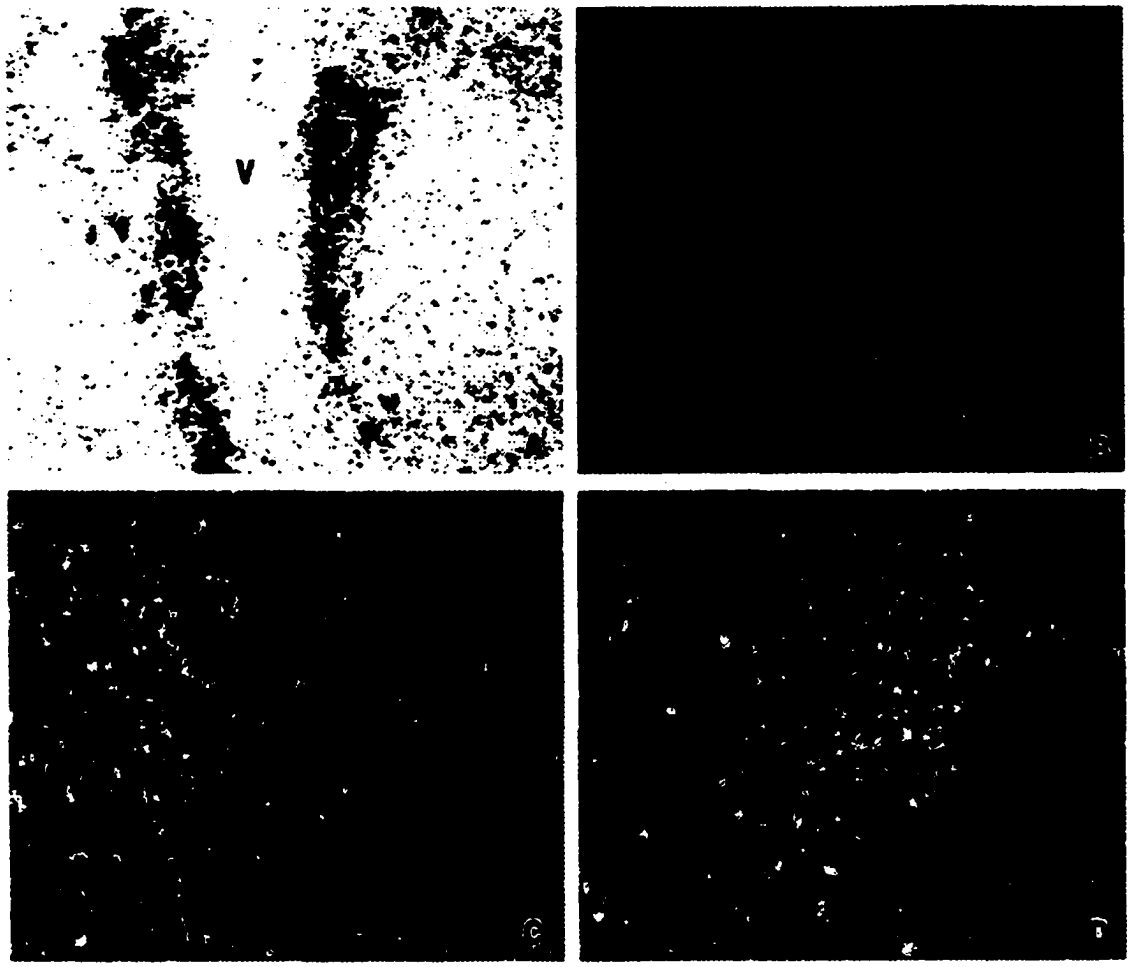

Figure 15. Localization of CRH mRNA in the PVN of the fetal baboon hypothalamus after *in situ* hybridization of coronal brain sections (V = third ventricle) with ^{35}S-labeled 48-mer antisense (A, C, D) or sense (B) oligonucleotide probes. A) Photomicrograph of x-ray film showing the distribution of CRH mRNA in the PVN on Day 165 of gestation (term = Day 184). B) Darkfield photograph of an emulsion autoradiogram of PVN on Day 165, labeled with sense probe and counterstained with cresyl violet. C and D). Darkfield photographs of emulsion autoradiograms showing cells in the PVN on Day 100 (C) and Day 165 (D) of gestation labeled with antisense probe.

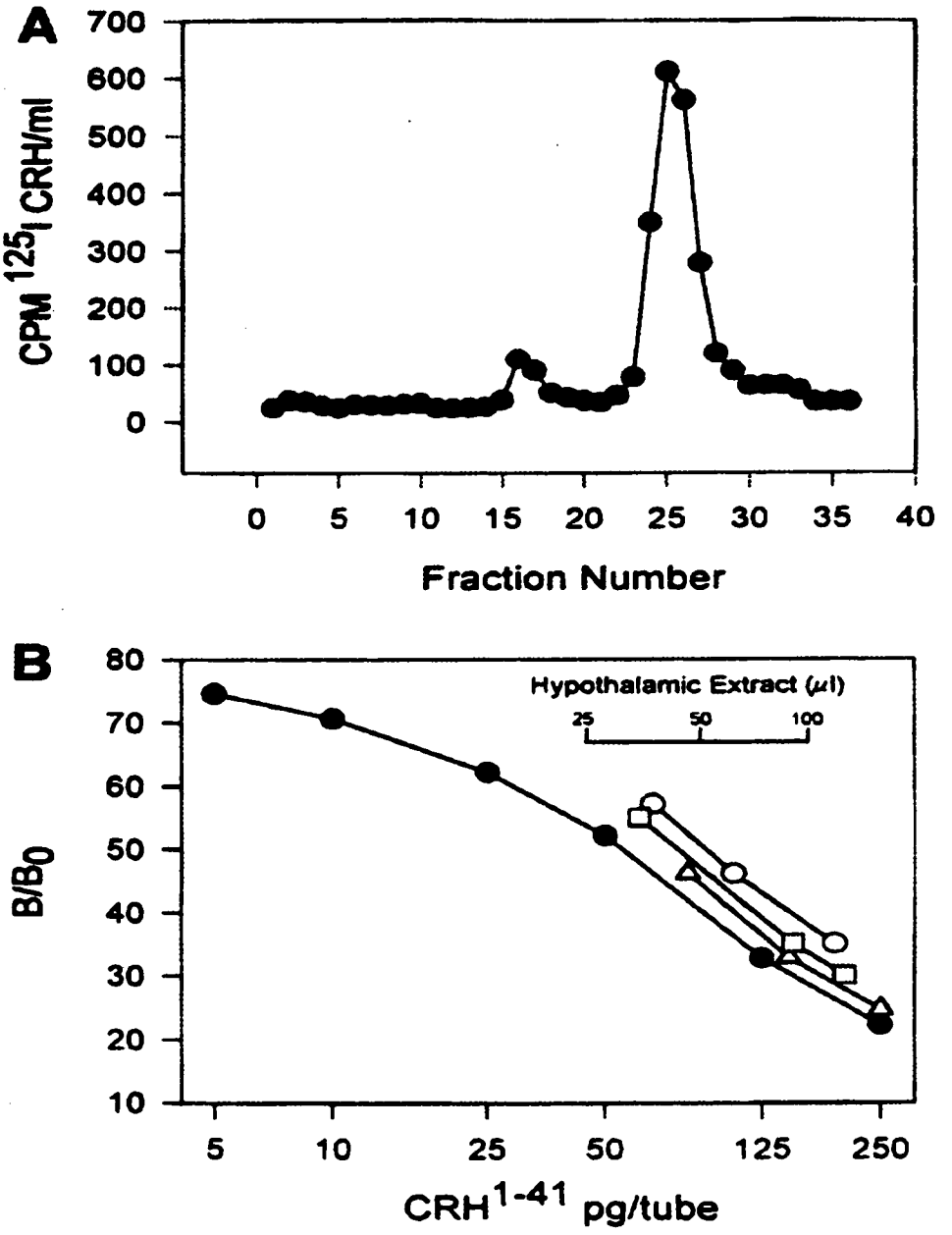

Figure 16. A) Representative Sephadex chromatography of hypothalamic extracts containing [^{125}I]-Tyr^{O}-CRH. B) RIA of CRH in representative serial dilutions of fetal hypothalamic extracts on day 100 (open circles) and day 165 (open triangles) in untreated baboons and on day 100 after maternal treatment with estradiol (open squares) compared with synthetic rat/human CRH standards (solid circles). B/B_O, amount of [^{125}I]-Tyr^{O}-CRH bound relative to that at zero hormone concentration.

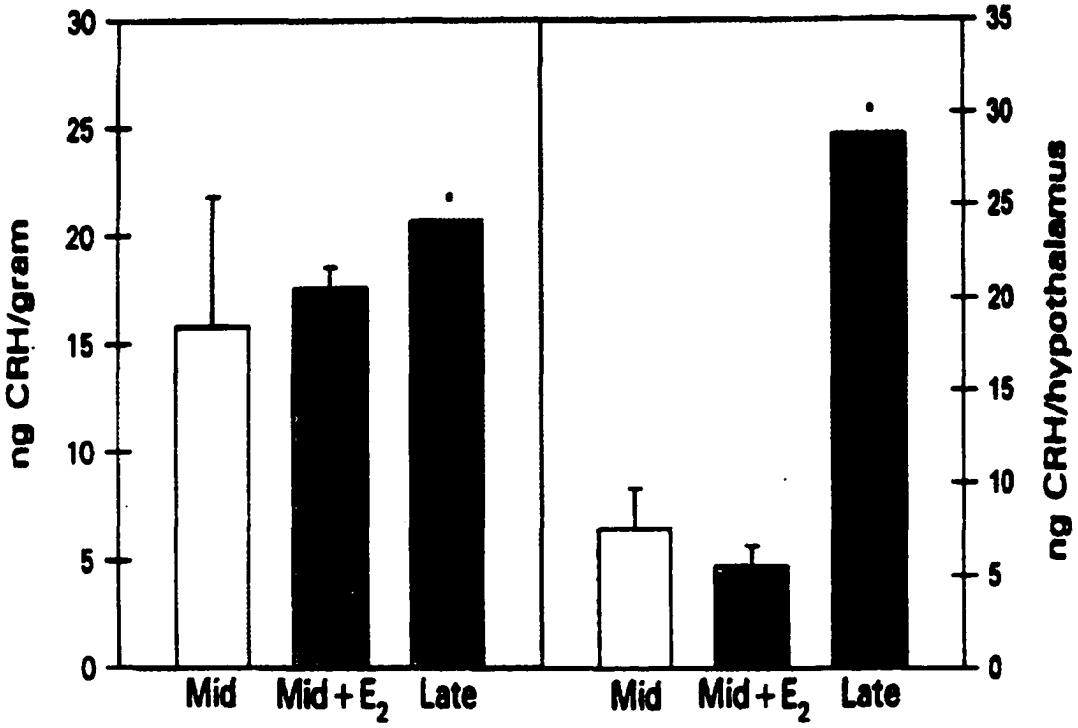

Figure 17. Mean (± SE) fetal hypothalamic CRH concentrations and CRH content determined by RIA at mid (day 100; n = 3)- and late (day 165; n = 2) gestation (term = day 184) and at mid-gestation in baboons treated with estradiol (E_2, n = 4). See Materials and Methods for methodological details. Content of CRH (ng CRH/ hypothalamus) in late gestation differs from that at mid-gestation ($P<0.05$; ANOVA and Student Newman Keuls Test).

sections with a labeled sense probe under identical conditions as described for the antisense probe gave no hybridization signal (Figure 15B). As seen in Figure 18, mean levels of CRH mRNA at mid-gestation, when expressed per cell (17.4 ± 1.3 grains/ cell) or per unit area of PVN (350 ± 55 grains/area), were similar to respective values in late gestation (18.3 ± 1.1 grains/cell; 375 ± 20 grains/area).

Mean CRH levels in uterine vein (Table V) were similar at mid- and late gestation and not significantly altered at mid-gestation by maternal treatment with estradiol. In umbilical serum samples of three mid-gestation baboons in which sufficient serum was available for assay, CRH levels were below the minimal detectable dose (≤ 50 pg/ml; data not shown).

DISCUSSION

The present study demonstrated that the steady-state concentration of CRH peptide in and CRH mRNA expression within the PVN of the baboon fetal hypothalamus were similar at mid- and late gestation. These findings support the suggestion that the increase in fetal pituitary POMC mRNA expression and ACTH peptide previously reported normally between mid-gestation and term are not associated with a concomitant increase in hypothalamic CRH peptide or CRH mRNA concentrations. The absence of change in fetal hypothalamic CRH peptide concentrations with gestational age has also been noted in humans studied between 12 and 27 weeks of gestation (Ackland *et al.*, 1986). Interestingly, the concentration of CRH measured in hypothalamic extracts of human fetuses (9.2 ± 11.4 ng/g) was similar to that measured in fetal baboons of the present study. In the sheep, fetal hypothalamic CRH concentrations, although relatively low very early in gestation, appear to be similar (Brooks *et al.*, 1989; Myers *et al.*, 1993) or rise only slightly (Watabe *et al.*, 1991; Brieu *et al.*, 1989) between days 100 and 135 of gestation. The present study also showed that CRH peptide concentrations were not

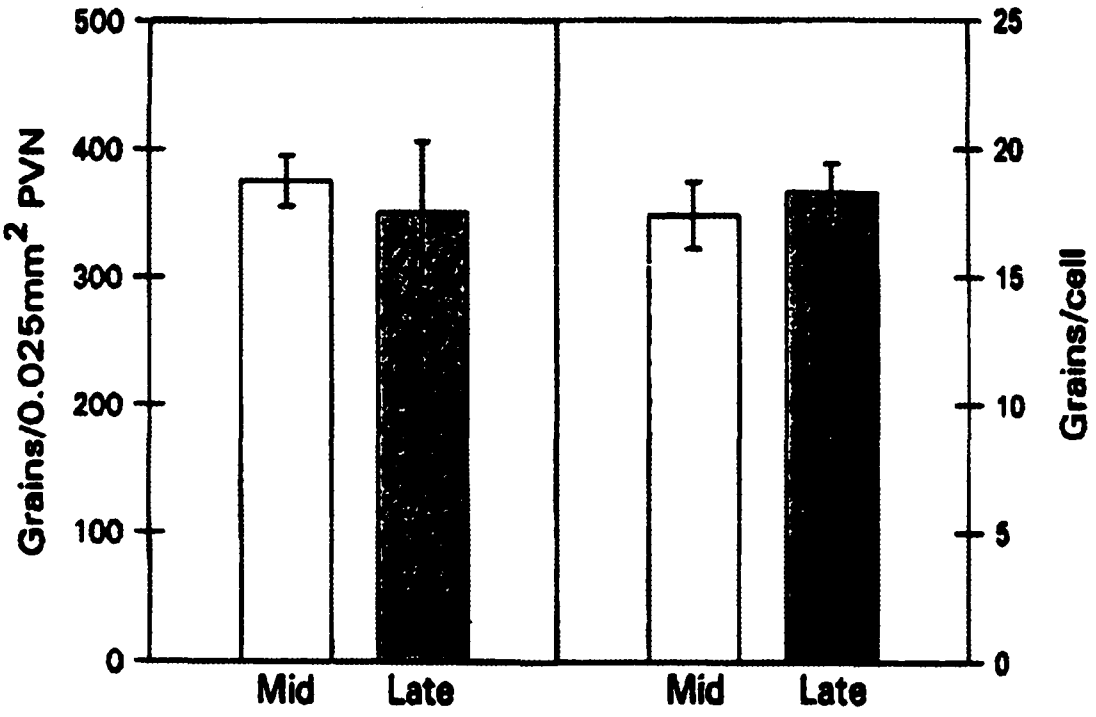

Figure 18. Mean (± SE) expression of CRH mRNA determined by *in situ* hybridization in sections of the paraventricular nucleus (PVN) of the fetal baboon hypothalamus at mid (day 100; n = 3)- and late (day 165; n = 3) gestation. See Materials and Methods for methodological details.

Table V. Mean (± SE) CRH levels (pg/ml) in uterine vein in baboons at mid- and late gestation and at mid-gestation after treatment with estradiol[a].

MIDGESTATION		LATE GESTATION
Control	+ Estradiol	Control
572 ± 21	479 ± 50	509 ± 59
(n=7)	(n=8)	(n=7)

[a]Uterine venous blood samples obtained on day 100 (mid) and day 165 from untreated animals and on day 100 from baboons treated between days 70 and 100 of gestation with a maximal dose of 1 mg estradiol benzoate.

altered at mid-gestation in animals in which the mother was treated with estradiol. In our previous studies, we demonstrated that the concentration of fetal pituitary POMC mRNA/ACTH expression was increased at term and at mid-gestation following maternal estrogen administration (Pepe *et al.*, 1994). It is possible therefore, that the estrogen-dependent increase in fetal pituitary POMC mRNA/ACTH peptide reflects activation of the fetal pituitary and not the hypothalamus. Additional studies are required to establish this point particularly since the total level of hypothalamic CRH at term was 2-3 fold greater than at mid-gestation. Nevertheless, it would appear, that by mid-gestation hypothalamic CRH is available in adequate concentrations to "drive" the fetal pituitary and that it is the levels of maternal cortisol arriving within the fetal circulation, as dictated by estrogen-regulated placental 11β-HSD-oxidase activity (Pepe and Albrecht, 1995), which establishes the extent to which the fetal pituitary responds to CRH. Our findings do not imply that fetal hypothalamic CRH is unimportant to fetal pituitary function and/or maturation. Indeed, in addition to the fact that CRH content in the fetal hypothalamus in late gestation was 2-fold greater than that at mid-gestation, in the absence of CRH as presumably occurs in anencephalic human fetuses, fetal pituitary corticotrophs appear to be less in number and smaller in size (Begeot *et al.*, 1977) and fetal adrenal growth and steroidogenic maturation essentially abolished (Pepe and Albrecht, 1990). In addition, in the sheep fetus, hypothalamic-pituitary disconnection (Antolovich *et al.*, 1990) or lesions of the hypothalamic PVN greatly retard maturation of fetal anterior pituitary corticotrophs (McDonald *et al.*, 1992) and prevent the increase in fetal ACTH levels associated with advancing gestation (McDonald *et al.*, 1992). Finally, although factors in addition to CRH (e.g. arginine vasopressin) have been shown to elicit release of ACTH from the fetal and adult pituitary *in vivo* (Hargrave and Rose, 1986; Berghorn *et al.*, 1991) and *in vitro* (Blumenfeld and Jaffe, 1986), it appears that CRH

primarily modulates POMC mRNA expression (Bruhn *et al.*, 1984). Although the ontogenesis and potential role of arginine vasopressin on fetal baboon pituitary function remains to be determined, we suggest that it is the level of cortisol in fetal blood which dictates the responsivity of the pituitary (e.g. POMC mRNA expression) to CRH. Indeed, the negative feedback action of cortisol on CRH-induced increases in fetal ACTH can occur directly at the fetal pituitary (Ozolins *et al.*, 1990). Moreover, infusion of cortisol into the sheep fetus blocked CRH- or hypotension-induced secretion of ACTH (Rose *et al.*, 1985). In the adult rat, adrenalectomy elicited a 2-fold increase in hypothalamic CRH mRNA expression whereas POMC mRNA expression was increased by more than 9-fold. Moreover, while treatment of the adrenalectomized rat with dexamethasone reduced CRH mRNA to levels which were similar to those in intact rats, POMC mRNA was reduced to a level which was only 19% of that in the controls (Jingami *et al.*, 1985). Thus, it is possible that the negative feedback effects of cortisol on the pituitary are exerted at the level of transcription. Consistent with this suggestion, it has been shown in AtT20 cells that glucocorticoids have a direct inhibitory effect on ACTH precursor mRNA levels (Nakamura *et al.*, 1978; Roberts *et al.*, 1979) and inhibit the transcription rate of the ACTH precursor gene (Birnberg *et al.*, 1983; Eberwine and Roberts, 1984). Thus, as suggested (Jingami *et al.*, 1985), glucocorticoids exert their negative feedback effect at the level of gene expression more dominantly on pituitary corticotrophs than on hypothalamic CRH neurons. It is likely therefore, that in the baboon fetus the developmental expression of POMC mRNA is regulated, in part, by cortisol and its direct effects on the fetal pituitary.

Because cortisol appears to play a significant direct role in regulating pituitary POMC mRNA/ACTH expression, it remains to be demonstrated by what means/mechanisms fetal pituitary POMC mRNA expression is increased late in gestation

concomitant with an increase in fetal adrenal maturation measured as increased production of cortisol. In this regard, we have shown that estrogen in addition to its action on placental cortisol metabolism also enhances cortisol oxidation to cortisone by the fetus (Albrecht *et al.*, 1981; Waddell *et al.*, 1988a) presumably by modulating tissue specific activity of 11β-HSD (Pepe and Albrecht, 1995). In sheep, the mRNA for 11β-HSD type 1 is present in several fetal tissues including the fetal pituitary (Yang *et al.*, 1992) while in human fetal tissues 11β-HSD type 2 is predominantly expressed (Pepe and Albrecht, 1995). It is possible, therefore, that the maintenance of POMC mRNA late in gestation presumably reflects increased fetal conversion of cortisol to its inactive metabolite cortisone which does not bind to the glucocorticoid receptor.

The human CRH gene contains both a consensus glucocorticoid response element and two half-palindromic estrogen response elements (Vamvakopoulos and Chrousos, 1994). Estrogen can enhance the activity of chloramphenicol acetyltransferase (CAT) in CV-1 cells in which CRH-CAT constructs were transiently expressed and supplemented with a co-transfected estrogen receptor cDNA expression plasmid (Vamvakopoulos and Chrousos, 1993). Conversely, estrogen has been shown to decrease hypothalamic CRH expression in ovariectomized rats (Grino *et al.*, 1995). Despite the fact that estrogen production and secretion into the fetus is increased with advancing gestation in baboons as in humans (Albrecht and Pepe, 1990), it seems unlikely that estrogen elicits a negative effect on CRH production in the baboon. Thus, although the concentration of hypothalamic CRH was not altered with advancing gestation, the total amount of CRH peptide and mRNA expressed was elevated. Consistent with this suggestion, results of the present study also showed that CRH concentrations in the uterine vein, and

presumably placental CRH production were similar at mid- and late gestation. Moreover, despite the fact that estrogen can enhance uterine blood flow, and the observation that the baboon does not synthesize a CRH binding protein (Pepe and Albrecht, 1995), uterine vein CRH concentrations in baboons of the present study were not decreased following treatment with estradiol. Indeed, estrogen did not alter CRH mRNA expression of CRH peptide released by human trophoblast cells/fragments studied *in vitro* (Jones *et al.*, 1989; Robinson *et al.*, 1988).

In summary, we have demonstrated that the concentration of CRH peptide and mRNA in the fetal baboon hypothalamus was similar at mid- and late gestation and that CRH peptide levels were not increased at mid-gestation following maternal treatment with estradiol. Therefore, we suggest that the increase in pituitary POMC mRNA and ACTH peptide expression at term and following maternal estrogen administration of estradiol is regulated, in part, by cortisol, the fetal serum levels of which are controlled by estrogen-regulated 11β-HSD-catalyzed oxidation to cortisone within placental and fetal tissues.

