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Glucocorticoid stimulates expression of corticotropin-releasing hormone gene in human placenta

(transcriptional initiation/pregnancy/positive feedback)

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ABSTRACT **Primary cultures of purified human cytotrophoblasts have been used to examine the expression of the corticotropin-releasing hormone (CRH) gene in placenta. We report here that glucocorticoids stimulate placental CRH synthesis and secretion in primary cultures of human placenta. This stimulation is in contrast to the glucocorticoid suppression of CRH expression in hypothalamus. The positive regulation of CRH by glucocorticoids suggests that the rise in CRH preceding parturition could result from the previously described rise in fetal glucocorticoids. Furthermore, this increase in placental CRH could stimulate, via adrenocorticotropic hormone, a further rise in fetal glucocorticoids, completing a positive feedback loop that would be terminated by delivery.**

Corticotropin-releasing hormone (CRH), one of the hypothalamic components of the hypothalamic–pituitary–adrenal axis (1, 2), is also present in human placenta, where its synthesis and secretion rise more than 20-fold in the 5 weeks preceding parturition (3, 4). The secretion of glucocorticoids by the adrenal gland of the human fetus also increases markedly during this same period (5), although the manner in which glucocorticoids interact with placental CRH is not known. Defining the nature of this interaction is a prerequisite to determining the role of these hormones in human pregnancy. To examine the effect of glucocorticoids on placental CRH gene expression, we have purified and characterized cultures of human cytotrophoblasts and performed blot hybridization analysis of RNA isolated from cultured cells.

METHODS

Cytotrophoblast Purification and Immunocytochemistry. Full-gestation human placentae were obtained at elective Caesarean section, and cytotrophoblasts were enzymatically dispersed, purified by Percoll gradient centrifugation, and cultured as described (6). For immunocytochemistry, cultures were washed in several changes of cold 0.01 M phosphate, pH 7.4/0.9% NaCl (PBS), and fixed for 10 min in cold acetone/methanol (1:1, vol/vol) or 2% paraformaldehyde in PBS. Slides were air dried and stored at −70°C until immunocytochemistry was performed. Acetone/methanol-fixed cells were allowed to react with anti-cytotrophoblast monoclonal antibody 18B/A5 (kindly provided by Y. W. Loke, Univ. of Cambridge) (7). Avidin–biotin–peroxidase (Vectastain, Burlingame, CA) and 0.05% diaminobenzidine (Sigma) were used to localize bound antibody. Paraformaldehyde-fixed cells were allowed to react with a polyclonal anti-placental alkaline phosphatase antiserum (Dako, Santa Barbara, CA) and horseradish peroxidase. All sections were lightly counterstained with Gill's hematoxylin.

Cell Culture. Cells were cultured at a density of 2×10^7 cells per 150-cm^2 flask and were exposed to 1 μM dexamethasone (Sigma) in absolute ethanol for either 4 hr (4-hr sample) or 24 hr (24-, 48-, and 72-hr samples) prior to trypsin digestion. Control cells were exposed to equal volumes of vehicle alone. All cells were released by brief exposure to 0.5% trypsin (Sigma) in calcium- and magnesium-free Hanks' balanced salt solution (GIBCO). Cells were spun at $2000 \times g$ at 4°C for 10 min and cell pellets were frozen at −70°C.

RNA Isolation and Blot Hybridization. RNA was prepared from cell pellets (8) after the addition of 400 pg per cell pellet of a 400 nucleotide-long synthetic sense-strand CRH complementary RNA standard (9), electrophoresed in 1.4% agarose containing 2.2 M formaldehyde, and blotted onto GeneScreen (DuPont). Hybridizations were performed using [α-^{32}P]UTP-labeled antisense complementary RNA probes (10). Riboprobe templates included the 375-base-pair (bp) *Xmn* I–*Pst* I fragment of the human CRH gene (9), the 746-nucleotide *Eco*RI fragment of a human glucocorticoid receptor (GR) cDNA encoding bases 1–2375 [isolated in our laboratory by screening a 34-week-gestation human placental cDNA library (Clontech Laboratories, Palo Alto, CA) (11)], and human β_2-microglobulin (β_2m) cDNA (12) [kindly provided by J. Seidman (Harvard Univ.)]. Blots were hybridized for 24 hr, washed as described (9), and exposed to Kodak X-AR film with an intensifying screen. Autoradiograms and photographs of ethidium bromide-stained gels were scanned with an LKB scanning densitometer. The amount of CRH and GR mRNA in each lane was corrected for differences in recovery based on the amount of β_2m mRNA, 18S rRNA, and synthetic CRH standard detected. All three recovery markers yielded equivalent results.