CHAPTER V

CLONING AND EXPRESSION ANALYSIS OF THE 11β-HYDROXYSTEROID DEHYDROGENASE (11β-HSD) -1 AND -2 GENES IN THE BABOON:EFFECTS OF ESTRADIOL ON PROMOTER ACTIVITY IN PLACENTAL JEG-3 CELLS

INTRODUCTION

Pepe and Albrecht have shown that estrogen, via regulation of placental 11β-hydroxysteroid dehydrogenase (11β-HSD) catalyzed metabolism of cortisol and cortisone (Pepe *et al.*, 1988) determines the amount of maternal cortisol arriving within the fetus (Pepe and Albrecht, 1990) and thus regulates the function of the fetal hypothalamic-pituitary-adrenocortical axis (Pepe and Albrecht, 1990; Pepe *et al.*, 1990; Pepe *et al.*, 1994). It is now known that cortisol-cortisone interconversion is regulated by two different 11β-HSD enzymes (Tannin *et al.*, 1991; Agarwal *et al.*, 1994; Monder and White, 1993; Pepe and Albrecht, 1995). The 11β-HSD type 1 originally cloned from the rat liver (Lakshmi and Monder, 1988) possesses both oxidative and reductive activity, prefers NADP/NADPH as a cofactor and has a relatively low (μM) affinity for glucocorticoid substrates. In contrast the 11β-HSD type 2 is unidirectional possessing only oxidative activity, uses only NAD^+ and has a high affinity (nM) for substrate cortisol (Brown *et al.*, 1993; Brown *et al.*, 1996a). While the 11β-HSD-1 gene appears to be expressed in several tissues (Tannin *et al.*, 1991; Monder and White, 1993; Pepe *et al.*, 1996b), the 11β-HSD-2 is expressed only in a few tissues including the kidney cortex, placenta and perhaps the fetal adrenal (Albiston *et al.*, 1994; Brown *et al.*, 1996b; Pepe *et al.*, 1996a). The molecular/ biochemical basis for selective tissue expression remains to be determined.

Recently, Pepe and Albrecht demonstrated that the mRNA and peptide levels of both 11β-HSD-1 and -2 in syncytiotrophoblast of the baboon placenta increased with advancing gestation (Pepe *et al.*, 1996a) and that baboon placental NAD^+-dependent 11β -HSD-2 activity was regulated by estrogen (Baggia *et al.*, 1990a). Because of the important role that both 11β-HSD-1 and –2 enzymes play in regulating fetal-placental function in the baboon (Pepe and Albrecht, 1995; Pepe *et al.*, 1996b), our more recent studies have been directed at elucidating the molecular basis by which syncytio-trophoblast expression of 11β-HSD-1 and -2 is regulated. The genes for 11β-HSD-1 and -2 have been isolated from several species (Tannin *et al.*, 1991; Agarwal *et al.*, 1994; Agarwal *et al.*, 1995; Cole, 1995) and sequence analysis indicates that although there is little homology between 11β-HSD-1 and -2, there is a high degree of inter-species homology of both genes. Because of the distinct tissue expression of the 11β-HSD genes, elucidation of the cell-specific transcription factors contributing to 11β-HSD-1 and -2 expression may require gene-transfection studies using primary cell culture in addition to analyses in transformed cell-lines. For example, the cAMP-responsive elements of placental growth hormone variant gene are preferentially activated in second and third trimester trophoblasts, a time when endogenous gene expression is elevated (Golos *et al.*, 1994). Because the promoter regions of 11β-HSD-1 and 11β-HSD-2 in the baboon have not been determined, the present study was designed to isolate both 11β-HSD-1 and 11β-HSD-2 genes from a baboon genomic library and sequence the promoter regions which would subsequently permit examination of the tissue specific factors contributing to the regulation of 11β-HSD-1 and 11β-HSD-2 gene expression in the baboon placenta. Moreover, we also produced luciferase reporter constructs of the

baboon 11β-HSD-1 and 11β-HSD-2 genes to determine whether the promoter regions of these two genes were activated in human placental JEG-3 cells and whether expression could be modified by estradiol. Finally, adenovirus constructs containing antisense human 11β-HSD-2 cDNA was analyzed for potential expression inhibition upon co-transfection with the cloned baboon sense 11β-HSD-2/luciferase construct.

MATERIALS AND METHODS

Isolation of genomic clones and sequence analysis of baboon 11βHSDs

Approximately 1.3 x 10^6 plaques of a baboon kidney genomic library (Stratagene, La Jolla, CA) were screened by hybridization with the human cDNA for 11β-HSD-1 (generously supplied by Carl Monder and Perrin White) labeled with [α-^{32}P] dCTP (Dupont-New England Nuclear Corp., Boston, MA) to a specific activity of 10^9 dpm/μg using the Random Primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN) as outlined by Feinberg and Vogelstein, 1983. An additional 1.3 x 10^6 plaques of a baboon kidney genomic library (Stratagene, La Jolla, CA) were screened in the same fashion with the human cDNA for 11β-HSD-2 (generously supplied by Zygmunt Krozowski). DNA from positive clones was isolated and analyzed by restriction endonucleases. The 5'-flanking region of the baboon 11β-HSD-1 gene was isolated by hybridization with a 5' end-specific human 11β-HSD-1 probe generated by EcoRI digestion of the human 11β-HSD-1 cDNA (+1 to +165bp) and subcloned into pBluescript SK (Stratagene). DNA sequences of both strands were determined by the dideoxy nucleotide chain termination method using a Sequenase (version 2) kit (United States Biochemical, Cleveland, OH). Based on the DNA sequence information obtained, new oligonucleotides were synthesized (Bio-Synthesis Inc, Lewisville, TX) and used as primers to continue the

sequencing process. In similar fashion, the 5'-flanking region of the baboon 11β-HSD-2 gene was also isolated by hybridization with a 5' end-specific human 11β-HSD-2 probe generated by restriction enzyme digestion of the human 11β-HSD-2 cDNA and subcloned into pBluescript SK (Stratagene). Again, DNA sequences of both strands were determined by the dideoxy nucleotide chain termination method above and new oligo-nucleotides were synthesized and used as primers to continue the sequencing process.

Northern analysis

Total RNA from term placenta as well as fetal and adult baboon liver and/or heart was isolated (Albrecht *et al.*, 1995). Briefly, tissues were homogenized in 4M guanidine isothiocyanate (Sigma Chemical Co., St. Louis, MO) containing 0.83% 2-mercapto-ethanol (Sigma), extracted with chloroform:isoamyl alcohol (24:1), layered over a 5.7M cesium chloride gradient and RNA pelleted by centrifugation (Chirgwin *et al.*, 1979). Following size-fractionation by electrophoresis in 1% agarose-formaldehyde gels, 30 μg total RNA was transferred to nylon membrane (Micron Separations, Inc., Westboro, MA) and samples hybridized with either the full-length (1368 bp) human 11β-HSD-1 cDNA (1 x 10^6 cpm/ml) or the full-length (1871 bp) human 11β-HSD-2 cDNA (1 x 10^6 cpm/ml) at 65°C in 6 x SSC, 2 x Denhardt's, 0.5% SDS overnight. The membranes was washed under stringent conditions (Pepe *et al.*, 1996) and then exposed (-70°C) for 96 h to Kodak X-AR film (Eastman Kodak, Rochester, NY).

Primer extension analysis

Primer extension analysis of the transcriptional start site(s) of the baboon 11β-HSD-1 clone was conducted using a baboon-specific 22 base DNA primer (5'- ACCTGGTCTGAATTCCTATATG -3') located at the 3' end of exon I. The primer

was end-labeled with [γ-^{32}P] ATP (Dupont-NEN) and purified from unincorporated label by separation on a Chroma Spin-10 Column (Clontech Laboratories, Inc., Palo Alto, CA). To analyze the start site(s) of baboon 11β-HSD-2, a 22 base DNA primer (5'-GGATCGGGGAGAAAGAGTCTGG-3') and a 23 base primer (5'-AGCGCGCGGGCAGCCACGAGCAG-3') located at the 3' end of exon I of our baboon 11β-HSD-2 clone, were end-labeled and purified as above. Approximately 30 μg of baboon liver total RNA and 30 μg yeast tRNA were precipitated with ^{32}P labeled 11β-HSD-1 primer (5.2×10^5 cpm) and resuspended in hybridization buffer composed of 40 mM PIPES, 1 mM EDTA (pH 8.0), 0.4M NaCl, and 80% formamide. In similar fashion, approximately 30 μg of baboon kidney and placenta total RNA and 30 μg yeast tRNA were precipitated with the two ^{32}P labeled 11β-HSD-2 primers (1.2×10^6 and 1.7×10^6 cpm) and resuspended in hybridization buffer as above. After heating to 85°C, the mixtures were incubated overnight at 30°C. Hybridized duplexes were precipitated and resuspended in reverse transcription buffer composed of 50 mM Tris-HCl (pH 8.3), 60 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM each dNTP, 20U RNasin (BRL Life Technologies Inc., Gaithersburg, MD) and 400 units Molony mouse leukemia virus reverse transcriptase (BRL). After incubation for 2 h at 37°C, reactions were terminated by addition of 1 μl 0.5 M EDTA (pH 8.0) and 1 μl RNase-A (20 U/ml; BRL) and incubation for 30 min at 37°C. The samples were extracted with phenol-chloroform, precipitated and then resuspended in 6 μl sequencing gel loading buffer, and electrophoresed on an 8M urea-acrylamide-DNA sequencing gel. A set of dideoxynucleotide DNA sequencing reactions were run alongside the samples to accurately size the extended product.

Solution hybridization – RNA nuclease protection assay

A 262 bp Bam H1-Eco R1 fragment of the 5'-flanking region of the baboon 11β-HSD-1 gene (-91 to +171 nt; Figure 20) encompassing both the upstream and downstream transcription start sites was isolated, purified and subcloned into pBluescript SK (Stratagene). After confirmation of orientation of the sequence by DNA sequencing analysis, probe and reference (sense) RNAs were transcribed and transcription terminated by digesting the template with RNase-free DNase (BRL). Solution hybridization-RNase protection was performed (Jakubowski and Roberts, 1992). Briefly, approximately 10 ng of RNA probes labeled with [α-^{32}P] UTP (Dupont-NEN) were mixed with 5 or 30 μg total RNA isolated from adult baboon liver or term baboon placenta in a final volume of 25 μl of hybridization buffer (4M guanidinium thiocyanate, 0.1 M EDTA, pH 7.5). Standard curves were established by mixing the probe with increasing amounts (0-37 pg) of the reference RNA. After hybridization overnight at 30°C, the mixture was incubated (30°C) for 60 min with 300 μl of a RNase solution composed of 10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 40 μg/ml RNase A (BRL) and 2 μg/ml RNase T1 (BRL). After treatment with proteinase K for 15 min at 45°C, samples were extracted with phenol-chloroform and the duplex RNA fragments protected from RNase digestion precipitated with ethanol and electrophoresed through a 6% nondenaturing polyacrylamide gel. Gels were dried, exposed to Kodak X-AR film for 12 h and bands quantified densitometrically (Pepe *et al*, 1994).

Luciferase reporter constructs and transient transfection assay

The 1.7 kb fragment of the 5'-flanking region of baboon 11β-HSD-1 and the 1.2 kb fragment of baboon 11β-HSD-2 were each subcloned into promoter-less luciferase

reporter pGL3-Basic vectors (Promega Corp., Madison, WI). Constructs were then transiently-transfected into human choriocarcinoma JEG-3 cells, 293 human embryonic kidney cells, Chang adult liver cells and HEP-G2 human adolescent liver hepatoblastoma cells (American Type Tissue Culture, Bethesda, MD) using calcium phosphate precipitation procedure. Briefly, cells were cultured on 60 mm plates in Dulbecco's Modified Eagle's medium (DMEM) containing phenol red and 10% fetal bovine serum (FBS). After washing, cells were transiently transfected in triplicate with 5 μg of plasmid-DNA construct in DMEM-10% FBS. Control cells were transiently transfected with the promoter-less pGL3-Basic vector. Following incubation for 12-14 h, all cells were washed three times with phosphate buffered saline (PBS) and then incubated in DMEM without phenol red and 10% charcoal-stripped FBS for an additional 16-18 h. Cells were scraped from dishes and then lysed in 25 mM glycylglycine (pH 7.8) buffer containing 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol, 1% Triton-X 100. Following centrifugation (13,000 x g, 4°C, 5 min), supernatants were diluted in 25 mM glycylglycine (pH 7.8) buffer containing 15 mM KPO_4, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, and 11 mM luciferin and immediately assayed for luciferase activity. Protein concentrations in cell lysates were determined by the bicinchoninic acid method (Sigma). To study the role of estrogen, JEG-3 cells were transfected with 11β-HSD-1/-2 constructs, washed and incubated overnight with $1x10^{-8}$ M 17β-estradiol or $1x10^{-8}$ M 17α-estradiol. To augment the low level of estrogen receptor (ER) in JEG-3 cells (Radovick *et al.*, 1991; Wierman *et al.*, 1992), studies of 11β-HSD-1 and -2 promoter activity were also performed in JEG-3 cells co-transfected with 1 μg of an expression vector containing the human ERα cDNA (pSG5ER, gift of P. Chambon) essentially as

described previously (Wierman *et al.*, 1992; Dong *et al.*, 1996). Control cells were co-transfected with 1 μg of an expression vector containing a beta galactosidase cDNA (Promega). Further analyses utilizing the membrane-permeant, lipophilic cAMP analog, CPT-cAMP, 8-(4-chlorophenylthio)-cAMP, were conducted on JEG-3 cells to determine the effect, if any, of cAMP-dependent protein kinase activation on the promoter regulation of 11β-HSD-1 and –2. Finally, the role of steroidogenic factor 1 (SF-1) was assayed using a human SF-1 expression vector cDNA (kindly donated by S. J. Beebe). To more accurately determine SF-1's role on 11β-HSD-1 and -2 promoter activity, cotransfections and luciferase determinations were conducted in 293 kidney cells, which do not contain endogenous SF-1 as do JEG-3 cells.

Adenovirus / 11βHSD-2 antisense construction

The University of Iowa Viral Vector Core laboratory constructed the adenovirus / 11β HSD-2 vector using non-oncogenic adenovirus serotype 5. The E1 gene region was deleted rendering it replication deficient and was replaced with the 1.9 kb human 11β HSD-2 cDNA inserted in an inverted fashion, i.e. antisense orientation. A 1.0 kb Rous Sarcoma Virus promoter was ligated 5' to the inverted 11βHSD-2 sequence to ensure proper nuclear targeting and high transcriptional expression. Constructed viruses were subsequently propagated in the E1-gene complementary 293 human embryonic kidney cell line. Prospective recombinant viral clones were screened by isolating and digesting their DNA to verify the presence of the 1.9 kb insert. Recombinant adenovirus clones containing the antisense human 11βHSD-2 cDNA insert were propagated and isolated to yield 1×10^{12} particles / ml.

Antisense gene therapy in JEG-3 cells

JEG-3 cells at 80% confluence on 60 mm plates were cotransfected either simultaneously with antisense human 11βHSD-2 cDNA adenovirus and calcium-phosphate-precipitated sense baboon 11βHSD-2/luc construct (as described above) or transfected with adenovirus alone 24 hrs prior to transfection via calcium phosphate precipitation with the sense baboon 11βHSD-2/luc DNA construct. With these later transfections, the adenovirus was introduced onto cells in DMEM and incubated for 1 hr before adding FBS to 10%. As a control, JEG-3 cells were cotransfected simultaneously with the adenovirus and calcium phosphate precipitated sense baboon 11βHSD-1/luc construct DNA to determine what role, if any, the adenovirus might play in transfection efficiency, luciferase expression and/or cell viability. The adenovirus was stored at –80°C and aliquots were thawed quickly just prior to use. Only adenovirus thawed once or twice were utilized.

RESULTS

The 11β-HSD-1 genomic DNA was isolated from a baboon kidney genomic library using the human 11β-HSD-1 cDNA as a probe. After three screenings, 7 recombinant phage exhibited strong hybridization to the human type 1 11β-HSD cDNA. The 5'-flanking region of the baboon 11β-HSD-1 gene was isolated using a portion of the 5' end of the human 11β-HSD-1 cDNA (+1 to +165 bp) prepared by restriction digestion with EcoRI. Using this probe, 2 of the 7 recombinant phage exhibited an extensive hybridization signal. A 1.7 kb fragment was subsequently isolated and subcloned into a pBluescript SK vector.

The 11β-HSD-2 genomic DNA was isolated from a baboon kidney genomic library using the human 11β-HSD-2 cDNA as a probe. After three screenings, 5 recombinant phage exhibited strong hybridization to the human type 2 11β-HSD cDNA. The 5'-

flanking region of the baboon 11β-HSD-2 gene was isolated using a Nco I fragment (+108 to +420 bp) containing a portion of the 5' end of the human 11β-HSD-2 cDNA. One of the 5 recombinant phage exhibited an extensive hybridization signal. A 1.2 kb Not I-Hind III fragment was subsequently isolated and subcloned into a pBluescript SK vector for DNA sequencing analysis.

To determine the transcription initiation site(s) of the baboon 11β-HSD-1 gene, primer extension analysis was performed using total RNA from adult baboon liver annealed with an antisense primer located at the 3' end of exon I (ACCTGGTCTGAATTCCTATATG). The location of the extended products was determined by parallel genomic sequencing reactions with the same primer. Two extended products 93 nucleotides apart were observed in baboon liver RNA but not in yeast transfer RNA indicating that two transcriptional start sites are present in baboon liver (Figure 19; A; upstream start site; B; downstream start site). The nucleotide sequence of the baboon 11β-HSD-1 and comparison with that for the published sequence in the human (Tannin *et al.*, 1991) is outlined (Figures 20 and 21). The nucleotide sequence of the cloned fragment (approximately 1737 bp) contains 440 bp of the 5'-flanking region, 183 bp of exon I (from the upstream transcription initiation site), 829 bp of intron I, 131 bp of exon II and 129 bp of intron II. Approximately 25 bp in the middle of intron I was not sequenced due to significant internal secondary structure. Repeated attempts to sequence this region via both manual and automated means were unsuccessful. DNA sequence comparison analysis confirmed a high degree of homology between the baboon and human 11β-HSD-1 in exons I and II (>95%) and in the proximal promoter (>98%). Overall similarity between the genes of these two species was >95%.

In order to verify the two transcriptional start sites of baboon 11β-HSD-1, as well as to compare the level of mRNA transcribed, ribonuclease protection assays were performed. Figure 22 shows the autoradiogram (Panel A) used to calculate a standard curve for the reference RNA (Panel C) and the two protected hybrids (Panel B) from

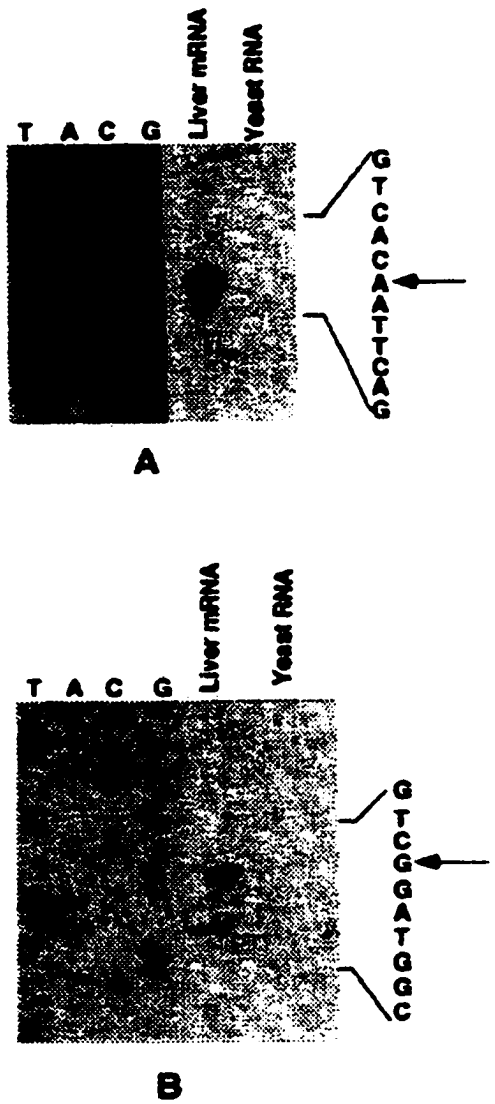

Figure 19. Mapping of the transcriptional start sites of baboon 11β-HSD-1 by primer extension of baboon liver RNA. Primer extension was performed using a 22 bp oligodeoxynucleotide probe complementary to nucleotides +164 to +185 of the 5'-flanking region. The arrow in panel A marks the upstream transcription start site; the arrow in Panel B marks the downstream transcription start site.

```
B   AAATGGTGTTAAGAGTAAGATGGACTtGGGTAGGGATGCTCAGGAATCCAGTCCTGT -194
H   AAATGGTGTTAAGAGTAAGATGGACTcGGGTAGGGATGCTCAGGAATCCAGTCCTGT -192

B   ACAGTCATGAGCTTGGCCATCTGGAAGTCTCCTCTTGCTCAATGAAATGGAGTAAAC -137
H   ACAGTCATGAGCTTGGCCATCTGGAAGTCTCCTCTTGCTCAATGAAATGGAGTAAAC -135

B   ATTGTCCATTATGAAATCCACCACACAGGCTGCCAGGgACGAATGGGATCCCACCCA -80
H   ATTGTCCATTATGAAATCCACCACACAGGCTGCCAGG-ACGAATGGGATCCCACCCA -79

B   AAGCCAATCGCTGCTCTGACAGGGAAATTGGCTAGCACTGCCTGAGACTACTCCAGC -23
H   AAGCCAATCGCTGCTCTGACAGGGAAATTGGCTAGCACTGCCTGAGACTACTCCAGC -22

                    EXON I
B   CTCCCCCcGTCCCTGATGTCACAATTCAGAGGCTGCTGCCTGCTTAGGAGGTTGTAG +35
H   CTCCCCC-GTCCCTGATGTCACAATTCAGAGGCTGCTGCCTGCTTAGGAGGTTGTAG +35

B   AAAGCTCTGTAGGTTCTCTCTGTGTGTCCTACAGGAGTCTTCAGGCCAGCTCCCTGT +92
H   AAAGCTCTGTAGGTTCTCTCTGTGTGTCCTACAGGAGTCTTCAGGCCAGCTCCCTGT +92

B   CGG ATGGCTTTTATGAAAAAATATCTCCTCCCCATTCTGGGGCTCTTCATGGCCTA +148
H   CGG ATGGCTTTTATGAAAAAATATCTCCTCCCCATTCTGGGGCTCTTCATGGCCTA +148

                                        INTRON I
B   CTACTACTATTCTGCAtAtGAGGAATTCAGACCAG GTAAGTACC- 713+25bp - +192
H   CTACTACTATTCTGCAaAcGAGGAATTCAGACCAG GTAAGTACC--- 727bp -- +192

B   TAACCTTTAAGTTACAAATTGaGATAAGCcTGCCTATATCCAGAGAGGGAGAAGGAA
H   TAACCTTTAAGTTACAAATTGcGATAAGCaTGCCTATATCCAGAGAGGGAGAAGGAA

                                                      EXON II
B   TTTTGCTGCCAACTTGGGTATGGTCCTCACTTCCTTTTGGgGTTCCtCAG AGATGCT
H   TTTTGCTGCCAACTTGGGTATGGTCCTCACTTCCTTTTGGaGTTCCcCAG AGATGCT

B   CCAAGGAAAGAAAGTGATTGTCACAGGGGCCAGCAAAGGGATCGGAAGAGAGATGGCT
H   CCAAGGAAAGAAAGTGATTGTCACAGGGGCCAGCAAAGGGATCGGAAGAGAGATGGCT

B   TATCATCTGGCGAAGATGGGAGCCCAcGTGGTGGTaACAGCGAGGTCAAAAGAAACTC
H   TATCATCTGGCGAAGATGGGAGCCCAtGTGGTGGTgACAGCGAGGTCAAAAGAAACTC

            INTRON II
B   TACAGAAG GTGAGGGTTCcATGCcCGCAGATAcaTGTACCcTCACATGCcCAGATGT
H   TACAGAAG GTGAGGGTTCtATGCtCGCAGATAtgTGTACCgTCACATGCtCAGATGT

B   GTTCTTATATATGCTCACATATACACAGAAGCTAGCATATCGCAGATC---
H   GTTCTTATATATGCTCACATATACACAGAAGCTAGCATATCGCAGATC---
```

Figure 20. Sequence of the baboon 11β-HSD-1 promoter. The nucleotide sequence of the 5'-flanking region of the 11β-HSD-1 gene in the baboon (B) and human (H) were aligned by GCG computer program. The transcriptional start sites are shown as a bent arrow. The CAAT boxes, the putative TATA box and putative glucocorticoid (-197 to -190) and estrogen-like (+60 to +65) response elements are underlined. The numbering is relative to the upstream transcription site; lower case symbols indicate a nucleotide difference between the two species.

```
ATTTTGCTGT TTTGTGCTGT TATTTTTAAT TGCTTCCGAC TGGGTAGGGG AGTAGGGGGA CTGAAAGTGA -371

GATTTGGCTT GAGTTTGGCT CTCTTTGCTA TTACTCACAT TTCCCCCCAG AAGCCCTACA TGCACTCCTC -301

TCTCTCTGTC TTTGACAAAT CACTTTTGAA AGATCATTGA TCCCTGGCGT AAATGGTGTT AAGAGTAAGA -231

TGGACTTGGG TAGGGATGCT CAGGAATCCA GTCCTGTACA GTCATGAGCT TGGCCATCTG GAAGTCTCCT -161

CTTGCTCAAT GAAATGGAGT AAACATTGTC CATTATGAAA TCCACCACAC AGGCTGCCAG GGACGAATGG -91

GATCCCACCC AAAGCCAATC GCTGCTCTGA CAGGGAAATT GGCTAGCACT GCCTGAGACT ACTCCAGCCT -21
                                  EXON I
CCCCCCGTCC CTGATGTCAC AATTCAGAGG CTGCTGCCTG CTTAGGAGGT TGTAGAAAGC TCTGTAGGTT +50

CTCTCTGTGT GTCCTACAGG AGTCTTCAGG CCAGCTCCCT GTCGGATGGC TTTTATGAAA AAATATCTCC +120
                                              MetAl aPheMetLys LysTyrLeuL

                                                                    INTRON I
TCCCCATTCT GGGGCTCTTC ATGGCCTACT ACTACTATTC TGCATATGAG GAATTCAGAC CAGGTAAGTA +190
euProIleLe uGlyLeuPhe MetAlaTyrT yrTyrTyrSe rAlaTyrGlu GluPheArgP roG

CCCATGTGTC TCACTTTGGA GGAATAGGTT TTAAAAACAC AGGGGTGCTT AGTGTTCCTG AGGACCAAGA +260

TGGTTCTTGA TCCTCAAAGT TGGTGAAAAT GAGGGAACCC TGAAGTAAAA CAGTACTTTT GTGTCCTCCA +330

GCTACAGGCT GTGGGCAGGG GGGAATGTGT AGGGAGGGTA ATCGTGTAGC CAAAATCTTG CCAAATAGGC +400

CATGCTTTAT TTGGCACCCT TAATGGAAAG CAATTTCAAA TATTCTACAA AAATGAGAGT AATACCCAAA +470

TTCACAACTC AGCCAGCTAA TTGGGCTCTA TGAAGAGACT GATAAGTGGG AGGCAAAGAA AAAAAAAAAT +540

CTCTGGAGAT GATGACTACA TAGAAGTGAG GGAAAATCCC TGGAATTTCT ATGAACACAT GTTGTTTTGA +610

TTCCTTGTTA AATATACTTT CAATTGGCAA AACTGAAAGA GTCCAGAAAT GTTCTGGAAC TCCA-25bp-

TGGAGCAAAA TGAAAGGATC CAGAAATTTT GGGAATTCAG CATTTTAGGT GGAACCATGT ATGAATTTCT

GGGCTTCATC CTATGAATTT CTTTCCATGG GAGGTTTTTA GGAAATGACA GTAGGGAGGC TGTCAGCCCA

AAACTTCCCA ATTCAGAGCA TGTTTATGAA AGACGTGGCC TGTTCCCAAC AGTGATTTAA CAACAGTTTG

TGACAAACTG ATCTGGGCTC ATAACCTTTA AGTTACAAAT TGAGATAAGC CTGCCTATAT CCAGAGAGGG
                                                                  EXON II
AGAAGGAATT TTGCTGCCAA CTTGGGTATG GTCCTCACTT CCTTTTGGGG TTCCTCAGAG ATGCTCCAAG
                                                                  lu MetLeuGlnG

GAAAGAAAGT GATTGTCACA GGGGCCAGCA AAGGGATCGG AAGAGAGATG GCTTATCATC TGGCGAAGAT
lyLysLysVa lIleValThr GlyAlaSerL ysGlyIleGl yArgGluMet AlaTyrHisL euAlaLysMe
                                                 INTRON II
GGGAGCCCAC GTGGTGGTAA CAGCGAGGTC AAAAGAAACT CTACAGAAGG TGAGGGTTCC ATGCCCGCAG
tGlyAlaHis ValValValT hrAlaArgSe rLysGluThr LeuGlnLys

ATACATGTAC CCTCACATGC CCAGATGTGT TCTTATATAT GCTCACATAT ACACAGAAGC TAGCATATCG

CAGATCCGAA TTCTTCGCCC TATAGTGAGT CGTATTAC
```

Figure 21. DNA sequence of the baboon 11β-HSD-1 gene. A 1.7 Kb Hind III/Not 1 fragment containing the 5'-flanking region, Exon 1, Intron 1, Exon II, and Intron II of the baboon 11β-HSD-1 gene was sequenced using the dideoxy nucleotide chain termination method. The transcriptional start sites are shown as a bent arrow. The CAAT boxes, the putative TATA box, and the putative glucocorticoid (-197 to -190) and estrogen-like (+60 to +65) response elements are underlined. The numbering is relative to upstream transcriptional start site of the baboon 11β-HSD-1 gene.

extracts of adult liver and near term baboon placenta. As can be seen, solution hybridization-RNase protection confirmed that 2 transcriptional start sites were utilized in the near term baboon placenta as well as in adult liver. In the liver, however, transcripts emanating from the upstream start site were more than 6-fold greater than those originating from the downstream transcription start site (Figure 22, Panel D). In contrast, in the placenta, transcripts originated primarily from the downstream start site.

To determine the transcription initiation site(s) of the baboon 11β-HSD-2 gene, primer extension analysis was performed using total RNA from adult baboon kidney and placenta annealed with two antisense primers located at the 3' end of exon I (311-332 and 207-229 bases upstream from the 3' end of exon I respectively). The location of the extended products was determined by parallel dideoxynucleotide DNA sequencing reactions. When the first antisense primer (311-332) was used, two major extended products, which migrate at approximately 219 nt and 244 nt, and a minor extended band migrating at 235 nt were observed both in baboon kidney and placenta RNA, but not in yeast transfer RNA, indicating that multiple transcriptional start sites are present in baboon kidney and placenta (Figure 23). To verify this observation, another antisense primer (207-229) 82 bases downstream from the first one was used for primer extension analysis. Again three extended bands of identical size were observed confirming multiple transcriptional initiation sites in the baboon 11β-HSD-2 gene. The nucleotide sequence of the cloned fragment (1,220 bp) of baboon 11β-HSD-2 contains 167 bp of the 5'-flanking region, 554 bp of exon I, and 499 bp of intron I (Figure 24). DNA sequence comparison analysis confirmed a high degree of homology between the baboon and human 11β-HSD-2 (Agarwal *et al.*, 1995) in exon I (96%) and in the proximal promoter (>90%). Overall similarity between the genes of these two species was >95%.

The expression of 11β-HSD-1 in different baboon tissues was assessed by Northern blot analysis using 30 μg total RNA prepared from adult heart and liver, liver from a fetus delivered at mid-gestation and from whole villous placenta of an animal delivered

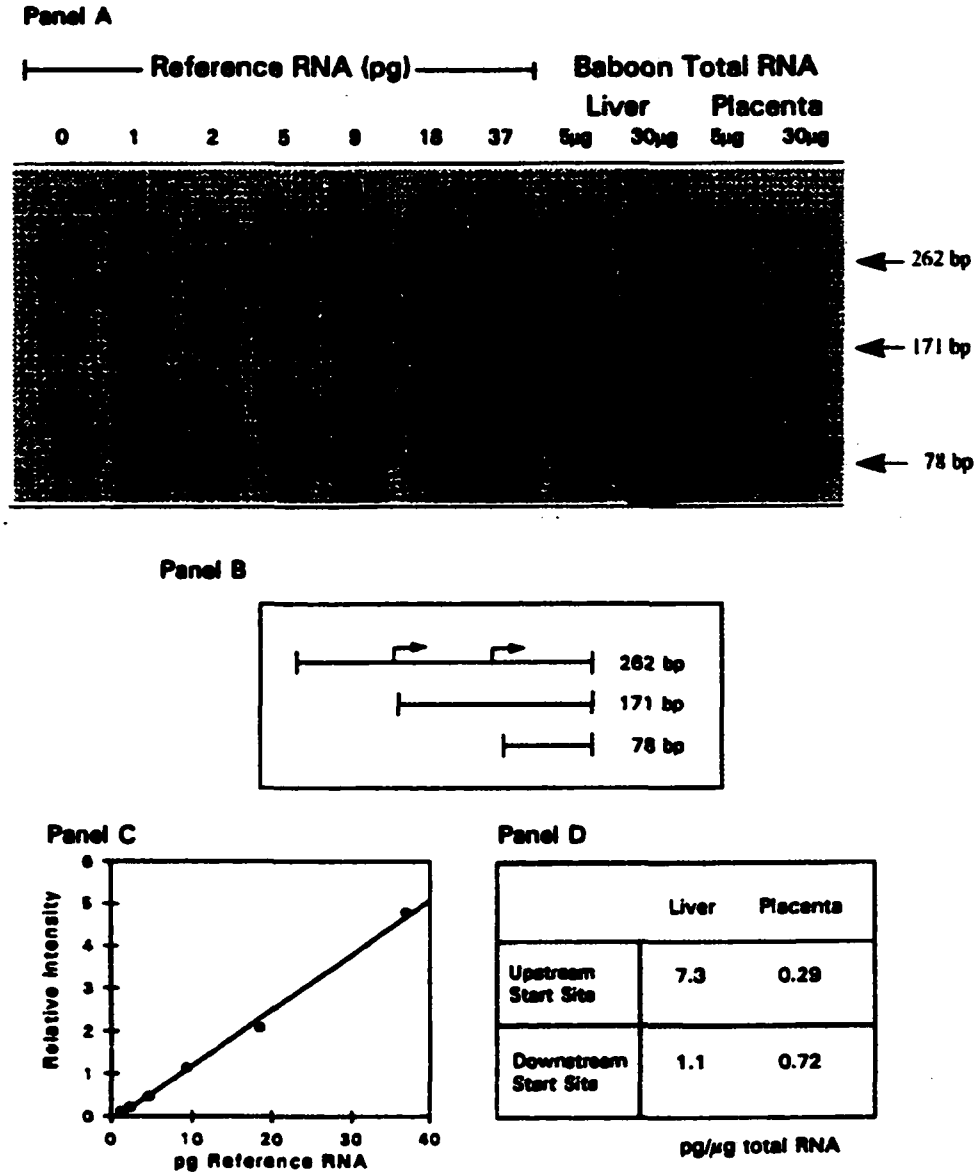

Figure 22. Mapping and quantification of the upstream and downstream transcriptional start sites of the baboon 11β-HSD-1 gene by ribonuclease protection assay. Panel A: Autoradiogram of a ribonuclease protection assay of total RNA from baboon liver and term placenta hybridized with a radiolabeled 262 bp probe. The mRNA protected from the upstream and downstream start sites are shown as protected bands of 171 bp and 78 bp, respectively. The standard reference (sense) RNA is shown as a protected 262 bp hybridized product. Panel B: The scheme of the 262 bp 5'-flanking region of 11β-HSD1 gene. The transcriptional start sites are shown as bent arrows and the protected sequence emanating from the upstream start site shown as a 171 bp product and that from the downstream start site as a 78 bp product. Panel C: Standard curve generated from the autoradiogram in Panel A. The relative density of the reference product plotted against the pg of reference added. Panel D: Levels (pg/µg total RNA) of the mRNA transcribed from the upstream and downstream transcription start sites in baboon liver and placenta.

near term. The mRNA for 11β-HSD-1 was detected as a single species of approximately 1.5 kb in adult liver and term placenta, but not fetal liver or adult heart (Figure 25). In all samples, β-actin mRNA was detected as a strong band of 2.0 kb (data not shown) indicating that our failure to find the 11β-HSD-1 mRNA in fetal liver and adult heart reflected minimal 11β-HSD-1 gene expression and not RNA degradation. This distribution of 11β-HSD-1 expression is similar to that seen in the human (Tannin *et al.*, 1991), sheep (Yang *et al.*, 1992) and rat (Moisan *et al.*, 1990) and confirms our previous observations (Pepe *et al.*, 1996b) demonstrating abundant expression of 11β-HSD-1 mRNA in baboon placenta, decidua and adult liver but not fetal liver of mid-gestation.

The expression of 11β-HSD-2 in different baboon tissues was assessed by Northern blot analysis using 30 μg total RNA prepared from adult heart, kidney and liver, as well as fetal liver and kidney and whole villous placenta of an animal delivered near term. The mRNA for 11β-HSD-2 was detected as a single species of approximately 1.9 kb in adult and fetal kidneys and near term placenta, but not adult and fetal liver or adult heart (Figure 26). The hybridization signal appeared highest in the adult kidney and lowest in the fetal kidney. In all samples, β-actin mRNA was detected as a strong band of 2.0 kb (1.8 kb in heart) indicating that absence of 11β-HSD-2 mRNA expression in fetal liver and adult heart and liver reflected minimal 11β-HSD-2 gene expression and not RNA degradation. This distribution of 11β-HSD-2 expression is similar to that seen in the human (Agarwal *et al.*, 1995) and sheep (Agarwal *et al.*, 1994) and confirms our previous observations (Pepe *et al.*, 1996a) demonstrating abundant expression of 11β-HSD-2 mRNA in baboon placenta.

The promoters of both 11β-HSD-1 and -2 were activated following transient transfection into JEG-3 cells (Figure 27 and Figure 28, Panel A). In the independent experiments performed, basal activity of the 11β-HSD-2 promoter (87 ± 21 RLU/μg protein) always exceeded ($P<0.05$) that of 11β-HSD-1 (37 ± 7) by approximately 2-fold ($n=5$). Similar promoter activation patterns ($P<0.05$) were demonstrated in 293

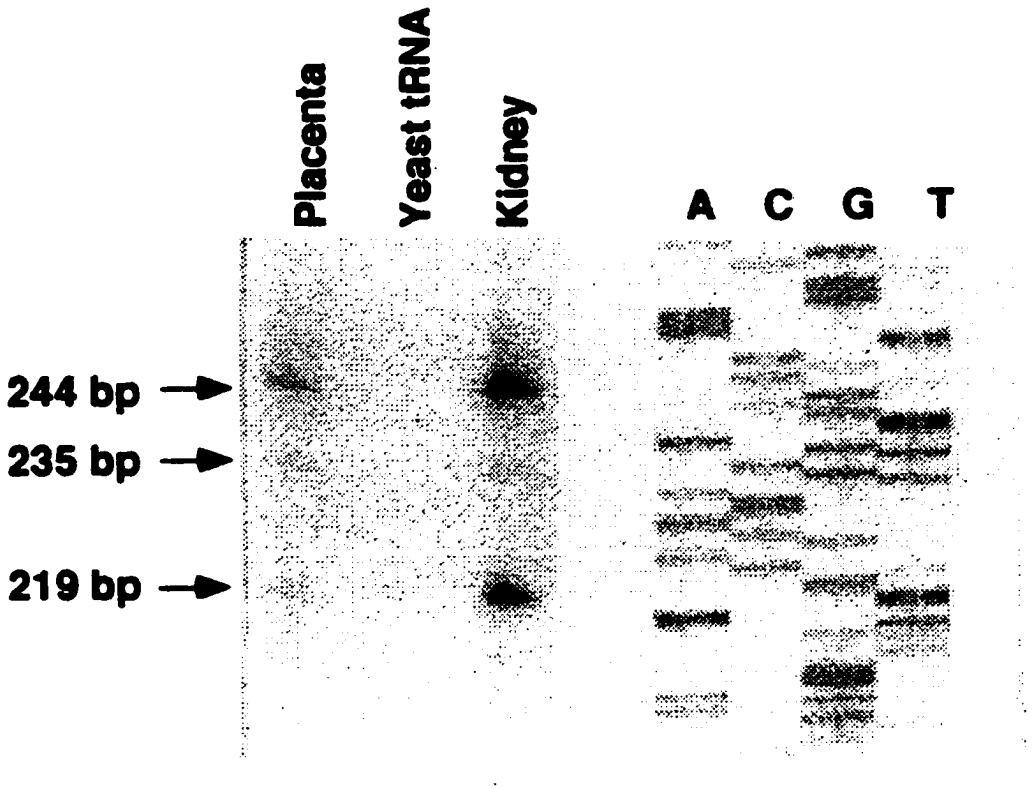

Figure 23. Mapping of the transcriptional start sites of baboon 11β-HSD-2 by primer extension of baboon placental and adult kidney total RNA and yeast tRNA. Primer extension was performed using a 22 base oligodeoxynucleotide probe complementary to nucleotides 311 to 332 bp away from the 3' end of Exon I. The arrows mark the transcription start sites.

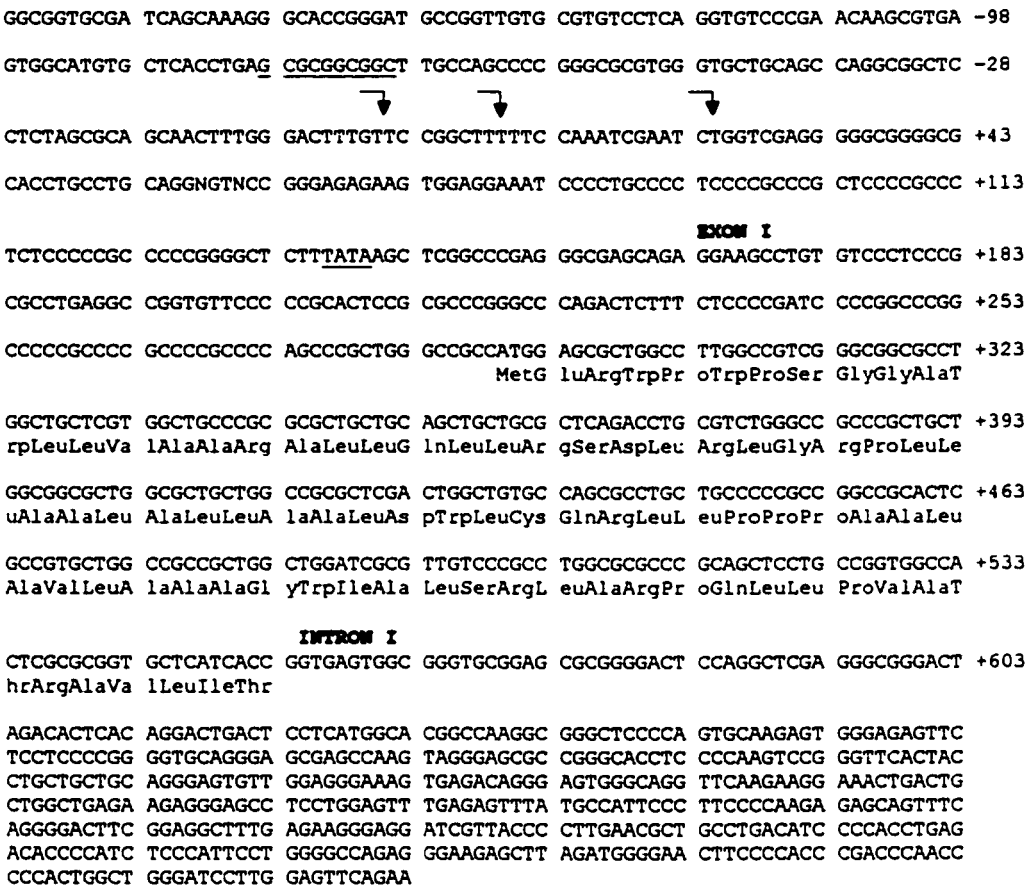

Figure 24. DNA sequence of the baboon 11β-HSD-2 gene. A 1.2 Kb Not I/Hind III fragment containing the 5'-flanking region, Exon 1 and Intron 1 of the baboon 11β-HSD-2 gene was sequenced using the dideoxynucleotide chain termination method. The transcriptional start sites are shown as bent arrows. The ATG codon, TATA box and putative GC box are underlined.

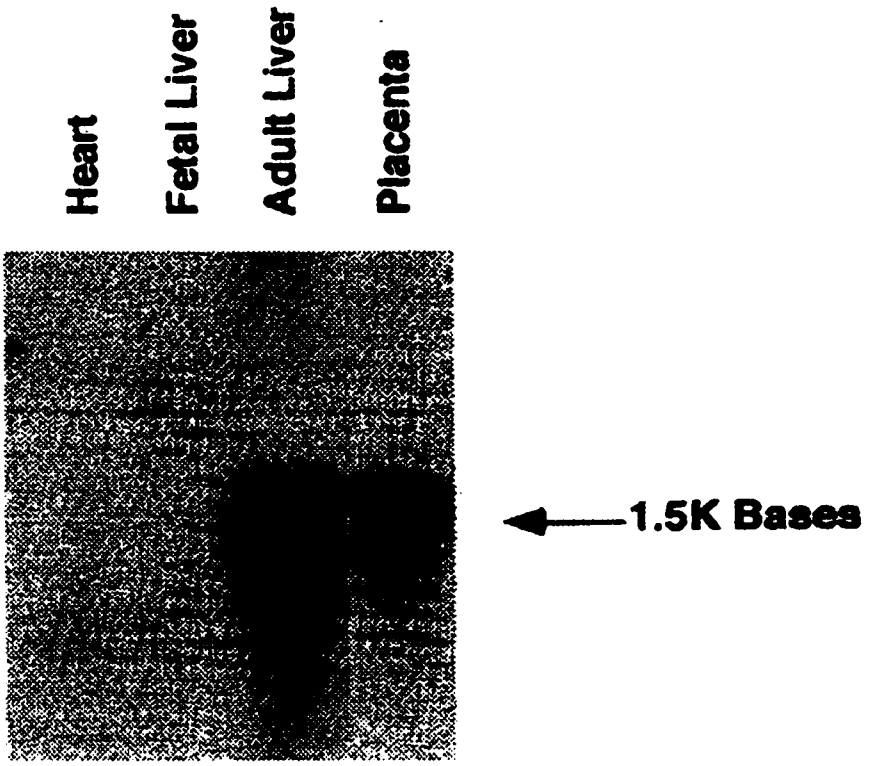

Figure 25. Northern blot analysis of 11β-HSD-1 mRNA expression in baboon term placenta, adult liver, adult heart and fetal liver of midgestation. Total RNA was isolated and probed with a human cDNA to 11β-HSD-1.

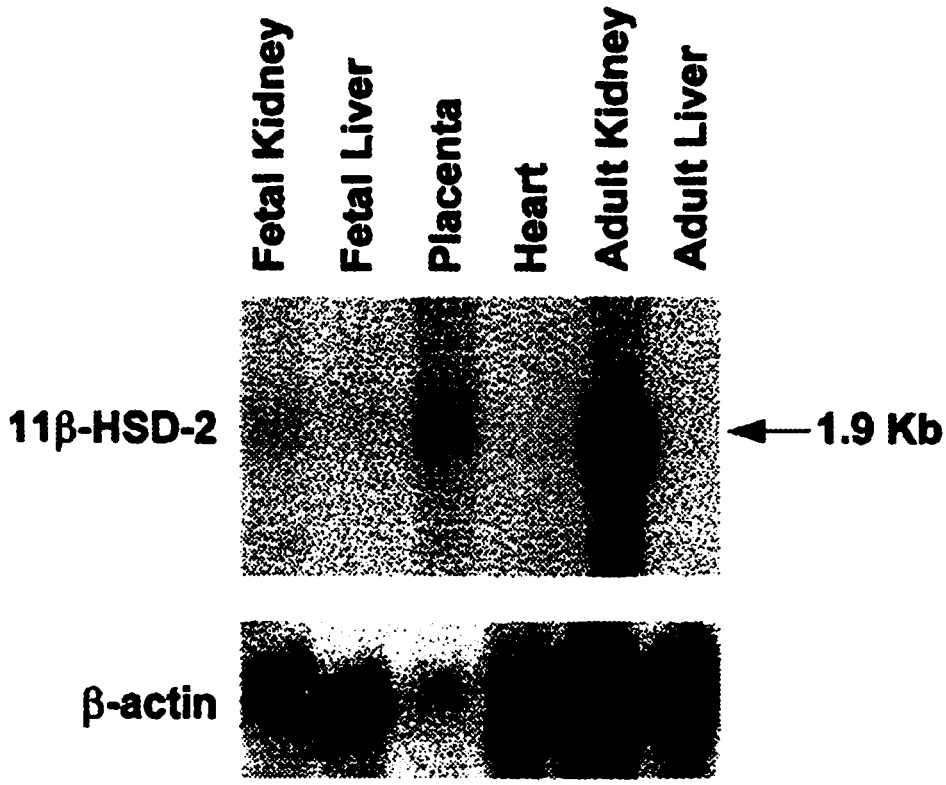

Figure 26. Northern blot analysis of 11β-HSD-2 mRNA expression in baboon term placenta and adult liver and adult heart and near term fetal liver and kidney. Total RNA was isolated and probed with a human cDNA to 11β-HSD-2 and then to β-actin.

embryonic kidney cells (1344 ± 810 RLU/μg protein for type 2 and 280 ± 139 RLU/μg protein for type 1; n=3) and Chang human adult liver cells (420 ± 381 RLU/μg protein for type 2 and 64.3 ± 35.6 RLU/μg protein for type 1; n=3; $P<0.01$). In three tissue culture cell lines, luciferase expression utilizing the promoter-less pGL3-Basic vector was minimal (13 ± 4 in JEG-3, 23 ± 0.5 in 293 and 9 ± 4 in HEP-G2; activity in Chang was not done; Figure 27). Repeated attempts (n=3) to transiently transfect HEP-G2 human adolescent liver hepatoblastoma cells with $CaPO_4$-precipitated-DNA were unsuccessful. In Panels B and C of Figure 28, the effects of estradiol in the presence or absence of a cotransfected ERα on 11β-HSD-1 and -2 in a representative of three experiments is described. As indicated, in the absence of cotransfected ERα, basal promoter activities of both 11β-HSD-1 and -2 were not altered by 17β-estradiol. Although the promoter activities of 11β-HSD-1 and -2 were enhanced by approximately 2-fold in cells co-transfected with pSG5ER alone, these increases were not statistically significant. However, in cells co-transfected with the ERα, incubation with 17β-estradiol, but not 17α-estradiol, increased ($P<0.05$) promoter activity of 11β-HSD-1 (177 ± 12) and 11β-HSD-2 (1309 ± 203) by approximately 4-fold over respective values in cells incubated with no steroid (or with 17α-estradiol). These results are presented in Panels D and E (Figure 28), as the fold induction of basal promoter activity, which was designated as 1.

Thus, in the presence of a co-transfected ERα, 17β-estradiol but not 17α-estradiol stimulated ($P<0.05$) the basal activities of the 11β-HSD-1 and 11β-HSD-2 promoters by 8.1 ± 1.5 and 8.3 ± 2.0 fold respectively, values which also exceeded ($P<0.05$) those in cells transfected with the ERα and incubated with no steroid or with 17α-estradiol. Basal activity of the promoterless pGL3 Basic vector (12 ± 3 RLU/μg protein; n = 3) was not

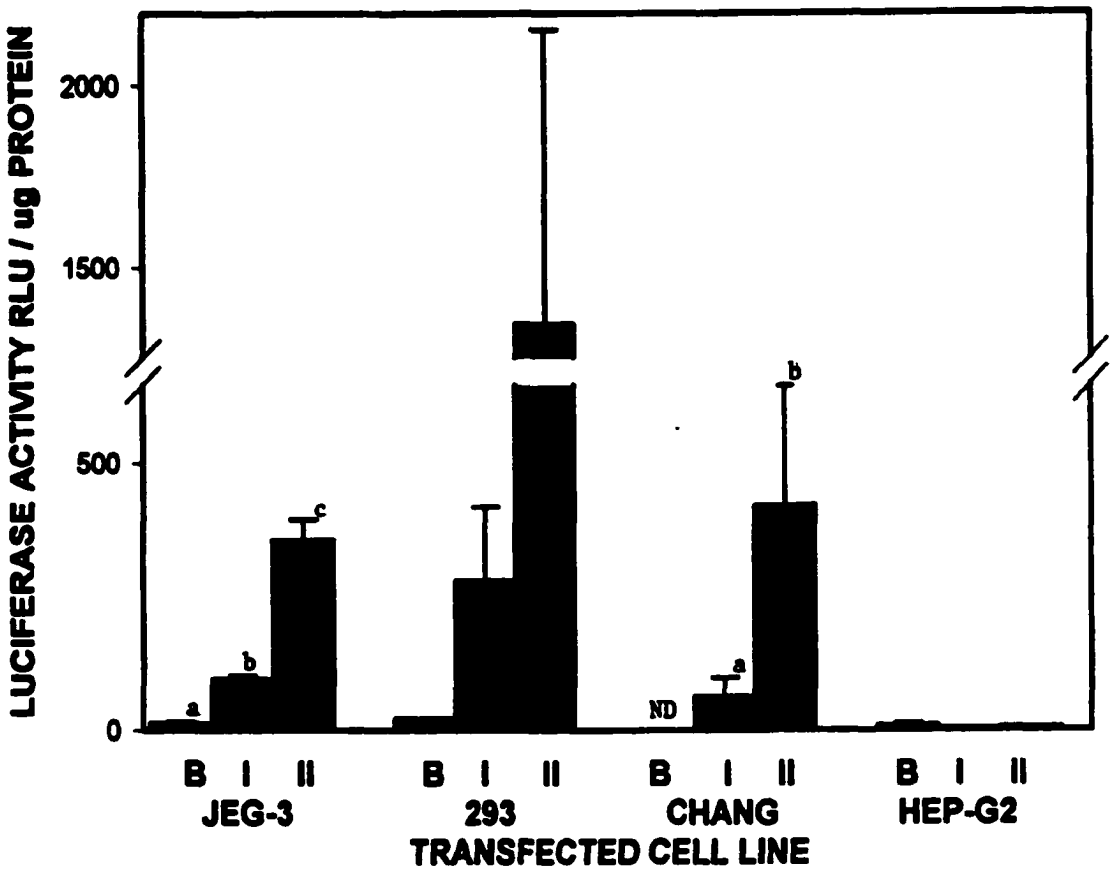

Figure 27. Luciferase activity in JEG-3 human choriocarcinoma cells, 293 human embryonic kidney cells, Chang human adult liver cells and HEP-G2 human adolescent liver hepatoblastoma cells of the 1.7 kb baboon 11βHSD-1/luciferase construct (I) and the 1.2 kb baboon 11βHSD-2/luciferase construct (II) after $CaPO_4$–precipitated-DNA transfection. Values (mean ± SE) expressed as luciferase promoter activity RLU / μg cell protein and were determined 18 h after incubation of constructs in cells (n = 4). Basic luciferase activity was determined following transient transfection under similar conditions with the promoter-less pGL3-Basic luciferase reporter vector (B). Values (mean ± SE) with different letter superscripts differ from each other at P=0.01 (by one-way ANOVA). Basic luciferase was not done in Chang cells.

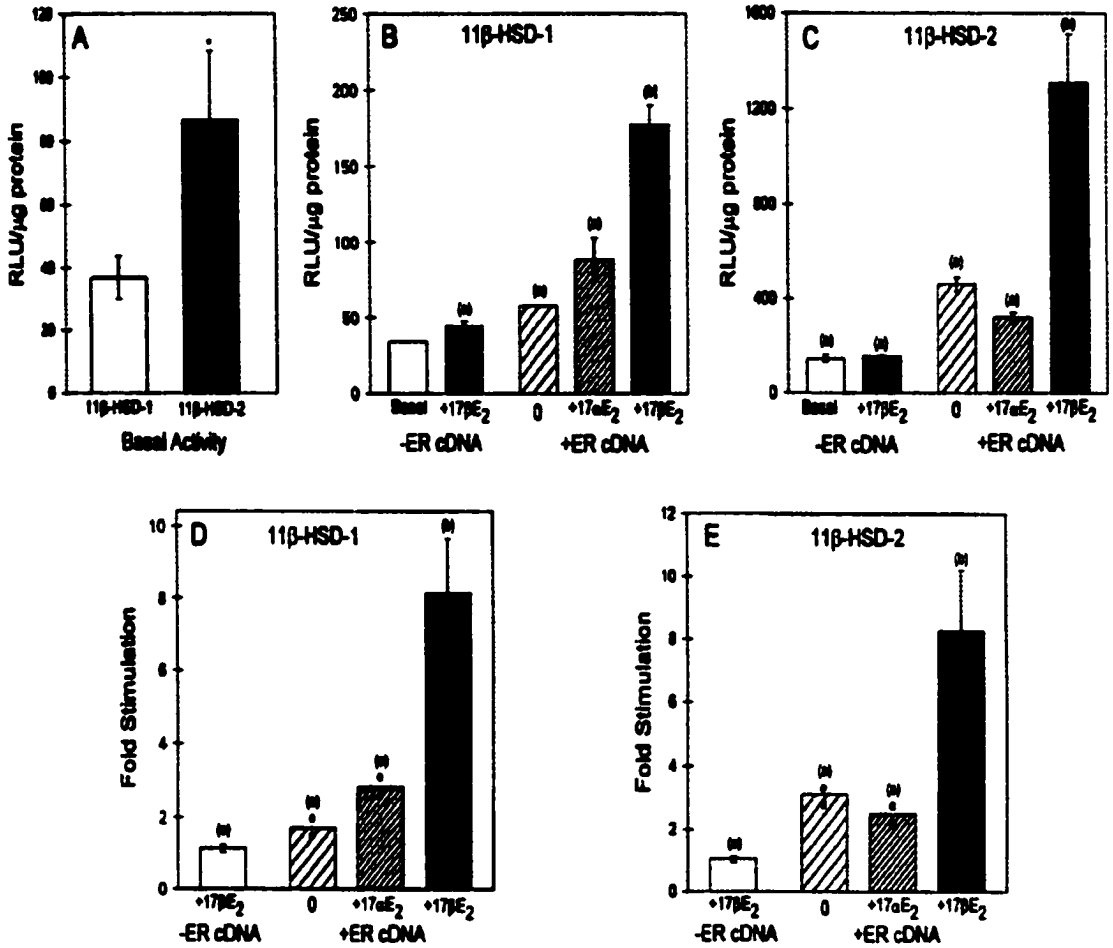

Figure 28. Promoter activity of the 1.7 and 1.2 kb fragments of the 5'-flanking regions of baboon 11β-HSD-1 and -2 subcloned into a luciferase promoter vector (pGL3) and transfected without (-) or with (+) a human estrogen receptor (ERα) cDNA (pSG5ER) into JEG-3 cells. Luciferase activity expressed as relative light units (RLU)/µg cell protein was determined 18 h after incubation of cells without or with 1 x 10^{-8} M 17β-estradiol (17β-E_2) or 17α-estradiol (17α-E_2). (A) Basal promoter activities corrected for luciferase activity in cells co-transfected with a promoterless pGL3 vector. Asterisk indicates values (mean ± SE of four experiments) differ at $P < 0.05$; paired *t*-test. (B, C) Effects of estradiol alone or in the presence of a co-transfected ERα on 11β-HSD-1 (B) and 11β-HSD-2 (C) promoter activities. Values (mean ± SE) of cell replicates (n = 2-5) in one representative experiment. (D, E) The effect of estradiol in the presence or absence of a co-transfected ERα on 11β-HSD-1 and 11β-HSD-2 promoter activities. Values expressed as the fold induction (mean ± SE of 3 experiments; where indicated, n=2) of basal promoter activity (Panel A) which was designated as 1. In panels B-E, values with different letter superscripts differ from each other at $P < 0.05$ (ANOVA; Neuman-Keuls multiple comparison test).

altered by 17β-estradiol in the presence or absence of a cotransfected ERα (data not shown).

Additional studies into the activation of the 11β-HSD-1 and 11β-HSD-2 promoters were undertaken using CPT-cAMP in JEG-3 cells. Initial studies demonstrated a linear increase in luciferase expression as CPT-cAMP increased from 5 μM to 50 μM (data not shown). It was decided to use 50 μM CPT-cAMP in subsequent treatments. As shown (Figure 29), CPT-cAMP significantly ($P<0.01$) increased 11β-HSD-1 promoter activation in JEG-3 cells almost 3-fold (2.54 ± 0.21; $n = 8$) over respective values in cells incubated with no CPT-cAMP, whereas 11β-HSD-2 promoter activity increased slightly (1.30 ± 0.07; $n=6$).

Finally, the basal promoter activities of 11β-HSD-1 and –2 with cotransfected steroidogenic factor 1 (SF-1), an orphan nuclear receptor, were investigated. Since SF-1 is expressed in adrenal, ovary, testes and placenta (Parker and Schimmer, 1997), 293 embryonic kidney cells, instead of JEG-3 choriocarcinoma cells, were used to determine the basal effect of SF-1. SF-1 in 293 kidney cells (Figure 30), decreased the basal promoter activity of 11β- HSD-1 by 24% RLU/μg protein ($n=1$) and increased the basal promoter activity of 11β-HSD-2 by 25% RLU/μg protein ($n=1$). The cAMP-stimulated activity of SF-1 was not investigated.

To determine the degree inhibition of luciferase expression by the adenovirus/11β HSD-2 antisense construct, JEG-3 cells were cotransfected with the adenovirus human antisense 11βHSD-2 cDNA construct and $CaPO_4$-precipitated baboon sense 11βHSD-2/luc DNA. As indicated (Figure 31), 20% inhibition ($n=3$) of luciferase activity was demonstrated in JEG-3 placental cells when the antisense and sense constructs were

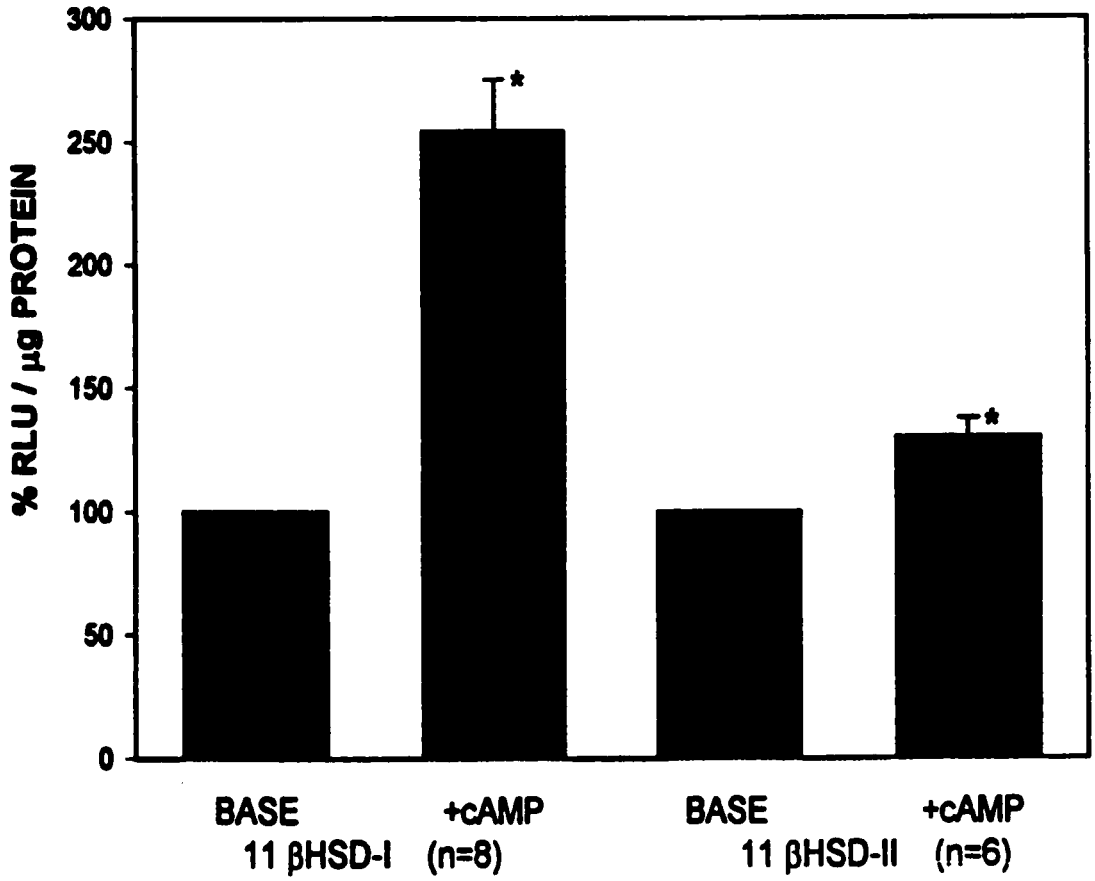

Figure 29. Luciferase activity in JEG-3 cells of the 1.7 kb baboon 11βHSD-1/luciferase construct and the 1.2 kb baboon 11βHSD-2/luciferase construct following treatment with CPT-cAMP (50 µM) 24 hr after $CaPO_4$–precipitated DNA transfection. Values (mean ± SE) expressed as % change in basal (11βHSD-1 or -2/luciferase DNA alone = 100%) luciferase promoter activity RLU / µg cell protein and were determined 18 h after incubation of CPT-cAMP in JEG-3 cells. The *asterisk* indicates that the mean value differs from the respective basal value ($P<0.001$, by unpaired *t*-test).

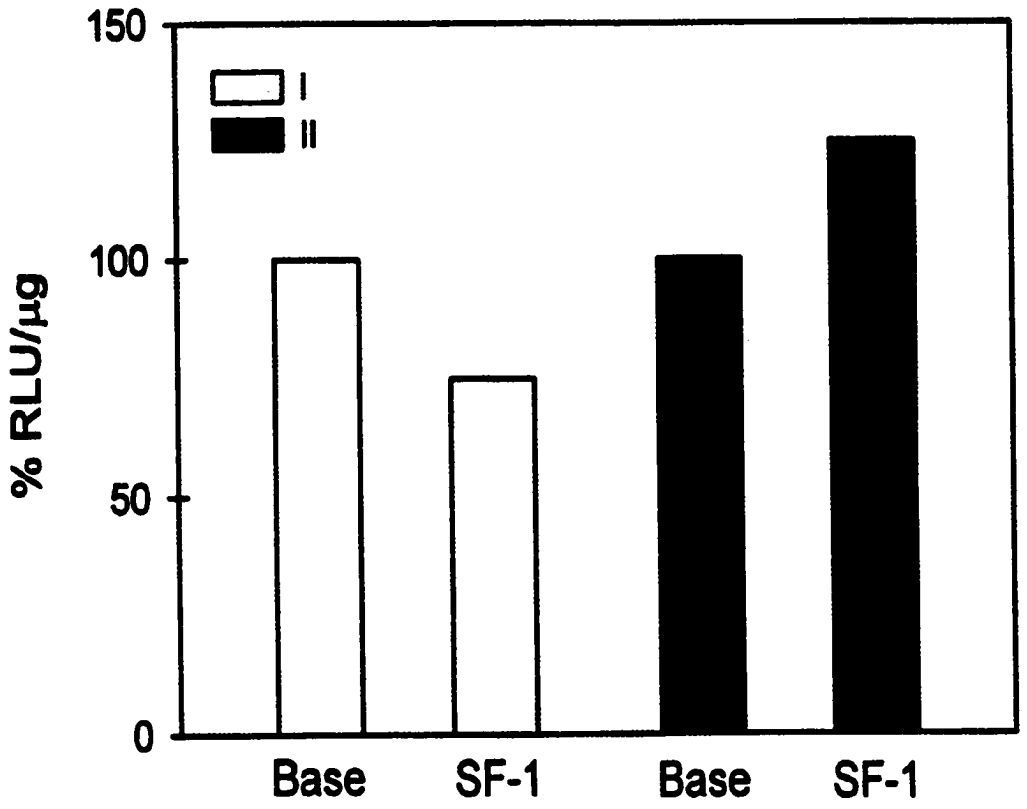

Figure 30. Luciferase activity in 293 embryonic kidney cells of the 1.7 kb baboon 11β-HSD-1/luciferase construct and the 1.2 kb baboon 11βHSD-2/luciferase construct following simultaneous $CaPO_4$-precipitated cotransfection with human cDNA Steroidogenic Factor 1 expression vector (n = 1). Values expressed as % change in basal (11βHSD-1 or -2/luciferase DNA alone = 100%) luciferase promoter activity RLU / µg cell protein and were determined 18 h after incubation of expression vectors in 293 cells.

cotransfected concomitantly. When the adenovirus/11βHSD-2 antisense construct was administered one day prior (in order to allow cellular accumulation of antisense mRNA), 51% inhibition (n=3) of luciferase expression/activity was demonstrated. As evidenced, (Figure 32), there was a consistent (n=4) linear decrease in luciferase expression as the adenovirus/11βHSD-2 antisense construct concentration increased. At the highest virus concentration tested (30×10^9 particles per 60 mm plate at 80% cell confluence), luciferase expression was reduced 51%. The virus particle to cell concentration ranged from approximately 10:1 to 100:1. At high virus concentrations when administered 24 hrs prior to the sense DNA, there was a diminution of total cellular protein by an average of 50% (data not shown). The cells did not lift off the plate but apparently did not replicate during the 72 hour incubation as rapidly as the controls. Even though there was a reduction in total cellular protein at high virus titers, the RLU/μg protein (Figure 32) were still significantly diminished ($P<0.05$). This decrease in total cellular protein did not occur when the adenovirus and sense 11βHSD-2/luc constructs were cotransfected concomitantly (data not shown) perhaps because the virus-infected cells were incubated for a shorter period of time (48 hrs vice 72 hrs). As a control (n = 2), baboon sense 11β HSD-1/luc construct DNA was cotransfected with the adenovirus/11βHSD-2 antisense construct. There was actually a 20% increase in luciferase expression (RLU/μg protein (data not shown). This increase in luciferase expression is most likely attributable to adenovirus enhanced uptake of $CaPO_4$-precipitated DNA. In light of this increased luciferase expression in the control cotransfections, the luciferase inhibition values as evidenced with the antisense gene are particularly striking.

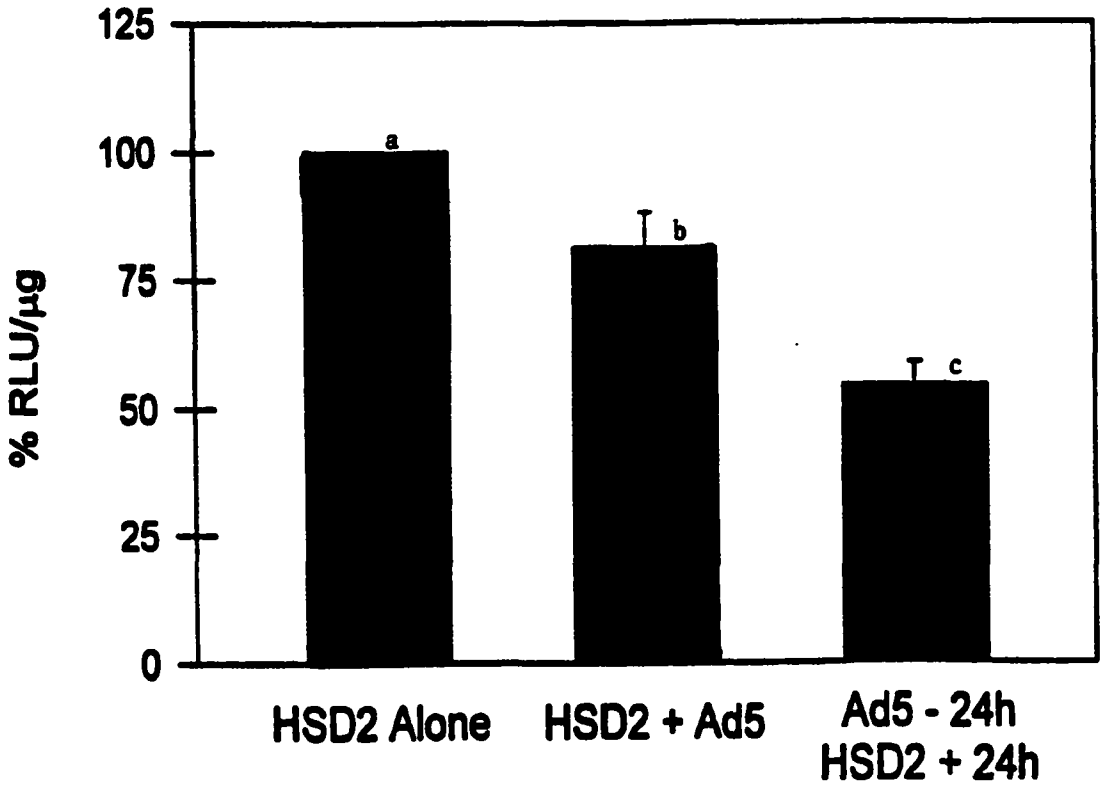

Figure 31. Luciferase activity in JEG-3 cells of the 1.2 kb flanking region of baboon 11β-HSD-2/luciferase construct following simultaneous transfection with adenovirus containing human 11βHSD-2 antisense cDNA and $CaPO_4$-precipitated sense 11βHSD-2/luciferase DNA (n = 3) or when the antisense adenovirus was administered one day prior to the addition of $CaPO_4$-precipitated sense 11βHSD-2/luciferase DNA (n = 3). Values (mean ± SE) expressed as reduction in basal (11βHSD-2/luciferase DNA alone = 100%) luciferase promoter activity RLU / µg cell protein (12K x g cytosol) and were determined 18 h after incubation of cells with adenovirus and baboon 11βHSD-2/luciferase construct. Values (mean ± SE) with different superscripts differ from respective values at $P<0.001$ (by one-way ANOVA).

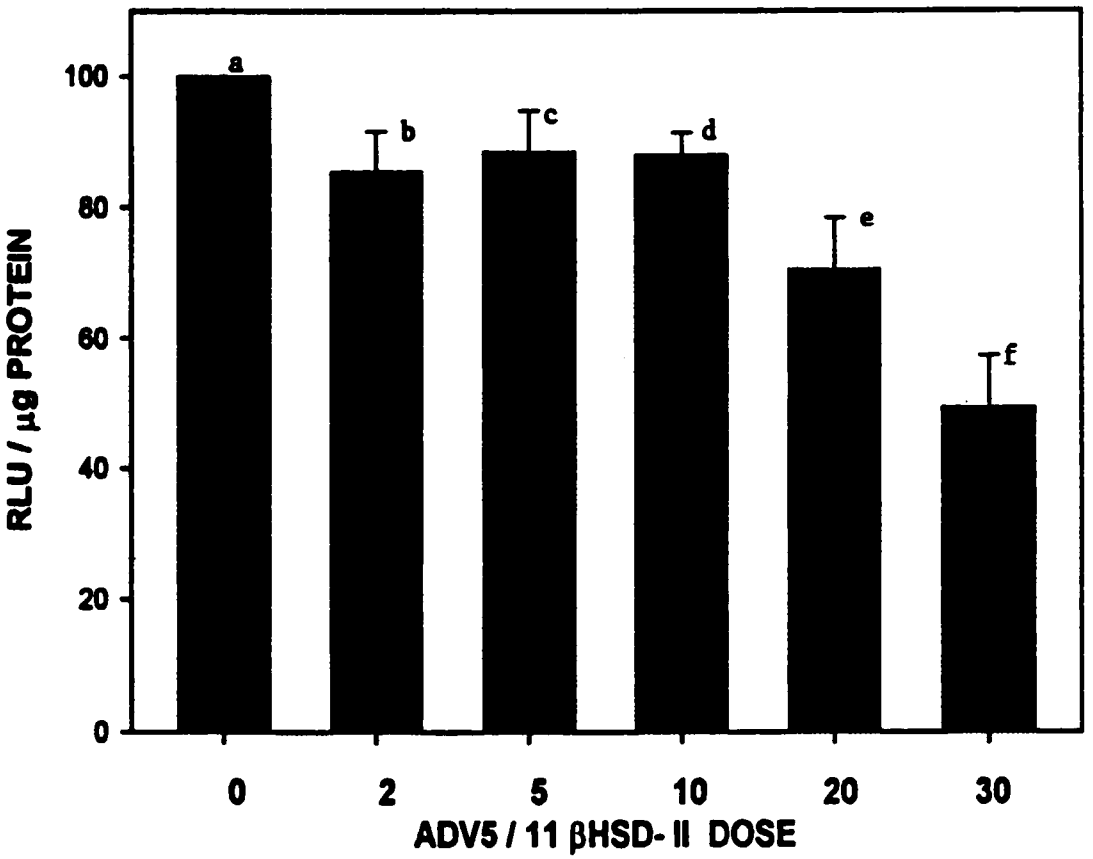

Figure 32. Luciferase activity in JEG-3 cells of the 1.2 kb flanking region of baboon 11β-HSD-2/luciferase construct following concomitant transfection with adenovirus (0 – 30 x 10^9 particles per 60 mm plate at 80% confluence) containing human 11β-HSD-2 antisense cDNA and $CaPO_4$-precipitated sense 11βHSD-2/luciferase DNA. Values (mean ± SE) expressed as reduction in basal (11βHSD-2 antisense cDNA alone = 100%) luciferase promoter activity RLU / µg cell protein (12K x g cytosol) and were determined 18 h after incubation of cells with adenovirus and baboon 11β-HSD-2/luciferase construct (n = 4). Values (mean ± SE) with different superscripts differ from respective values at $P<0.001$ (by one-way ANOVA).

RT-PCR confirmed that estrogen receptor α mRNA was expressed in trophoblast and nontrophoblast cells of the late gestation baboon placenta at levels that appeared lower than in baboon uterus (Figure 33). In placenta, estrogen receptor mRNA expression was lowest in the syncytiotrophoblast.

DISCUSSION

In the present study, a baboon kidney genomic library was screened with a full length cDNA of the human 11β-HSD-1 to isolate and characterize the 5'-flanking region as well as to identify the transcriptional start sites for baboon 11β-HSD -1. Screening of 1.3×10^6 plaques followed by three rounds of plaque purification, yielded seven verified genomic clones which were partially mapped with restriction enzymes and confirmed to contain insert DNA of 12-19 kb which hybridized with the human 11β-HSD-1 cDNA. Additional results indicated that only two clones hybridized to the exon I cDNA fragment and it was the 1,737 bp Hind III-Not I fragment which we subsequently subcloned and sequenced. Overall, the nucleotide sequence of the 5'-flanking region of baboon 11β-HSD-1 analyzed in this study was more than 95% homologous to that in the human (Tannin *et al.*, 1991). This high degree of homology was particularly striking in the region of exons I and II and in the proximal promoter region (>98%). Using baboon liver RNA and a 22 bp primer complimentary to nucleotides +164 to +185 exon I, primer extension studies confirmed that the baboon 11β-HSD-1 gene has two transcriptional start sites 93 nucleotides apart. Similarly, as in the human gene, the 5'-flanking region of the baboon 11β-HSD-1 does not contain a TATA box but there are two consensus CAAT boxes. Because there is no consensus TATA box, this transcriptional start site is presumably regulated by the consensus CAAT box 70 bp upstream from the start of

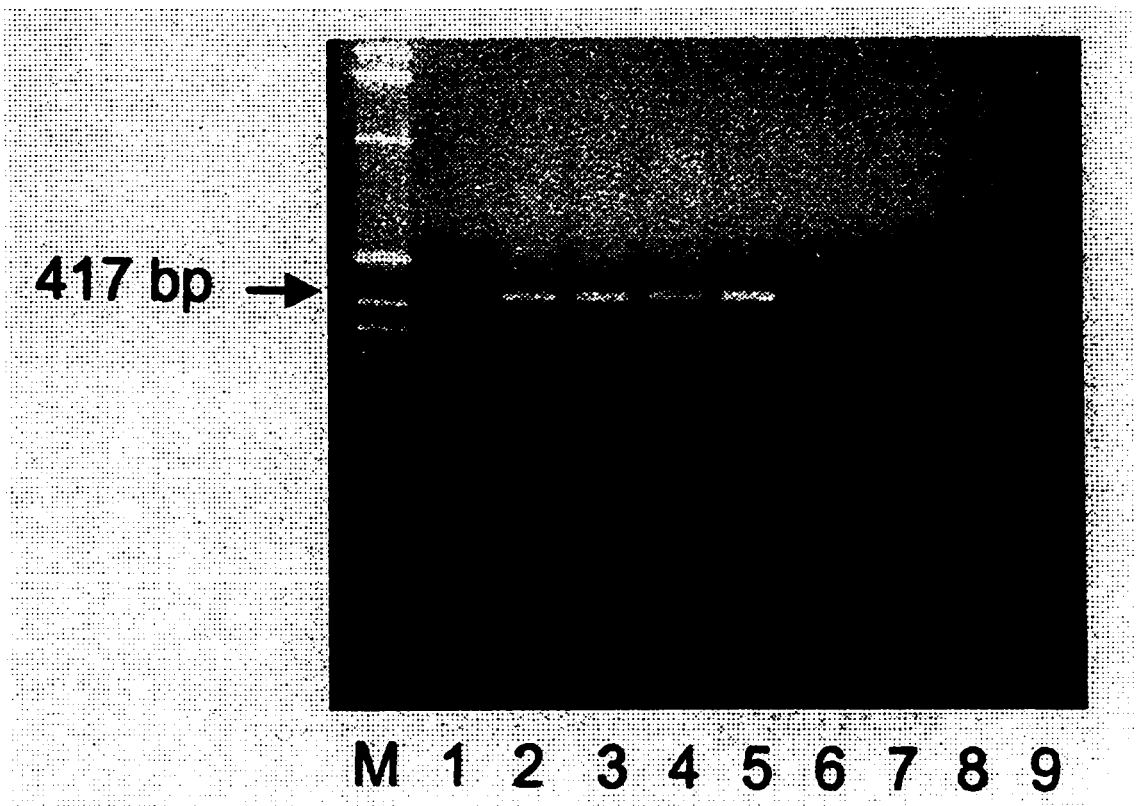

Figure 33. Relative distribution of estrogen receptor (ERα) mRNA in baboon placenta. Total RNA (3 μg) from syncytiotrophoblast (Lane 1), a heterogeneous non-trophoblast cell population (Lane 2), cytotrophoblasts (Lane 3) and whole villous tissue (Lane 4) from a late gestation baboon placenta were reverse transcribed and amplified in the presence of primers specific for ERα. The 417 bp ERα PCR product from nonpregnant baboon uterus (75 ng total RNA) is shown in Lane 5. RT was omitted from the reaction in Lanes 6, 7, and 8. RNA was omitted from the reaction in Lane 9. M = 1kb ladder.

transcription. Significantly, this upstream transcription start site is identical to that of the human 11β-HSD-1 gene in both its sequence and its location (Tannin *et al.*, 1991). The second transcription start site is 93 bases downstream from the first one and this site is two bases up-stream from the translation initiation site for the human/ baboon 11β-HSD-1 gene. This data indicates that the transcripts from both transcription initiation sites may encode the same 11β-HSD-1 protein. Again, there is no TATA box in the up-stream region of the second promotor; rather there is a typical CAAT box 90 base pairs up-stream from the second start site. Our studies also indicated that in the baboon, as in the rat and human (Tannin *et al.*, 1991; Agarwal *et al.*, 1989), there is a TATA box in intron I which may serve to regulate an alternative translation initiation site at Met 31 (Met 27 in the rat) of exon II. Indeed, in the rat kidney it has been demonstrated that as a result of differential promoter utilization, multiple 11β-HSD-1 mRNAs and presumably proteins are expressed *in vivo* (Krozowski *et al.*, 1990). Whether the utilization of this initiation site plays any role physiologically seems unlikely since transcripts originating in Intron I of the rat 11β-HSD-1 apparently encode a truncated polypeptide(s) that is enzymatically inactive (Obeid *et al.*, 1993). Finally, a glucocorticoid response element and an estrogen-like-response-element (86% homology) were identified in the 5'-flanking region of the baboon 11β-HSD-1 gene as has been reported for the human gene (Tannin *et al.*, 1991), as well as the rat gene (Moisan *et al.*, 1992).

Solution hybridization RNase protection assays confirmed that baboon 11β-HSD-1 mRNA transcripts emanated from both transcription start sites in the near term baboon placenta as well as the liver. We have recently demonstrated that in the baboon placenta as in the human, the protein and mRNA for 11β-HSD-1 are expressed primarily in the

syncytiotrophoblast and not in non-trophoblast cells (Pepe *et al.*, 1996a). Therefore the observation that the relative abundance of mRNA transcripts produced in the adult liver exceeded that produced in the placenta per unit total RNA most likely reflects the fact that nontrophoblast cells comprise a large proportion of the primate placenta. Interestingly, however, our studies are the first to demonstrate that the 11β-HSD-1 mRNA transcripts emanating from the baboon placenta and liver utilize different transcriptional start sites. Thus, mRNA transcripts originating from the upstream initiation start site dominated in the liver whereas in the placenta these transcripts originated primarily from the downstream initiation start site. Since these transcriptional start sites are only 93 bp apart, it remains to be determined using, for example, DNA sequence knock-out studies whether the two transcripts are transcribed from one or two promoters.

The observation that the 11β-HSD-1 promoter exhibits glucocorticoid and estrogen-like response elements would suggest that these hormones play a role in regulating cell-specific expression of 11β-HSD-1. However, the regulatory effects of these two hormones appear extremely complex and highly dependent upon the physiologic status of the animal. For example, in sheep, 11β-HSD-1 mRNA expression is enhanced in adult liver but reduced in fetal liver following glucocorticoid administration *in vivo* (Yang *et al.*, 1994), whereas in the hippocampus, glucocorticoids and chronic-stress up-regulate neural 11β-HSD-1 (Low *et al.*, 1994a). In the rat, hepatic 11β-HSD-1 is down-regulated by estrogen directly and/or via growth hormone (Low *et al.*, 1994b; Low *et al.*, 1993). The hormonal milieu to which a cell is exposed *in vivo* is not only complex but also modified during various physiologic states (i.e., pregnancy). This factor, plus the

observation that the two transcriptional start sites of baboon 11β-HSD-1 are utilized in a tissue-specific manner, suggests that the exclusive utilization of immortalized cell lines for the study of regulation of the 11β-HSD-1 promoter, although valuable, may not permit unequivocal extension of findings to the *in vivo* situation. Thus, cultures of primary cells obtained from animals under varying physiologic situations or following treatment *in vivo* with selected secretogogues may provide a necessary adjunct to elucidate the factors operating to modulate to regulate the cell-specific factors important to the activation of the 11β-HSD-1 gene.

Additionally, a baboon kidney genomic library was screened with a full length cDNA of the human 11β-HSD-2 to isolate and characterize the 5'-flanking region as well as to identify the transcriptional start sites for baboon 11β-HSD-2. Screening of 1.3×10^6 plaques yielded five verified genomic clones that were confirmed to contain insert DNA of 12-19 kb, which hybridized with the human 11β-HSD-2 cDNA. One clone hybridized to the exon I cDNA fragment and it was the 1220 bp Not I fragment which we subsequently subcloned and sequenced. Overall, the nucleotide sequence of the 5'-flanking region of baboon 11β-HSD-2 analyzed in this study was more than 95% homologous to that in the human (Agarwal *et al.*, 1995). This high degree of homology was particularly striking in the regions of exon I and the proximal promoter.

Using RNA from adult baboon kidney and near term placenta, primer extension studies confirmed that transcription is initiated at -311 nt from the ATG encoding methionine. In both the baboon placenta and adult kidney a second transcription initiation site was identified at -286 nt while a minor transcription initiation site was observed at -306 nt. Interestingly, a GC box, which typically regulates multiple

transcription start sites, was identified 41 and 72 bases upstream from the first and second major transcription initiation sites respectively in the baboon 11β-HSD-2 gene. Multiple start sites were also identified for 11β-HSD-2 in the human placenta (Agarwal *et al.*, 1995) although the major start site was at -74 nt and the minor one at -116 nt. In the human kidney, however, only the -116 nt start site was observed (Agarwal *et al.*, 1995). The transcription start site for 11β-HSD-2 in the sheep kidney (Agarwal *et al.*, 1994) also appears to differ slightly from that in the human (Agarwal *et al.*, 1995) and baboon (Pepe *et al.*, 1999). Despite the fact that the first initiation transcription site of baboon 11β-HSD-2 is approximately 263 bases upstream from that in the human, the size and nucleotide composition of the 11β-HSD-2 encoded by baboon and human tissues is virtually identical. Although we have not determined the amino acid composition of the baboon 11β-HSD-2 protein, the deduced structure of this protein is highly conserved in several mammalian species (Agarwal *et al.*, 1994; Agarwal *et al.*, 1995; Cole, 1995; Campbell *et al.*, 1996).

Although we have only sequenced a relatively small component of the 5'-flanking region of both 11β-HSD genes, the promoters of both 11β-HSD-1 and -2 were activated when transfected into JEG-3 cells. Moreover, the results of the current study also indicate that estradiol in the presence of estrogen receptorα enhances the promoter activity of the 5'-flanking regions of the genes for baboon 11β-HSD-1 and 11β-HSD-2. In the baboon, 11β-HSD-1 and -2 mRNA and protein are expressed in and localized specifically to the syncytiotrophoblast (Pepe *et al.*, 1996a). The fact that the promoter activities of both 11β-HSD-1 and -2 were enhanced by approximately 2-fold in cells co-transfected with pSG5ER alone in the absence of 17β-estradiol (Figure 28), supports the

finding that these increases may be attributable to ER phosphorylation. ER phosphorylation occurs as part of both ligand-induced activity and ligand-independent transcriptional activation (Pettersson and Gustafsson, 2001). It has been shown that ER, in the absence of ligand, can be activated following phosphorylation by growth factors (EGF) or kinases (PKA, MAPK or CDK2; Hall and McDonnell, 1999)

Moreover, syncytiotrophoblast expression of both genes increases with advancing gestation in association with the progressive rise in estrogen (Albrecht and Pepe, 1990). The results of the present study also demonstrated that estrogen receptorα is expressed in the cytotrophoblast and syncytiotrophoblast of baboon placenta, results consistent with previous observations of estrogen receptorα mRNA (Chibbar *et al.*, 1995) and protein (Billiar *et al.*, 1997) expression in human placental trophoblast. Collectively, these results support the hypothesis that estrogen regulates the developmental expression of both 11β-HSD-1 and -2 in baboon syncytiotrophoblast. However, because putative transcription factor binding sites may also be present outside the most proximal regions of these genes, it remains to be determined whether the estrogen-estrogen-receptorα activation of 11β-HSD-1 and -2 reflects the magnitude of the response of the endogenous gene. Moreover, it remains to be determined whether estrogen regulates the promoter activity of the 11β-HSD-1 and -2 genes following transfection into primary cultures of baboon placental trophoblast. Nevertheless, the results of the current study are consistent with our previous studies demonstrating that placental NAD-dependent 11β-HSD-2 enzyme activity (Baggia *et al.*, 1990a) as well as oxidation of cortisol to cortisone (Pepe *et al.*, 1988; Baggia *et al.*, 1990b), were increased at mid-gestation in baboons in which

placental estrogen production was prematurely elevated *in vivo* and thus support a role for estradiol in regulating 11β-HSD-2 expression.

However, similar studies in cultured human term placental syncytiotrophoblast demonstrated estradiol (1μM) diminished 11β-HSD-2 activity and mRNA (Sun *et al.*, 1998). Progesterone (1μM) and nitric oxide also inhibited 11β-HSD-2 activity and mRNA, whereas the stimulation of the cAMP pathway with forskolin increased 11β-HSD-2 activity and mRNA. Treatment of these cultured human syncytiotrophoblasts with the phorbol ester PMA (PK-C signal transduction pathway activator) had no effect on 11β-HSD-1 or –2 activity or mRNA (Sun *et al.*, 1998). None of the five aforementioned compounds affected 11β-HSD-1 activity or mRNA. *In vitro* analysis of baboon syncytiotrophoblast 11β-HSD-2 activity demonstrated that both progesterone and cortisone were enzymatically inhibitory (Baggia *et al.*, 1990a).

Our transient transfection analyses in JEG-3 cells showed cAMP slightly increased 11β-HSD-2 promoter activity while 11β-HSD-1 activation increased 3 fold. These results coincide directly with the number of putative CREs (cAMP Response Elements; palindromic octamer TGACGTCA) present in each 5'-flanking region. The promoter of the baboon 11β-HSD-1 gene contains 5 putative CREs, while the baboon 11β-HSD-2 5'-flanking region contains only 1 putative CRE. The modicum increase in cAMP-stimulated 11β-HSD-2 promoter activity therefore reflects a promoter (i.e., at basal) which was approaching maximum possible stimulation, whereas cAMP-stimulated 11β-HSD-1 promoter activity was more susceptible to increased levels of activation (i.e., by cAMP). Perhaps the comparatively high basal activity of 11β-HSD-2 reflected constitutive and/or fetal bovine serum growth factor-related activation and the low basal

11β-HSD-1 activity indicates that this promoter required additional stimulants (i.e., cAMP). Another interesting note is that the cAMP analog, CPT-cAMP, used in these studies has been shown to bind with high affinity to the cAMP-binding site type B (vice type A) of the type II (vice type I) regulatory subunit (Ogreid *et al.*, 1985) and perhaps the 11β-HSD-1 promoter is more selectively activated by this specific PK-A form (Rydel and Greene, 1988).

Our work demonstrates (Davies *et al.*, 1997) that the 5'-flanking region of the baboon 11β-HSD-1 gene contains an estrogen receptor-like response element thereby providing a mechanism for estrogen to regulate expression of this gene. However, the promoter region of the gene also contains a glucocorticoid receptor response element and others have shown that glucocorticoids modulate 11β-HSD-1 mRNA expression in liver of fetal and adult sheep (Yang *et al.*, 1994) and in rodent neural tissue (Low *et al.*, 1994a). Although additional studies remain to be performed, perhaps using a larger component of the 11β-HSD-1 promoter region, it is unlikely that cortisol regulates baboon placental 11β-HSD-1 expression since syncytiotrophoblast mRNA and protein levels of 11β-HSD-1 (Pepe *et al.*, 1996a) increase concomitant with increased local catabolism of cortisol to cortisone by baboon syncytiotrophoblast (Baggia *et al.*, 1990a; Pepe and Albrecht, 1995). It would appear therefore, that the regulation of 11β-HSD-1 is tissue specific. The rat 11β-HSD-1 gene utilizes tissue-specific differential promoter usage as evidenced by the detection of multiple mRNA species in different tissues (Moisan *et al.*, 1992). Indeed, we also demonstrated (Davies *et al.*, 1997) that there are two transcriptional start sites in the 5'- flanking region of the baboon 11β-HSD-1 gene

and that mRNA transcripts emanating from the downstream start site dominated in baboon placenta whereas in the liver, transcripts originated from the upstream start site.

The results of the present study also indicate that, in contrast to 11β-HSD-1, the promoter region of the baboon 11β-HSD-2 gene, like that in the human (Agarwal *et al.*, 1995) and sheep (Yang *et al.*, 1994), does not contain an estrogen response element. Rather, in the baboon, as in the human, the 5'-flanking region of 11β-HSD-2 has a high GC base content, regions which are known to exhibit binding for transcription factors including Sp1 (Briggs *et al.*, 1986) and AP-2 (Williams and Tjian, 1991). Both Sp1 and AP-2, as well as SF-1, have been shown to be required for cAMP-dependent transcription (Liu and Simpson, 1997). Recently, it was demonstrated that native estrogen receptor protein or an estrogen receptor mutant lacking the DNA binding domain formed a complex with Sp1 (Porter *et al.*, 1997). However, although these estrogen-receptor-Sp1 complexes resulted in enhanced Sp1-binding to DNA in the presence or absence of estradiol, transactivation of promoter-reporter constructs was totally estrogen-dependent. These authors proposed (Klinge, 2000) the existence of an estrogen-dependent transactivation pathway that involves estrogen receptor-protein interaction and which is estrogen-response element independent. Although further study is required, the latter would provide a mechanism by which estrogen regulates 11β-HSD-2 expression in the baboon placenta.

Steroidogenic Factor-1, (SF-1), an orphan nuclear receptor/transcription factor, has emerged as a key regulator of endocrine function (cytochrome P450 enzymes) and sex differentiation (Parker and Schimmer, 1997). Because of endogenous SF-1 expression in JEG-3 cells, SF-1 expression vector-activation of 11β-HSD-1 and –2 were investigated in

293 embryonic kidney cells. Basal activation of both promoters by SF-1 was minimal, perhaps because required endocrine-tissue-specific factors were not present in kidney cells. SF-1's promoter activation in response to cAMP was not determined although many studies have demonstrated that SF-1 can mediate cAMP responsiveness (Liu and Simpson, 1997).

Finally, we have also demonstrated that a cotransfected adenovirus containing antisense cDNA can significantly diminish the expression of the complimentary sense cDNA cotransfected via calcium-phosphate precipitation into JEG-3 cells. It is uncertain how this inhibition occurs, but it is postulated to concern RNA triplex structures and/or increased mRNA destabilization/degradation. Nonetheless, 11β-HSD-2/luciferase expression diminished in the presence of antisense 11β-HSD-2 promoter DNA/mRNA. Further analyses to determine whether the reduction in luciferase activity was attributable to reductions in luciferase mRNA or protein would have proved interesting.

In summary, we have isolated and sequenced the 5'-flanking regions the baboon 11 β-HSD-1 and 11β-HSD-2 genes and shown that the baboon genes exhibit extensive nucleotide homology to their respective human genes. The baboon 11β-HSD-1 gene has two transcriptional start sites 93 nucleotides apart and it appears that the upstream start site is the major one utilized in liver whereas the placenta appears to utilize the downstream start site. The baboon 11β-HSD-2 gene also exhibited multiple transcriptional start sites in placenta and kidney, analogous to that in the human placenta, but not the human kidney (Agarwal *et al.*, 1995). The baboon 11β-HSD-1 promoter contains no TATA boxes, but two consensus CAAT boxes, which presumably regulate transcription initiation. The baboon 11β-HSD-2 promoter contains multiple GC boxes

that probably regulate transcription. The 5'-flanking region of the baboon 11β-HSD-1 and -2 genes exhibit extensive nucleotide homology to that of the human and are activated when transfected into JEG-3 cells. Moreover, it would appear that the 5'-flanking regions of both the baboon 11β-HSD-1 and -2 genes are responsive to 17β-estradiol in the presence of a co-transfected estrogen receptorα cDNA. Because of the importance of placental 11β-HSD-1 and 11β-HSD-2 in regulating cortisol transfer to the fetus and thus the function of the fetal hypothalamic-pituitary axis in the baboon (Pepe and Albrecht, 1995), studies of the regulation of the promoter in primary cultures of baboon trophoblasts obtained from animals at various stages of gestation with/without hormonal treatment *in vivo* should enhance our understanding of the mechanism(s) by which this gene is regulated in the placenta.

CHAPTER VI

EXPRESSION OF 11β-HYDROXYSTEROID DEHYDROGENASE (11β-HSD) TYPES –1 AND –2 IN BABOON PLACENTAL SYNCYTIOTROPHOBLAST: EFFECT OF PLACENTAL P450-AROMATASE ENZYME INHIBITION *IN VIVO*

INTRODUCTION

Pepe and Albrecht have shown that estrogen, via regulation of placental 11β-hydroxysteroid dehydrogenase (11β-HSD) catalyzed metabolism of cortisol and cortisone (Pepe *et al.*, 1988), determines the amount of maternal cortisol arriving within the fetus (Pepe and Albrecht, 1990) and thus regulates the function of the fetal hypothalamic-pituitary-adrenocortical axis (Pepe and Albrecht, 1998; Albrecht and Pepe, 1999). It is now known that cortisol-cortisone interconversion is regulated by two different 11β-HSD enzymes (Tannin *et al.*, 1991; Agarwal *et al.*, 1994; Monder and White, 1993; Pepe and Albrecht, 1995). The 11β-HSD type 1 originally cloned from the rat liver (Lakshmi and Monder, 1988) possesses both oxidative and reductive activity, prefers $NADP^+$/NADPH as a cofactor and has a relatively low (μM) affinity for glucocorticoid substrates. In contrast, the 11β-HSD type 2 is unidirectional, possessing only oxidative activity, uses only NAD^+, and has a high affinity (nM) for substrate cortisol (Brown *et al.*, 1993; Brown *et al.*, 1996a). While the 11β-HSD-1 gene appears to be expressed in several tissues (Tannin *et al.*, 1991; Monder and White, 1993; Pepe *et al.*, 1996b), the 11β-HSD-2 is expressed only in a few tissues including the kidney cortex, placenta (Hirasawa *et al.*, 2000) and the fetal adrenal (Albiston *et al.*, 1994; Brown *et al.*, 1996b; Pepe *et al.*, 1996a; McMillen *et al.*, 1999). The molecular/ biochemical basis for selective tissue expression remains to be determined.

The observation that the baboon and human 11β-HSD-1 gene promoter exhibits glucocorticoid and estrogen-like response elements would suggest that these hormones play a role in regulating cell-specific expression of 11β-HSD-1. However, the regulatory

effects of these two hormones appear extremely complex and dependent upon the physiologic status of the animal. For example, in sheep, 11β-HSD-1 mRNA expression is enhanced in adult liver but reduced in fetal liver following glucocorticoid administration *in vivo* (Yang *et al.*, 1994), whereas in the hippocampus, glucocorticoids and chronic-stress up-regulate 11β-HSD-1 (Low *et al.*, 1994a). Rat hepatic 11β-HSD-1 is down-regulated by estrogen directly and/or via growth hormone (Low *et al.*, 1994b; Low *et al.*, 1993) while estrogen upregulates both 11β-HSD-1 and -2 expression in the rat uterus (Burton and Waddell, 1999). It is unlikely that cortisol regulates baboon placental 11β-HSD-1 expression since syncytiotrophoblast mRNA and protein levels of 11β-HSD-1 (Pepe *et al.*, 1996a) increase concomitant with increased local catabolism of cortisol to cortisone by baboon syncytiotrophoblast (Baggia *et al.*, 1990a; Pepe and Albrecht, 1995). The rat 11β-HSD-1 gene exhibits tissue-specific differential promoter usage as evidenced by the detection of multiple mRNA species in different tissues (Moisan *et al.*, 1992). Indeed, we also demonstrated (Davies *et al.*, 1997) that there are two transcriptional start sites in the 5'- flanking region of the baboon 11β-HSD-1 gene and that mRNA transcripts emanating from the downstream start site dominated in baboon placenta whereas in the liver, transcripts originated from the upstream start site. It would appear therefore, that the regulation of 11β-HSD-1 is tissue specific.

In cultured human term placental syncytiotrophoblast, studies demonstrated that estradiol (1μM) diminished 11β-HSD-2 activity and mRNA levels (Sun *et al.*, 1998). Moreover, progesterone (1μM) and nitric oxide also inhibited 11β-HSD-2 activity and mRNA *in vitro*, whereas stimulation of the cAMP pathway with forskolin increased 11β-HSD-2 expression. In contrast, treatment of cultured human syncytiotrophoblasts with the phorbol ester PMA, a PK-C signal transduction pathway activator, had no effect on 11β-HSD-2 activity or mRNA (Sun *et al.*, 1998) and none of the five aforementioned compounds affected 11β-HSD-1 expression. Studies of baboon syncytiotrophoblast 11β-HSD-2 enzymatic activity showed that both progesterone and cortisone were competitive

inhibitors for substrate cortisol (Baggia *et al.*, 1990a). Our transient transfection analyses in JEG-3 cells showed cAMP slightly increased 11β-HSD-2 promoter activity while 11β-HSD-1 activation increased 3 fold (Figure 29).

Our recent study (Pepe *et al.*, 1999b) also indicated that, in contrast to 11β-HSD-1, the promoter region of the baboon 11β-HSD-2 gene, like that in the human (Agarwal *et al.*, 1995) and sheep (Yang *et al.*, 1994), does not contain an estrogen response element. Rather, in the baboon, as in the human, the 5'-flanking region of 11β-HSD-2 has a high GC base content, regions which are known to exhibit binding for transcription factors including Sp1 (Briggs *et al.*, 1986) and AP-2 (Williams and Tjian, 1991). The 5'-flanking regions of both the baboon 11β-HSD-1 and -2 genes are responsive to 17β-estradiol in the presence of a co-transfected estrogen receptorα cDNA (Pepe *et al.*, 1999b; Figure 28).

Because of the importance of placental 11β-HSD-1 and 11β-HSD-2 in regulating cortisol transfer to the fetus and thus the function of the fetal hypothalamic-pituitary adrenal axis (Pepe and Albrecht, 1995), it is essential to ascertain whether the developmental increase in mRNA of these two enzymes in baboon syncytiotrophoblast is regulated by estrogen. This study was therefore undertaken to determine the levels of 11 β-HSD-1 and –2 mRNA in syncytiotrophoblast isolated from placenta of baboons following treatment on day 100 – day 170 with CGS-20267, a highly-specific placental P450-aromatase enzyme inhibitor.

MATERIALS AND METHODS

Baboon treatment with aromatase inhibitor CGS-20267

Baboons were randomly selected to receive CGS-20267 (4,4'-[1,2,4-triazol-1-*yl*-methylene]-*bis*-benzonitrite, Letrozoe; Novartis Pharma AG, Basel, Switzerland) injected

subcutaneously beginning on either day 30, day 60 or day 100 of gestation with a dose of 0.1 mg/d in sesame seed oil. The dose of CGS-20267 was gradually increased by 0.1 mg/d increments to a maximum of 2.0 mg/d, the level of which was maintained until cesarean-section delivery on days 160 through 178 of gestation. Other randomly assigned baboons received either no treatment or CGS-20267 (0.1 – 2.0 mg/day as above) plus estradiol benzoate (0.1 – 2.0 mg/d subcutaneously in sesame seed oil) at doses comparable to the normal ontogenetic pattern of maternal serum estradiol concentration.

Placenta harvesting and syncytiotrophoblast cellular RNA purification

Syncytiotrophoblast-enriched and cytotrophoblast-enriched fractions were isolated by collagenase dispersion (Kliman *et al.*, 1986) as modified by Baggia *et al.*, 1990b and Pepe *et al.*, 1999a. Essentially, the placenta was weighed immediately upon harvest and placed in a baked steel tray (8" x 12" x 2") and rinsed repeatedly with sterile saline or Hanks (GIBCO #310-4185AJ; filter sterilized) at 4°C on ice to remove as much blood as possible. The maternal and fetal membranes were pulled/dissected away while on ice in cold Hanks and discarded along with calcifications and any infarcts. The placenta was then weighed again (50 g mid-gestation, 80 g term gestation) and Dispersion Media (4 ml/g tissue) in a 500 ml baked Erlenmeyer flask was prepared. Dispersion Media for 40 g tissue; add to 160 ml sterile Hanks 1.6 ml FBS, 160 mg collagenase (0. 1%; Sigma C9891 or 200 mg Sigma C8051), 160 mg hyaluronidase (0. 1 %; Sigma H3506), 16 mg DNAse (0.01%; Sigma D5025), 40 mg trypsin inhibitor (0.025%; Sigma T9003) and 54 g $NaHCO_2$ (Sigma S5761). The placental tissue was minced 5 g at a time in a petri dish on ice with 5 -10 ml cold Hanks using a sharp, curved scissors and then the minces were collected/pooled into 50 ml tubes (polypropylene, Fisher #05-539-5 and –6). The tubes

were filled to the top with cold Hanks (4 tubes mid, 8 tubes term) and spun at 300 x g for10 min at 4°C. The pellet was loosened by hitting the centrifuge tube on the counter top and then pouring it into warmed Dispersion Media. Digestion occurs at 37°C for 35 min with agitation.

Following collagenase dispersion, the digestion was strained through autoclaved Nitex cloth into a baked glass beaker on ice. The cloth was squeezed to maximize recovery. The cell suspension, now in 50 ml tubes, was diluted about 50% with cold Hanks and then centrifuged 500 x g 15 min at 4°C. The supernatant was aspirated or poured off carefully and the pellets were then resuspended and combined using sterile plastic pasteur pipettes to two 50 ml tubes. The volume was brought to 50 ml with cold Hanks and then recentrifuged 500 x g 30 min at 4°C. Again the supernatant was carefully removed and only the top white and middle pink (not the bottom RBC pellet) placental cells layers were collected and resuspended well in cold Hanks. (5-8 ml per Percoll gradient)

Trophoblasts were isolated via 5 - 70% Percoll (Sigma P1644) gradient centrifugation. Baked 12 x 75 mm glass test tubes were labeled 1 to 14 (3 sets midterm placenta, 6 sets for term placenta). 90% Percoll was prepared by adding 6 ml of 10 x Hank's to 54 ml of Percoll for a midterm (12 ml 10 x Hanks + 108 ml Percoll for a term). A 70% to 5% gradient in 5% increments was prepared using proportions charted below. The tubes were vortexed for 3 – 5 sec and the gradient was then prepared in 50 ml conical clear polystyrene centrifuge tubes (Corning orange top #25339-50) placed at a 45° angle. Starting with tube #1, the Percoll mixture was applied to the 50 ml tube using a Rainin Rabbit 8-channel peristaltic pump (Rainin, Woburn, MA) with small diameter tubing

(Rainin # 39-625) at a rate of 100-150 ml/hr (setting 444). (The tubing was flushed first with sterile H_2O then 90% Percoll). This process was repeated for tubes 2 through 14. Interfaces between each 5% step in the gradient can be seen. 5-10 ml cell suspension was carefully pipetted onto the gradient and centrifuged in a Beckman TJ-6, speed 7, 1125 x g, 2400 RPM, for 20 min at RT; CAPS OFF, BRAKE OFF!

Tube #	90% Percoll (ml)	1 x Hank's (ml)	Final % Percoll
1	2.33	0.67	70
2	2.17	0.83	65
3	2.00	1.00	60
4	1.83	1.17	55
5	1.67	1.33	50
6	1.50	1.50	45
7	1.33	1.67	40
8	1.17	1.83	35
9	1.00	2.00	30
10	0.83	2.17	25
11	0.67	2.33	20
12	0.50	2.50	15
13	0.33	2.67	10
14	0.17	2.83	5

On a 5-70% Percoll gradient, 3 white cell layers were collected and pooled/washed. Band 1 (37-32 ml/mark) was very thick so it was separated into 2 - 3 50 ml tubes and diluted to 50 ml with Hanks. Bands 2 & 3 (32-23 ml/mark) and Band 4 (22-12 ml/mark) were also collected and diluted a lot with Hanks (at least 3 – 4 tubes). An aliquot of band 4 cytotrophoblast was counted as the total # cells recovered per tissue weight. 10% of band 1 and 4 cells were saved to -80°C in sterile 15 ml polypropylene tubes after snap freezing in liquid nitrogen. The cells were pelleted at 500 x g for 15 min 4°C. The supernatants were discarded. At this point, one could either snap-freeze cells in liquid nitrogen and store them -80°C or proceed.

Total RNA was isolated as stated above (Chirgwin *et al.*, 1979) using β-mercapto-ethanol and guanidine isothiocyanate (prepared as follows: 70.9 g GITC (Gibco) in a 250-300 ml baked volumetric cylinder. DEPC H_2O was added to the 140 ml mark and then 1.25 ml 3M NaAcetate pH 6.0 was added. The volume was brought to 150 ml with DEPC H_2O and filter sterilized with a 150 ml Nalgene 125-0080 filter; store room temperature). To the band 1 cell pellet, 19 ml GITC buffer (plus 158.7 µl βm-EtOH) was added. Bands 2/3 cell pellet received 7.2 ml or 10.8 ml GITC + 60 or 90 µl βm-EtOH. The Band 4 cell pellet received 3.6 ml GITC + 30 µl βm-EtOH. The cell lysate (in 50 ml polypropylene tubes) was then vortexed and polytroned (Polytron PT10, Kinematica, Switzerland; setting at 9.5) on ice for 15 sec x 4 with a 10 sec rest in between. The tubes were then centrifuged 1000 x g for 10 min at RT and the supernatants were poured into new polypropylene tubes and extracted with an equal volume of 24:1 chloroform:isoamyl alcohol. The tubes were inverted slowly and repeatedly for 1 min and then centrifuged 2300 x g for 15 min at RT. The top layer was carefully pipetted to new tubes, leaving the interface behind. Band 1 was extracted at least once or twice more. Genomic DNA was sheared by pulling the supernatants up and down once through a 23 gauge needle. At this junction, the lysates could be frozen/stored -80°C or gently layered with a 1 ml pipetteman onto 1.8 ml CsCl buffer (prepared as follows: 95.97 g CsCl (Sigma; 5.7 M) + 830 µl 3M NaAcetate pH 6.0 are added and brought to 100 ml with DEPC H_2O in a 100 ml volumetric cylinder, vacuum filter/sterilize as GITC above) in Beckman tubes #344057. Total RNA is pelletted 38.6K RPM (179,000 x g;SW 50.1 rotor), 21 hrs, 23°C.

Following the overnight spin, the supernatants were carefully removed incrementally with a plastic transfer pipette. The supernatant to the CsCl shelf was

discarded and then the CsCl was removed until about 0.5 ml remained. Then the tube was inverted to remove the last 0.5 ml. The tube was wiped with a sterile cotton Q-tip and allowed to dry for a few minutes. The RNA pellet was resuspended in 100 µl 0.3 M NaAcetate pH 6.0 by pipetting repeatedly and transferred to sterile 1.5 ml eppendorf tubes. This was repeated twice. 750 µl cold EtOH (100%) was then added and the eppendorf-containing-RNA preparations were stored indefinitely at -80°C. When needed, the RNA was pelletted via centrifugation at 13K x g for 30 min, 4°C, washed with 1 ml cold 70% ETOH in DEPC H_2O, repelletted, dried in a Savant Speed Vac Concentrator (Savant Instruments, Farmingdale, NY), dissolved in sterile H_2O and analyzed spectrophotometrically prior to RT-PCR experimentation.

RT-PCR determination of estrogen receptor α, 11βHSD-1 and 11βHSD-2 mRNA and 18S rRNA expression

The expression of ERα, 11βHSD-1 and -2 mRNA as well as 18S rRNA in sundry tissues and in enriched placenta cell fractions were determined by reverse transcription - polymerase chain reaction (RT-PCR) using procedures recently developed in our laboratories (Albrecht *et al.*, 1995; Babischkin *et al.*, 1997). Oligonucleotide primers synthesized by Life Technologies, Inc. were selected from the cDNA sequence of human ERα (Green *et al.*, 1986) and flanked a portion of the cDNA sequence that spans exons 4-7 and overlaps introns D, E and F. ERα forward primer (upstream): 5'GATCCTACCAGACCCTTCAG-3' (position 1226-1245). ERα reverse primer (downstream): 5'-TTCCAGAGACTTCAGGGTGC-3' (position 1642-1623). Position numbers correspond to the nucleotide sequence of human ERα (Green *et al.*, 1986) cDNA where the initiation codon begins at position 233. The ERα RT-PCR product

generated should be 417 bp in size. For 11β-HSD-1, primers were also selected from the human sequence (Tannin *et al.*, 1991) and flanked a portion of the cDNA sequence that spans exons 1-5 and overlaps introns A, B, C and D. 11β-HSD-1 forward primer (upstream): 5'-GAAAAAATATCTCCTCCCCATTCTG-3'. This sequence is 100% homologous to the baboon gene sequenced previously (position 107-131; Figures 20 and 21). 11β-HSD-1 reverse primer (downstream): 5'-GACACTGAATATTCCTTTCTGATGG-3' (position amino acid 204-196). The human sequence predicts an RT-PCR product 600 bp in size. For 11β-HSD-2, primers were again selected from the human sequence (Agarwal *et al.*, 1995) and flanked a portion of the cDNA sequence that spans exons 2-5 and overlaps introns B, C and D. 11 β-HSD-2 forward primer (upstream): 5'-TGCTGCAGATGGACCTGACCAA-3' (position amino acids 134-141). 11β-HSD-2 reverse primer (downstream): 5'-GTAGTAGTGGATGAAGTACATGAGC-3' (position amino acids 354-346). The human sequence predicts an RT-PCR product 659 bp in size. Finally, for 18S, primers were selected from the human 18S ribosomal RNA gene sequence (Torcynski *et al.*, 1985). 18S forward primer (upstream): 5'-TCAAGAACGAAAGTCGGAGG-3' (position 1126-1145). 18S reverse primer (downstream): 5'-GGACATCTAAGGGCATCACA-3' (position 1614-1595). The human sequence predicts an RT-PCR product of 489 bp in size.

Total RNA from sundry samples (1 - 3 μg) were reversed transcribed at 42°C for 60 min in a reaction mixture (20 μl) containing 1 mM each of dATP, dCTP, dGTP, dTTP (Promega) 1 mM dithiothreitol, 200 U SUPERSCRIPT RNase H RT (Life Technologies), 40 U RNAguard ribonuclease inhibitor (Pharmacia Biotech Inc., Piscataway, NJ), 50 mM

Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 250 ng of Random Primers (Life Technologies). After 60 min, the RT mixture was heated to 70°C for 15 min and then cooled to 4°C. DNA amplification was carried out in a 100 µl reaction volume containing 20 µl of the RT reaction, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 – 2.0 mM $MgCl_2$, 1.25 U cloned *Thermus aquaticus* DNA polymerase (Amplitaq, Applied Biosystems, Foster City, CA) and 20 pmoles each of the forward and reverse primers. On many occasions, a RNA PCR Core Kit (Perkin Elmer #N808-0143, Applied Biosystems, Foster City, CA) was utilized. PCR was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA). Samples were amplified from 23-40 sequential cycles at 94°C for 20-60 sec (melting), 48°-60°C for 30-60 sec (annealing) and 58°-72°C for 30-120 sec (extension), depending upon the cDNA amplification parameters. After the last cycle, samples were incubated for an additional 7 min at 72°C. Two negative controls, in which either RNA or RT was omitted from the reaction, were also performed. The PCR products were fractionated by electrophoresis in a 1% agarose gel and stained in ethidium bromide. The PCR target products were visualized with a UV transilluminator and photographed using type 665 positive/negative film or captured on a Stratagene Eagle Eye II Still Video System (Stratagene U.S.A.).

All the PCR reactions were preliminarily analyzed kinetically to ensure exponential amplification of the PCR product, since it is only in this phase that amplification is extremely reproducible (data not shown). The cycle number, $[Mg^{+2}]$ and cycle parameters were optimized to ensure all amplification reactions were within the linear range and had not entered the plateau phase. The cycle number was also appropriately

optimized to ensure the PCR product band width was suitable for quantitative SigmaGel Gel Analysis (Sigma, St. Louis, MO). SigmaGel Gel Analysis software in the lane analysis mode measures and integrates the product lane area "under the curve" statistically accurately only if the band-width is appropriately sized and is not saturated. Quantitative analysis of the PCR products for 11β-HSD-1 and -2, as a ratio of 18S rRNA expression, were determined using SigmaGel Gel Analysis of agarose gel images captured on a Stratagene Eagle Eye II Still Video System (Stratagene U.S.A.). PCR-generated bands were extracted from the agarose gel (QIAGEN, Chatsworth, CA) and verification of the products was accomplished by dideoxy-chain termination sequencing (San Diego State University MicroChemical Core Facility, San Diego, CA) using Big Dyes Kit with AmpliTAQ SS (Applied Biosystems, Foster City, CA) on an ABI 377 DNA automated sequencer.

RESULTS

Maternal peripheral serum estradiol concentrations in CGS-20267-treated baboons decreased to and remained at a level significantly lower (P <0.001) than that in the untreated control baboons (Figure 34, A). This decrease was evident within 1 to 3 days of the initiation of CGS-20267 administration. The pattern of maternal serum estradiol concentrations in baboons treated concomitantly with CGS-20267 and estradiol was similar to that demonstrated in control untreated baboons (Figure 34, B). The overall level of estradiol, however, was two-fold greater than that in untreated control baboons.

The placental cell fraction obtained by 5 – 70 per cent Percoll gradient centrifugation of collagenase-dispersed placenta tissue appeared to be comprised primarily of cellular aggregates with small highly basophilic nuclei (data not shown) that was shown to

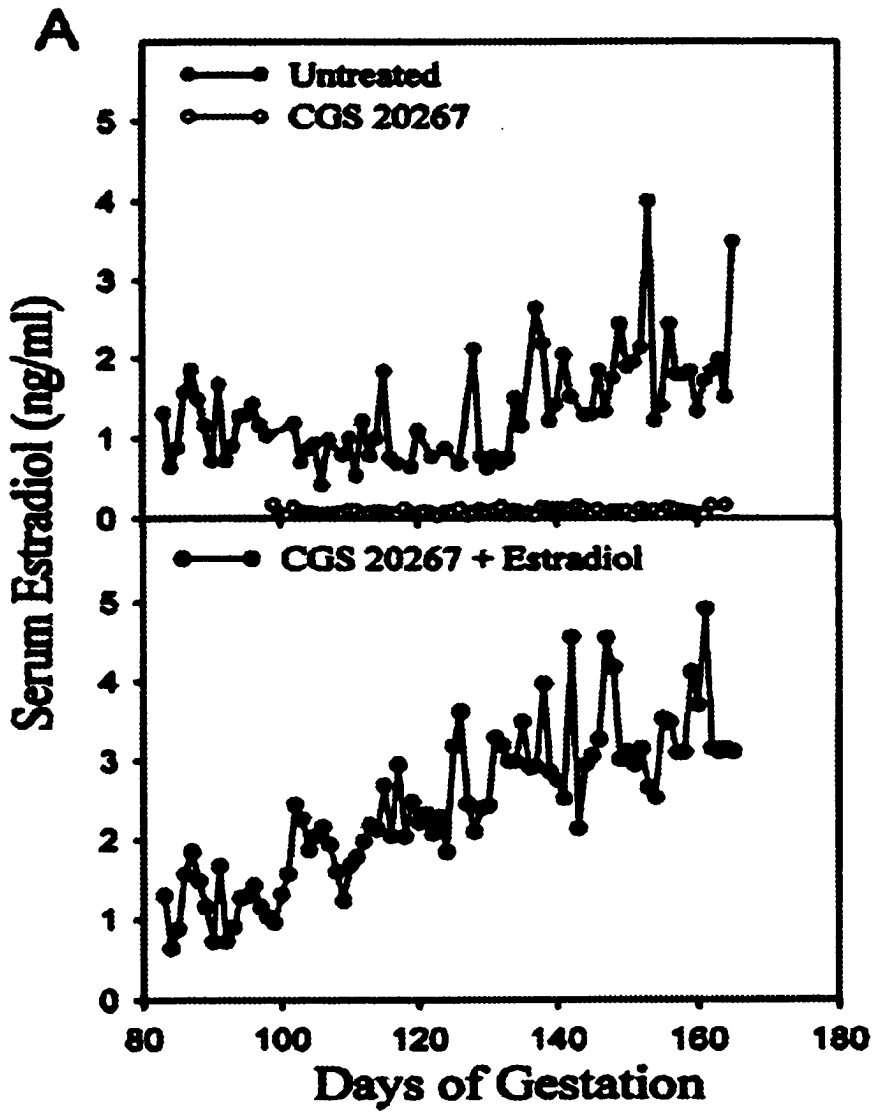

Figure 34. Mean maternal serum estradiol concentrations in CGS-20267-treated baboons. **A.** Baboons untreated (n = 20, *filled circles*) or treated daily subcutaneously with CGS-20267 at 0.1 to 2.0 mg/day beginning on day 100 of gestation (n = 24, *open circles*; term is 184 days). **B.** Baboons concomitantly treated subcutaneously with CGS-20267 at 0.1 to 2.0 mg/day and estradiol benzoate at 0.1 to 2.0 mg/day beginning on day 100 of gestation (n = 9, *filled circles*).

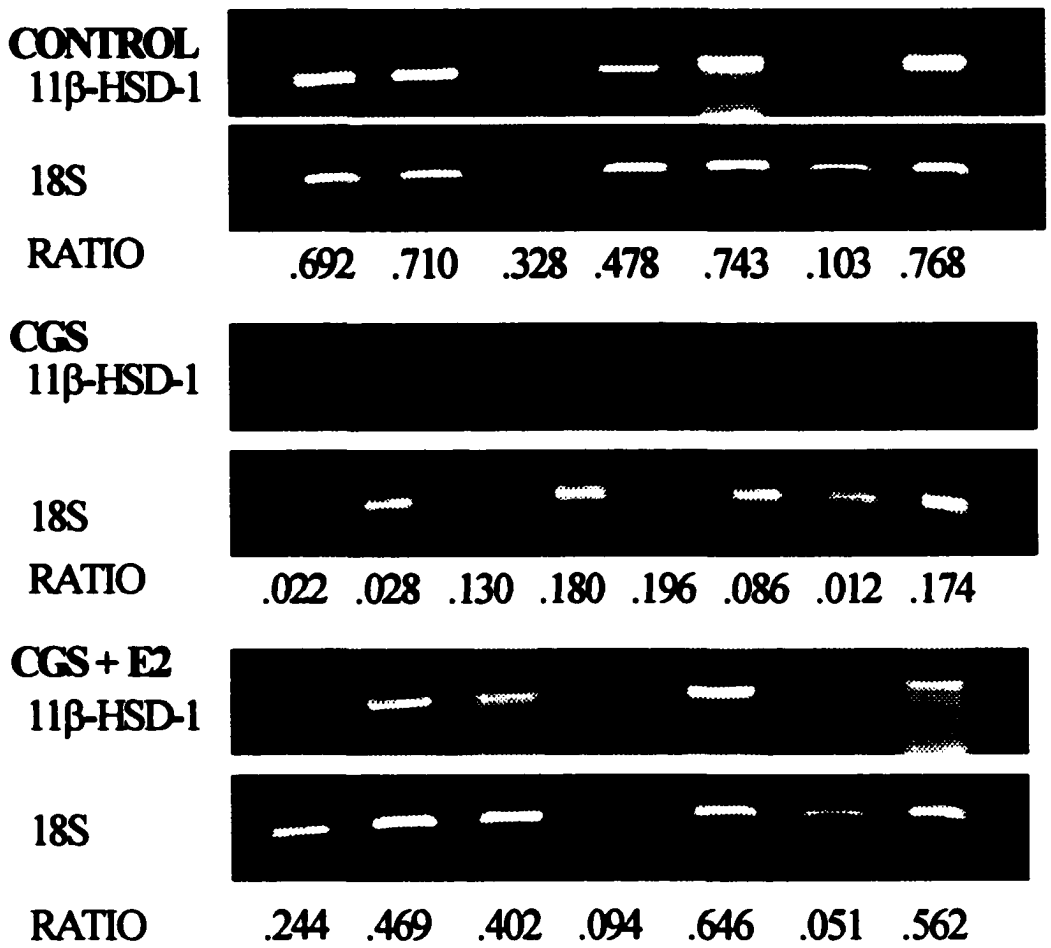

Figure 35. Expression of 11β-HSD-1 mRNA and 18S rRNA in placental syncytiotrophoblast isolated on day 165 – 170 of gestation from baboons which were untreated (N = 7) or treated on days 100 – 170 with CGS-20267 (N = 8) or CGS-20267 plus estradiol (N = 7). Total RNA (1 μg) was reversed transcribed and 90% of the RT reaction mixture PCR-amplified for 31 cycles with 11β-HSD-1 specific primers (600 bp product), and 10% PCR-amplified for 23 cycles with 18S specific primers (489 bp product). The ratios of the densitometric analyses of 11β-HSD-1 to 18S are displayed. All amplifications were performed in the exponential phase. Control samples utilizing RNA (1 μg) not reverse transcribed were also PCR-amplified with negative results to verify the absence of contaminating genomic DNA (data not shown).

exhibit extensive cytoplasmic immunoreactivity with antisera against SP_1 (Babischkin *et al.*, 1996). In contrast, this cellular fraction showed little of no staining for vimentin which labels endothelial cells and fibroblasts or α-antichymotrypsin which labels macrophages (Babischkin *et al.*, 1996). These results indicate that syncytiotrophoblasts were the predominant cell type isolated by Percoll gradient centrifugation.

Baboon syncytiotrophoblast cells isolated from near-term gestation (day 160-178, term = day 184) cesarean-section delivered placentas express the mRNA for 11β-HSD-1 and –2 enzymes, as well as 18S ribosomal RNA, as determined by RT-PCR (Figures 35 and 36). Using human sequence-specific primers to 11β-HSD-1 (Tannin *et al.*, 1991), RT-PCR generated a single product of 600 base pairs (bp), the correct size for the targeted region. The identity of this product was further confirmed by dideoxy nucleotide chain termination sequence analysis (data not shown) of both strands. The sequence analysis of the baboon 11β-HSD-1 RT-PCR product, in comparison to the published human sequence (Tannin *et al.*, 1991) indicated remarkable homology; 99.5 % identity at the amino acid level (199 of 200) and 98.7 % identity at the nucleotide level (592 of 600).

Similarly, RT-PCR analysis of 11β-HSD-2 (Figure 36), using human sequence-specific primers (Agarwal *et al.*, 1995), generated a single product of 659 bp. Sequence analysis (data not shown) of both strands of this product further confirmed its identity. In comparison to the human sequence (Agarwal *et al.*, 1995), the baboon 11β-HSD-2 mRNA in this region is also highly homologous; 98.6 % identity at the amino acid level (217 of 220) and 97.9 % identity at the nucleotide level (645 of 659).

Thirdly, RT-PCR amplification of the baboon 18S rRNA, utilizing human sequence-specific primers (Torczynski *et al.*, 1985), yielded a single 489 bp product, the size of the

targeted region. Once again, sequence analysis of both strands of this product (data not shown) further confirmed its identity, and, in comparison to the human sequence (Torczynski *et al.*, 1985), the baboon 18S rRNA in this region is 100 % homologous at the nucleotide level (489 of 489).

The PCR reactions for both 11β-HSD-1 and –2, as well as 18S ribosomal RNA, were analyzed kinetically in preliminary studies to ensure exponential amplification of the PCR product (data not shown). The cycle number, $[Mg^{+2}]$ and cycle parameters were optimized to ensure all amplification reactions had not entered the plateau phase. Control samples utilizing comparable RNA (1 μg) quantities were not reverse transcribed but PCR-amplified with negative results to verify the absence of contaminating genomic DNA (data not shown). The cycle number was also appropriately optimized to ensure the PCR product band width was "under the curve". SigmaGel Gel Analysis software (SIGMA, St. Louis, MO) in the lane analysis mode measures and integrates the product lane area "under the curve" statistically accurately only if the band width is appropriately sized and is not saturated. A typical RT-PCR reaction, using 18S rRNA as an endogenous internal standard, is demonstrated for 11β-HSD-1 (Figure 35) and 11β-HSD-2 (Figure 36). As evidenced, the 18S standard remains relatively constant whereas the 11 β-HSD-1 and –2 RT-PCR product bands demonstrate variability among the sundry treatment groups.

Quantitative analyses of the PCR products for 11β-HSD-1 and –2, as a ratio to 18S rRNA expression, were determined using SigmaGel Gel Analysis of agarose gel images captured on a Stratagene Eagle Eye II Still Video System (Stratagene U.S.A.). These results were highly reproducible, and therefore the RT-PCR ratios displayed in Figures

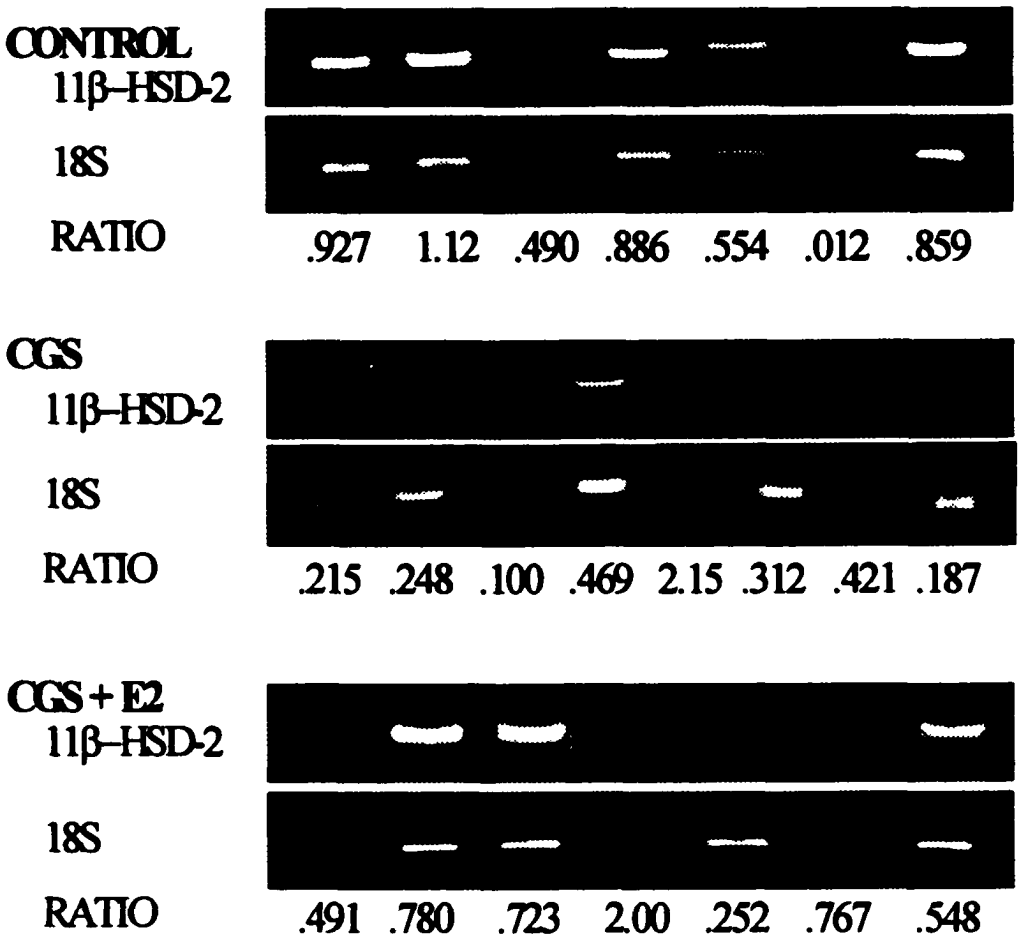

Figure 36. Expression of 11β-HSD-2 mRNA and 18S rRNA in placental syncytiotrophoblast isolated on day 165 – 170 of gestation from baboons which were untreated (N = 7) or treated on days 100 – 170 with CGS-20267 (N = 8) or CGS-20267 plus estradiol (N = 7). Total RNA (1 μg) was reversed transcribed and 90% of the RT reaction mixture PCR-amplified for 32 cycles with 11β-HSD-2 specific primers (659 bp product), and 10% PCR-amplified for 23 cycles with 18S specific primers (489 bp product). The ratios of the densitometric analyses of 11β-HSD-2 to 18S are displayed. All amplifications were performed in the exponential phase. Control samples utilizing RNA (1 μg) not reverse transcribed were also PCR-amplified with negative results to verify the absence of contaminating genomic DNA (data not shown).

35 and 36 were analyzed (Figure 37) using ANOVA with multiple comparison of the means by the Student-Newman-Keuls statistic test. When expressed as a ratio to 18S rRNA expression, baboon 11β-HSD-1 RT-PCR generated products was 0.546 ± 0.096 at term control and significantly reduced 5-fold to 0.104 ± 0.027 in syncytiotrophoblasts from baboons treated with CGS-20267 ($P<0.01$). The expression of 11β-HSD-1 in syncytiotrophoblasts from baboons treated with CGS-20267 and estradiol was 0.353 ± 0.087, a value significantly different from the CGS-20267 treatment alone ($P<0.05$), but not from the control ($P>0.05$; Figure 37).

Baboon syncytiotrophoblast expression of 11β-HSD-2 RT-PCR generated products was 0.692 ± 0.140 at term control and reduced, albeit not significantly to 0.512 ± 0.237 upon CGS-20267 treatment ($P>0.05$). With concomitant CGS-20267 treatment and estradiol supplementation, 11β-HSD-2 expression was 0.794 ± 0.213, again not a significant difference from either the CGS-20267 treatment alone ($P>0.05$) or from the control group ($P>0.05$; Figure 37).

With regard to 11β-HSD-2, two values, one from the CGS-20267 treatment alone (2.15) and the other from the CGS-20267 plus E_2 (2.00), appeared to be significantly removed from the main body of data (Figure 36). These two extreme observations were analyzed and proven to be statistical outliers ($P<0.005$) and not from the same population as the other observations. Upon removal of these two 11β-HSD-2 values and reanalysis of the data (Figure 38), syncytiotrophoblast expression of 11β-HSD-2 (ratio to 18S) mRNA in untreated animals (0.692 ± 0.140) was reduced ($P<0.001$) more than 2-fold (0.279 ± 0.049) in syncytiotrophoblast of baboons treated with CGS-20267. With concomitant CGS treatment and estradiol supplementation, 11β-HSD-2 mRNA

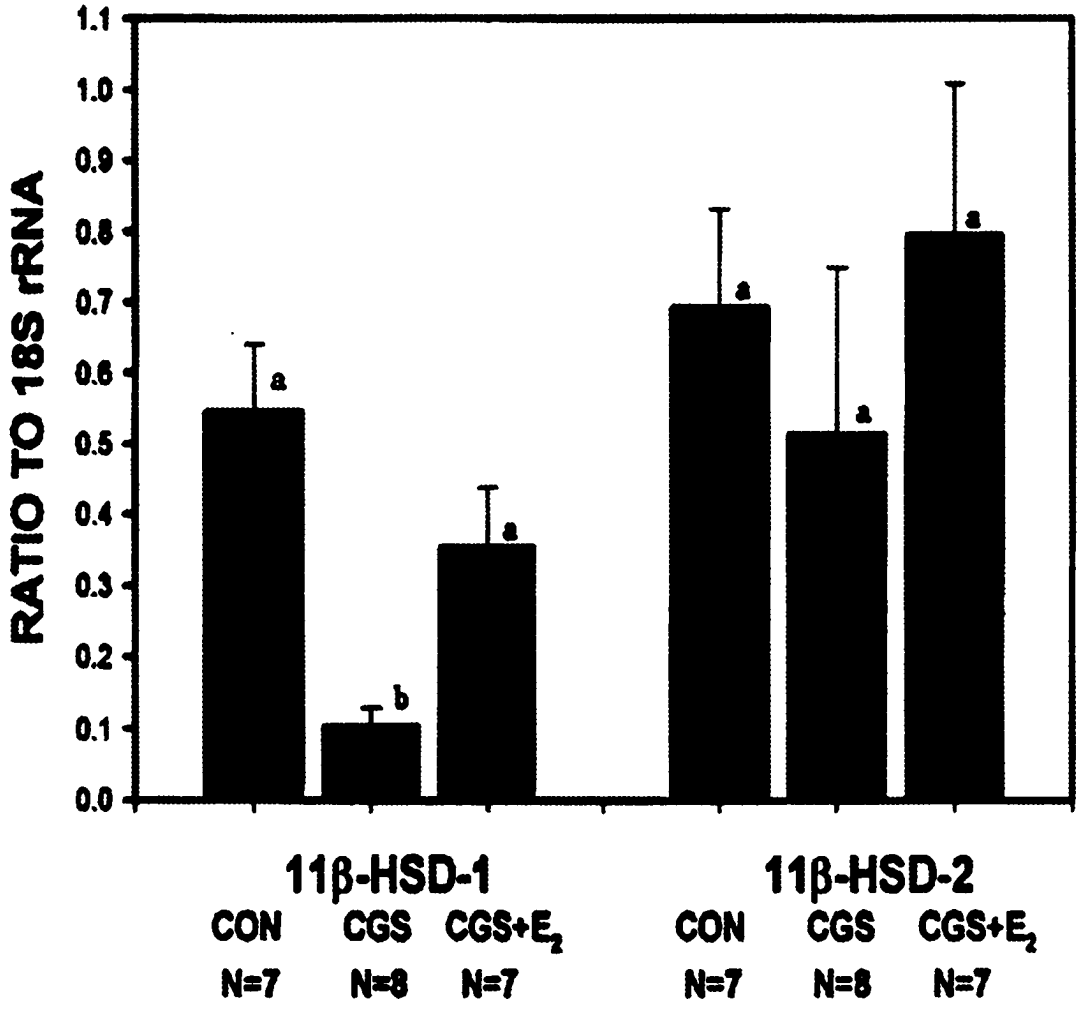

Figure 37. Relative expression of 11β-HSD-1 and -2 mRNA in isolated baboon placental syncytiotrophoblast cells from the agarose gels displayed in Figures 35 and 36. Data expressed as the ratio (mean ± SE) of 11β-HSD-1 or -2 message compared to its corresponding 18S rRNA signal. Baboon placental syncytiotrophoblast preparations were each assayed six times and the PCR product band areas densitometrically analyzed using SigmaGel Gel Analysis (Sigma, St. Louis, MO) of Stratagene Eagle Eye II Still Video System (Stratagene U.S.A)-photographed images. Values (mean ± SE) with different letter superscripts differ from each other at $P<0.01$ (ANOVA with multiple comparison of means by Student-Newman-Keuls Test).

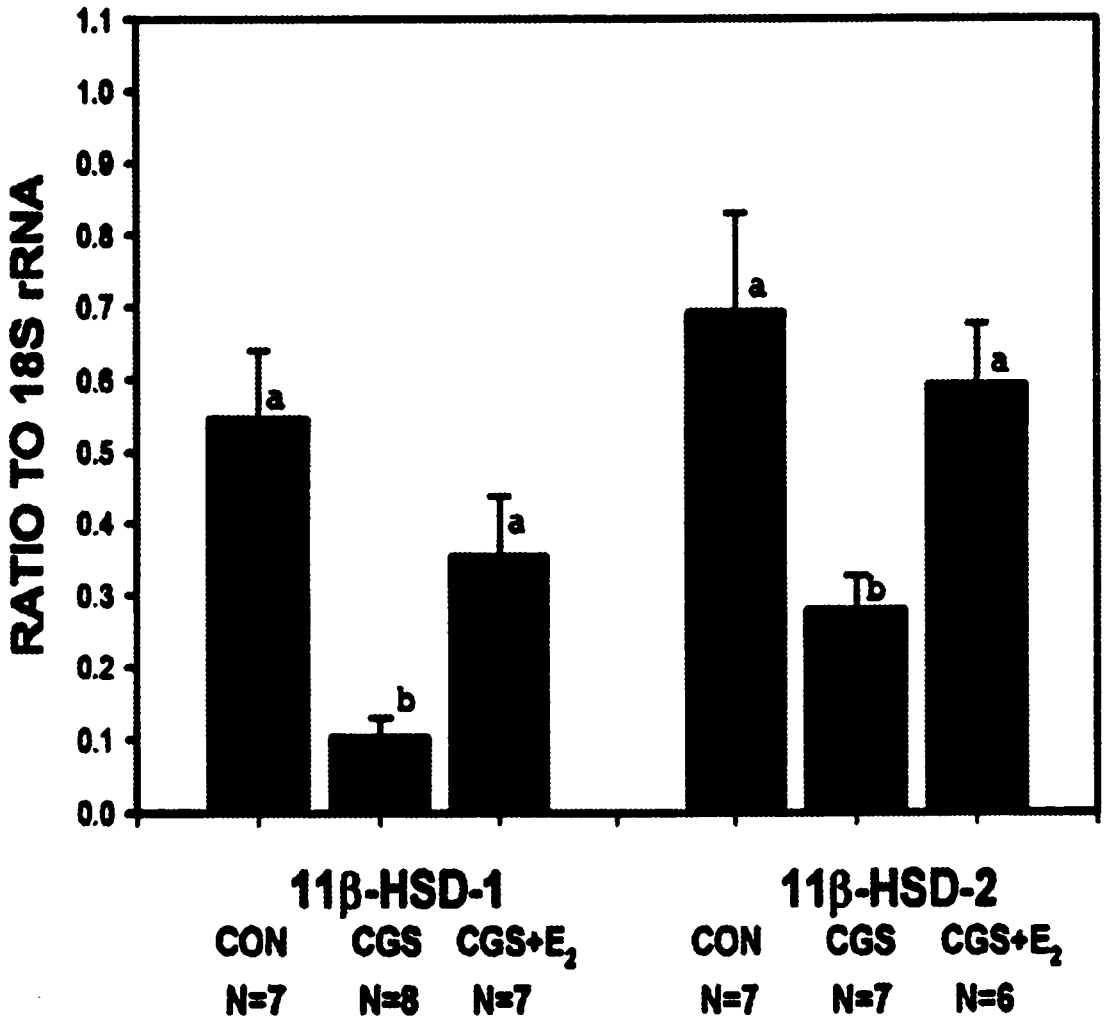

Figure 38. Relative expression of 11β-HSD-1 and -2 mRNA in isolated baboon placental syncytiotrophoblast cells from the agarose gels displayed in Figures 35 and 36. Values for 11β-HSD-1 are the same as in Figure 37. Values for 11β-HSD-2 are also from Figure 37 determined after deletion of statistical outliers. Values (mean ± SE) with different letter superscripts differ from each other at $P<0.01$ (ANOVA with multiple comparison of means by Student-Newman-Keuls Test).

expression was 0.594 ± 0.084, again a significant difference only from the CGS-20267 treatment alone ($P<0.01$), but not from the control group ($P>0.05$).

DISCUSSION

The results presented here are the first to unequivocally demonstrate that estrogen, *in vivo*, is essential for the transcriptional regulation of 11β-HSD-1 and -2 genes in the baboon syncytitrophoblast. CGS-20267, a potent and specific placental P450-aromatase enzyme inhibitor, when administered during the latter half of pregnancy, reduced maternal estrogen levels from approximately 2 ng/ml to less than 0.1 ng/ml (Figure 34). This estradiol diminution was associated with a significant decrease in placental syncytiotrophoblast mRNA levels of 11β-HSD-1 and –2.

Recently, Pepe and Albrecht demonstrated that the mRNA (via Northern analysis) and peptide levels (via Western analysis) of both 11β-HSD-1 and -2 in syncytiotrophoblast of the baboon placenta increased with advancing gestation (Pepe *et al.*, 1996a) and that baboon placental NAD^+-dependent 11β-HSD-2 activity was regulated by estrogen (Baggia *et al.*, 1990a). We also demonstrated that the promoter regions of both baboon 11β-HSD-1 and –2 genes are activated by estradiol in JEG-3 cells following transient transfection analyses *in vitro* (Pepe *et al.*, 1999b; Figure 28). The hormonal milieu to which a cell is exposed *in vivo* is not only complex but also modified during various physiologic states (i.e., pregnancy). This fact, plus the observation that the two transcriptional start sites of baboon 11β-HSD-1 are utilized in a tissue-specific manner, suggests that the exclusive utilization of immortalized cell lines for the study of regulation of the 11β-HSD-1 promoter, although valuable, may not permit unequivocal extension of findings to the *in vivo* situation. This *in vivo* demonstration of diminution of

baboon 11β-HSD-1 and –2 mRNA levels following the almost complete absence of estrogen is therefore remarkable.

Because of the distinct tissue expression of the 11β-HSD genes, elucidation/ verification of the cell-specific factors (i.e., estrogen) contributing to 11β-HSD-1 and -2 expression *in vivo* required unique cell-specific isolation/analysis studies to determine whether the promoter regions of these two genes are activated in baboon placenta following maternal/fetal estradiol reduction and/or enhancement. Because of the important role that both syncytiotrophoblast 11βHSD-1 and –2 enzymes play in regulating fetal-placental function in the baboon, i.e. activation of the fetal HPAA and subsequent fetal adrenal maturation (Pepe and Albrecht, 1995; Pepe *et al.*, 1996b), this study provides further amplifying evidence for the regulation of both baboon 11β-HSD-1 and –2 genes by estrogen. Following extended maternal/fetal estrogen reduction *in vivo* (day 100 – day 170) by CGS-20267, a highly-specific placental P450-aromatase enzyme inhibitor, semi-quantitative RT-PCR demonstrated a significant reduction in both 11β-HSD-1 and –2 mRNA in baboon placental syncytiotrophoblasts.

To spite the extremely large reduction in maternal serum estradiol level (about 20 fold decrease), the concentration of bioactive estradiol remained in the 10^{-9} mol/L range. Because the dissociation constant of estradiol binding to its placental receptor approximates 10^{-9} mol/L (Younes *et al.*, 1981), the level of estrogen here in CGS-20267-treated baboons may be sufficient to bind its receptor and elaborate a physiologic response (Albrecht *et al.*, 2000). At least one estrogen receptor, (ERα), has been localized to the baboon placenta syncytiotrophoblast (Figure 33). The presence/absence of the second receptor, (ERβ), has yet to be definitively resolved. Further studies utilizing gel-shift mobility assays

with/without ERα and/or ERβ antibodies, and DNAse I footprinting analyses of the 11β-HSD-1 and -2 promoter regions would prove valuable.

On the basis of cofactor preference (i.e., NAD^+), 11β-HSD-2 appears to be the major form expressed in the human placenta (Brown *et al.*, 1993; Stewart *et al.*, 1994; Stewart *et al.*, 1995; Krozowski *et al.*, 1995; Brown *et al.*, 1996a; Brown *et al.*, 1996b, Sun *et al.*, 1996, Sun *et al.*, 1998), baboon placenta (Pepe *et al.*, 1996a; Pepe *et al.*, 1996b; this research) and rat placenta (Li *et al.*, 1996; Roland *et al.*, 1996; Burton *et al.*, 1996). Expression of 11β-HSD-2 has been specifically localized by *in situ* hybridization (Roland *et al.*, 1996; Waddell *et al.*, 1998), immuncytochemistry (Krozowski *et al.*, 1995; Sun *et al.*, 1996; Pepe *et al.*, 1996b) and Northern analysis (Pepe *et al.*, 1996a) to placental syncytiotrophoblast cells. Despite this clear evidence for 11β-HSD-2 expression in the placenta, 11β-HSD-1 expression in the human placenta remains controversial (Burton and Waddell, 1999) and has only been demonstrated by Pepe and Albrecht in the baboon and human (Pepe *et al.*, 1996b; Pepe *et al.*, 1999a) and by Yang in the sheep placenta (Yang *et al.*, 1992; Yang, 1995). Despite readily apparent 11 β-HSD-1 bioactivity utilizing $NADP^+$ as a cofactor in human placental tissue fragments (Lakshmi *et al.*, 1993), Stewart (Stewart *et al.*, 1994b; Stewart *et al.*, 1995a) and Sun (Sun *et al.*, 1997) were unable to detect 11β-HSD-1 immunoreactivity and /or mRNA in human placental syncytiotrophoblast or whole villous tissue. Utilizing sensitive and specific RT-PCR analysis of human placental cell-enriched fractions, we have demonstrated a modicum amount of 11β-HSD-1 mRNA in cytotrophoblasts and an even smaller amount in syncytiotrophoblasts. On the other hand, detection of 11β-HSD-2 in both cell types was readily apparent at significantly larger quantities.

In summation, utilizing sensitive and reproducible comparative RT-PCR technology, we have definitively demonstrated a significant reduction in the *in vivo* expression of both 11β-HSD-1 and –2 mRNAs in baboon placental syncytiotrophoblast-enriched fractions following reduction of maternal serum estrogen by a highly-specific P450-aromatase enzyme inhibitor, CGS-20267. This discovery provides further amplifying evidence that estrogen, via regulation of placental 11β-hydroxysteroid dehydrogenase (11 β-HSD) catalyzed metabolism of cortisol and cortisone (Pepe *et al.*, 1988), determines the amount of maternal cortisol arriving within the fetus (Pepe and Albrecht, 1990) and thus regulates the ontogenetic development/function of the fetal hypothalamic-pituitary-adrenocortical axis (Pepe and Albrecht, 1998; Albrecht and Pepe, 1999).

CHAPTER VII

SUMMARY AND FUTURE DIRECTIONS

The goal of this research was to determine whether estrogen regulated the expression of the 11βHSD enzymes controlling secretion of maternal-derived cortisol into the fetus, and whether the latter regulated fetal adrenal maturation by enhancing hypothalamic and/or pituitary CRH/ACTH production. One of our studies clearly demonstrated that there was a developmental increase in fetal pituitary POMC mRNA expression between mid- and late gestation, and this increase could be induced at mid-gestation by maternal estrogen administration at mid-gestation, presumably via the estrogen-induced placental decrease in cortisol release into the fetal compartment. The increase in pituitary POMC mRNA was associated with a concomitant increase in pituitary ACTH peptide production. Collectively these results support the hypothesis that the decrease in placental-derived cortisol removes the feedback inhibition on the fetal pituitary and thereby permitting fetal pituitary ACTH to drive fetal adrenal maturation. These results also support the phenomenon that feedback inhibition by cortisol on the midterm fetal pituitary is a reality.

These results do generate some questions. For example, where and how early in fetal development are GRα and/or GRβ expressed in the baboon fetal pituitary and are the receptors co-localized with POMC? Where and how early in fetal development are PC1 and PC2 expressed in the baboon fetal pituitary? These questions could easily be ascertained by *in situ* hybridization and/or RT-PCR analysis of laser capture microscopy tissue of the fetal adenohypophysis. The absence of ERα and/or ERβ could also be determined to insure that the upregulation of POMC mRNA/ACTH peptide was indeed

indirect via the placental 11βHSD-1/-2 system. What role, if any, do placental CRH and placental ACTH play in the endocrine development of the fetal pituitary and/or adrenal? This could be ascertained by determining whether placental peptides are secreted into the fetus and whether levels are increased by estrogen.

A second significant discovery was that the concentration of CRH peptide and mRNA in the fetal baboon hypothalamus was similar at mid- and late gestation and that hypothalamic CRH peptide levels were not increased at mid-gestation following maternal treatment with estradiol. This indicated that the increase in pituitary POMC mRNA and ACTH peptide expression at term, and following maternal estrogen administration of estradiol at mid-gestation, is regulated, in part, by cortisol, acting on the pituitary and not the hypothalamus.

Thirdly, it was demonstrated that fetal adrenal PK-A activity is greater in late gestation when estrogen levels are elevated than at mid-gestation. Moreover, PK-A activity was experimentally increased at mid-gestation following premature elevation of estrogen to levels typically observed in late pregnancy, treatment paradigms altered placental cortisol metabolism and activated fetal pituitary ACTH release resulting in the onset of *de novo* cortisol production by the fetus (Pepe *et al.*, 1990). Additional studies in our laboratories demonstrated (Leavitt *et al.*, 1997; Aberdeen *et al.*, 1998) that treatment of baboon fetuses with betamethasone in late gestation suppressed fetal pituitary POMC mRNA expression, fetal serum ACTH levels and ACTH-R mRNA concentrations and 3β-HSD expression in the definitive zone of the fetal adrenal gland, whereas ACTH administration promoted maturation of the fetal adrenal transitional zone, but not the definitive zone (Leavitt *et al.*, 1999). Combined, these results suggest that

activation of fetal adrenal steroidogenic maturation requires, in part, an estrogen-regulated shift in placental conversion of maternal cortisol to biologically inactive cortisone, resulting in increased pituitary ACTH elaboration. Moreover, since estrogen production depends upon androgens of fetal adrenal origin, it would appear that the fetus is ultimately in control of the timing of the maturation of its own HPAA. A future research effort would be to investigate which transcription factors are responsible for the down-regulation of the ACTH-R mRNA in the fetal zone and the up-regulation of the ACTH-R mRNA in the definitive zone.

This work does not definitively preclude a direct effect of estrogen on the fetal pituitary corticotropes and/or fetal adrenal cells since fetal estrogen levels do increase ontogenetically, and prematurely at mid-gestation following maternal estrogen supplementation. Additionally, recent evidence indicates that ERα and/or ERβ are expressed in the fetal pituitary and adrenal glands (Hirst *et al.*, 1992; Enmark *et al.*, 1997; Brandenberger *et al.*, 1997; Albrecht *et al.*, 1999). ERα/β has also been demonstrated in the hypothalamic paraventricular nucleus (Couse *et al.*, 1997; Shughrue *et al.*, 1997; Hrabovszky *et al.*, 1998). Moreover, it (Albrecht and Pepe, 1995) has been shown that a negative feedback system exists *in utero* whereby placental product estrogen regulates maternal and fetal adrenal C_{19}-androgen production to maintain a physiologically normal balance of estrogen biosynthesis during primate pregnancy. Additionally, estrogen suppressed the responsivity of the baboon fetal gland to ACTH with respect to the formation of fetal-zone specific DHA, but not of transitional-zone specific cortisol, as determined *in vitro* (Albrecht and Pepe, 1987; Albrecht *et al.*, 1990) and *in vivo* (Pepe *et al.*, 1989).

In the second major part of this dissertation, the 5'-flanking regions of both baboon 11β-HSD-1 and 11β-HSD-2 genes were isolated and sequenced and subsequently shown to exhibit extensive nucleotide homology to their respective human genes. The baboon 11β-HSD-1 gene has two transcriptional start sites 93 nucleotides apart and it appears that the upstream start site is the major one utilized in liver whereas the placenta appears to utilize the downstream start site. The baboon 11β-HSD-2 gene also exhibited multiple transcriptional start sites in placenta and kidney, analogous to that in the human placenta, but not the human kidney (Agarwal *et al.*, 1995). The baboon 11β-HSD-1 promoter contains no TATA boxes, but two consensus CAAT boxes, which presumably regulate transcription initiation. The baboon 11β-HSD-2 promoter contains multiple GC boxes that probably regulate transcription. Moreover, gene transfection analyses in JEG-3 cells indicated that the 5'-flanking regions of both the baboon 11β-HSD-1 and -2 genes are responsive and transcriptionally activated by 17β-estradiol in the presence of a co-transfected ERα cDNA. These *in vitro* findings agree with our previous results (Pepe *et al.*, 1996a) which demonstrated an ontogenetic increase in 11β-HSD-1 and -2 mRNA and protein in baboon placental syncytiotrophoblasts.

Additionally, further support for the regulation of both baboon 11β-HSD-1 and -2 genes by estrogen was demonstrated utilizing sensitive and specific RT-PCR technology. The results presented here are the first to unequivocally demonstrate the *in vivo* transcriptional regulation of 11β-HSD-1 and -2 genes in the baboon placenta by estrogen. CGS-20267, a potent and specific placental P450-aromatase inhibitor, when administered during the latter half of pregnancy, reduced maternal estrogen levels from approximately 2 ng/ml to less than 0.1 ng/ml. This estradiol diminution resulted in a significant

decrease in placental syncytiotrophoblast mRNA levels of both 11β-HSD-1 and –2. This data is also consistent with recent observations in the nonpregnant rat uterus where estrogen potently up-regulated both 11β-HSD-1 and -2 expression (Burton and Waddell, 1999).

Because of the importance of placental 11β-HSD-1 and 11β-HSD-2 in regulating cortisol transfer to the fetus and thus the function of the fetal hypothalamic-pituitary adrenal axis in the baboon (Pepe and Albrecht, 1995), studies of the regulation of both 11 β-HSD-1 and 11β-HSD-2 promoters in primary cultures of baboon placental trophoblasts obtained from animals at various stages of gestation with/without hormonal treatment *in vivo* should enhance our understanding of the mechanism(s) by which these genes are regulated in the placenta. Further studies employing quantitative RT-PCR analyses of estrogen-supplemented and control baboons during early gestation would lend further support for the *in vivo* regulation of 11β-HSD-1 and –2 genes by estrogen. Additional studies utilizing gel-mobility shift assays (EMSA), DNase I foot-printing protection assays and promoter deletion transient transfection studies should be accomplished to determine which syncytiotrophoblast and/or JEG-3 nuclear proteins (ERα, ERβ, Sp1, AP-2, SF-1, SRC-1, SRC-2, CBP/p300, SMRT, NcoR, etc.) are binding and regulating specific 11β-HSD-1 and 11β-HSD-2 promoter regions. Also, *in vitro* transient transfection analyses of the interactions between co-transfected ERα and ERβ and the 11 β-HSD-1/luciferase and 11β-HSD-2/luciferase promoter constructs would prove valuable. What effect does ERβ have on the activation of 11β-HSD-1/luciferase and 11β -HSD-2/luciferase promoter constructs by ERα? Hall and McDonnell (1999) determined that ERβ functions as a transdominant inhibitor of ERα transcriptional activity and that

ERβ decreases the cellular sensitivity to estradiol. Utilizing EMSA technology, it can be learned which area of the 11β-HSD-1/luciferase and 11β-HSD-2/luciferase 5'-flanking regions bind ERα and/or ERβ. Is ERβ expressed in baboon placental syncytiotrophoblasts? Interestingly, many endocrine tissues, including the testes and ovaries, coexpress both ER subtypes (Hall and McDonnell, 1999). Also, the levels of ER α and/or ERβ mRNA could be determined quantitatively (RT-PCR) in syncytiotrophoblasts at different gestational ages with/without estrogen treatment. Are ERα and/or ERβ transcriptionally regulated by estrogen?

Another unanswered question concerns the role of cortisol and its receptors (GRα and GRβ) on the regulation of baboon 11β-HSD-1. In sheep, 11β-HSD-1 mRNA expression is enhanced adult liver but reduced in fetal liver following glucocorticoid administration *in vivo* (Yang *et al.*, 1994), whereas in the hippocampus, glucocorticoids and chronic-stress upregulate neural 11β-HSD-1 (Low *et al.*, 1994). Are GRα and/or GR β receptors expressed in baboon placental syncytiotrophoblasts? What are the local cortisol levels ontogenetically within the placenta? How do these levels compare to maternal and fetal serum cortisol levels?

Analogous studies would be to quantitatively determine (RT-PCR) the mRNA levels of 11β-HSD-1 and –2 in fetal tissues from baboons treated *in utero* with/without estrogen supplementation /amelioration. Are ERα and/or ERβ receptors and/or GRα and/or GRβ receptors expressed in these tissues? Perhaps both ERα and/or ERβ receptors are expressed in the liver and down-regulating 11β-HSD-1 and –2 expression, whereas in syncytiotrophoblast, only the ERα receptor is present and contributing to 11β-HSD-1 and –2 up-regulation.

Finally, the adenovirus gene therapy studies demonstrate an exciting tool to essentially "knock-down" an expressed gene in order to understand that gene's unique physiological role. Our preliminary work demonstrates the capability to repress gene expression *in vitro*. Whether this can be accomplished *in vivo* remains to be determined.

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PERSONAL

Birth: November 25, 1954 in Lansdale, Pennsylvania
Married; Father of two children, ages 15 and 12

EMPLOYMENT

Research Associate II
Medical College of Hampton Roads
Norfolk, Virginia 1987 to date

Responsibilities and accomplishments: Oversee laboratory operations employing four people conducting NIH-funded research in reproduction biology. Active in baboon husbandry, enzymology, molecular biology, *in situ* hybridization, cloning, RT-PCR and immunocytochemistry. Authored eight publications. Served on Radiation and Chemical Safety Advisory Committees.

Aviation Maintenance Officer, Naval Reserve
Norfolk, Virginia 1981 to date

Responsibilities and accomplishments: Currently serving as the Executive Officer with the Commander-in-Chief, U. S. Atlantic Fleet. Recent tours completed with the Naval Safety Center and Helicopter Mine Countermeasures Squadrons. Served Active Duty 1981 to 1987 in Norfolk and the Philippines. Supervised up to seventy personnel, prioritized/organized maintenance actions, managed programs, equipment and budgets. Schooled/experienced in TQM, logistics, hazardous material, personnel and financial management.

EDUCATION

Ph.D. in Biomedical Sciences
Old Dominion University / Eastern Virginia Medical School
Norfolk, Virginia
1991 – 2001 GPA 3.7 (168 credits)
Inducted into the National Academic Honor Society Phi Beta Phi

Master of Business Administration candidate
Old Dominion University
Norfolk, Virginia
1982 - 1987 GPA 3.5 (26 credits completed in 60 credit program)

Master of Science in microbiology and immunology
Temple University
Philadelphia, Pennsylvania
1978 - 1980 GPA 3.0 (44 credits)
Trained in all spectrophotometric and chromatographic procedures, bacterial and viral diagnostic techniques, protein and nucleic acid purification, enzymology and tissue culture. Thesis was published in Photochemistry and Photobiology in 1981.

Bachelor of Science in biology
Delaware Valley College of Science & Agriculture
Doylestown, Pennsylvania
1972 - 1977 GPA 3.3 (133 credits)
Earned the Burstein Award for the best senior research project. Named to the Dean's List six times.