Radioimmunoassay. CRH in media was extracted by using C_{18} Sep-Pak cartridges (Waters Division of Millipore) and measured by a radioimmunoassay based on the method of Suda *et al.* (13), using the human/rat anti-CRH antiserum R12 (kindly provided by T. Suda, Tokyo Women's Medical College). This antiserum is not known to cross-react with any peptide other than intact CRH. This assay has a sensitivity of 2 pg per tube and an intra-assay coefficient of variation of 10%. Recovery of exogenously added CRH was 84.3% ± 6.4%.

Transcription Initiation Sites. Transcriptional initiation was determined by hybridizing CRH mRNA to a synthetic oligodeoxynucleotide complementary to a portion of the 5′ region of CRH mRNA. This hybrid was treated with RNase H (which specifically digests RNA in the region of DNA·RNA hybridization), yielding two CRH mRNA fragments: a 5′ fragment extending from the transcriptional initiation site to the beginning of the region of hybridization and a 3′ fragment

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Abbreviations: CRH, corticotropin-releasing hormone; GR, glucocorticoid receptor; β_2m, β_2-microglobulin; ACTH, adrenocorticotropic hormone.
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extending from the end of the hybrid to the 3′ terminus of CRH mRNA (Fig. 4*a*). The site of CRH mRNA transcriptional initiation was deduced from the size of the 5′ CRH mRNA fragment, the location of the region of DNA·RNA hybridization, and the human CRH gene DNA sequence (14). Ten micrograms of RNA was dissolved in 0.025 ml of 100 mM KCl/0.1 mM EDTA and mixed with 500 pmol of a synthetic oligodeoxynucleotide 23 bases in length and complementary to bases 470–492 of the human CRH gene (14). The mixture was heated to 65°C for 2 min and incubated at room temperature for 30 min. RNase H (Bethesda Research Laboratories) at 40 units/ml in 0.025 ml of 10 mM $MgCl_2$/80 mM KCl/1 mM dithiothreitol/0.5 mg of bovine serum albumin per ml/50 mM Tris·HCl, pH 7.5, was added, and the samples were incubated at 37°C for 30 min. RNA was extracted with phenol/chloroform, electrophoresed on a 5% acrylamide gel containing 7 M urea, and electroblotted onto GeneScreen (DuPont). RNA size markers were synthesized by using an RNA transcriptional vector into which was inserted a template encoding a rat CRH cDNA corresponding to nucleotides 175–2106 of the rat CRH gene (15) (which was cut with restriction enzymes to generate length markers 131, 283, 426 bases). The filter was probed with the ^{32}P-labeled 375-bp *Xmn* I–*Pst* I fragment of the human CRH gene as described.

Statistics. Statistical analyses were performed by using the unpaired Student's *t* test for comparisons between two groups and one-way analysis of variance with post hoc comparison of means for more than two groups. All tests of significance were two-tailed. Uncertainties are expressed as ± SEM.

RESULTS

Human cytotrophoblast cells were purified (6) and cultured for up to 96 hr, and the morphological transformation from cytotrophoblast to syncytiotrophoblast was confirmed by immunocytochemical staining. Staining with the cytotrophoblast-specific monoclonal antibody, 18B/A5 (7), was restricted predominantly to single cells and diminished as cells differentiated into syncytiotrophoblast cells (Fig. 1 *Left*). In contrast, staining with a polyclonal antiserum to placental alkaline phosphatase, which is specific for syncytiotrophoblast cells (16), increased progressively during the period of culture, being absent from single cytotrophoblast cells and increasing in intensity as syncytiotrophoblast cells formed (Fig. 1 *Right*).

Total mRNA was prepared from cultured trophoblasts and analyzed by blot hybridization. Blots were probed for mRNAs encoding CRH, GR, and β_2m, the latter being the constitutively expressed cell surface component of the major histocompatibility locus (17). CRH mRNAs in cultured trophoblast and term placenta were similar in size (Fig. 2). In three separate placental preparations, CRH mRNA was pres-

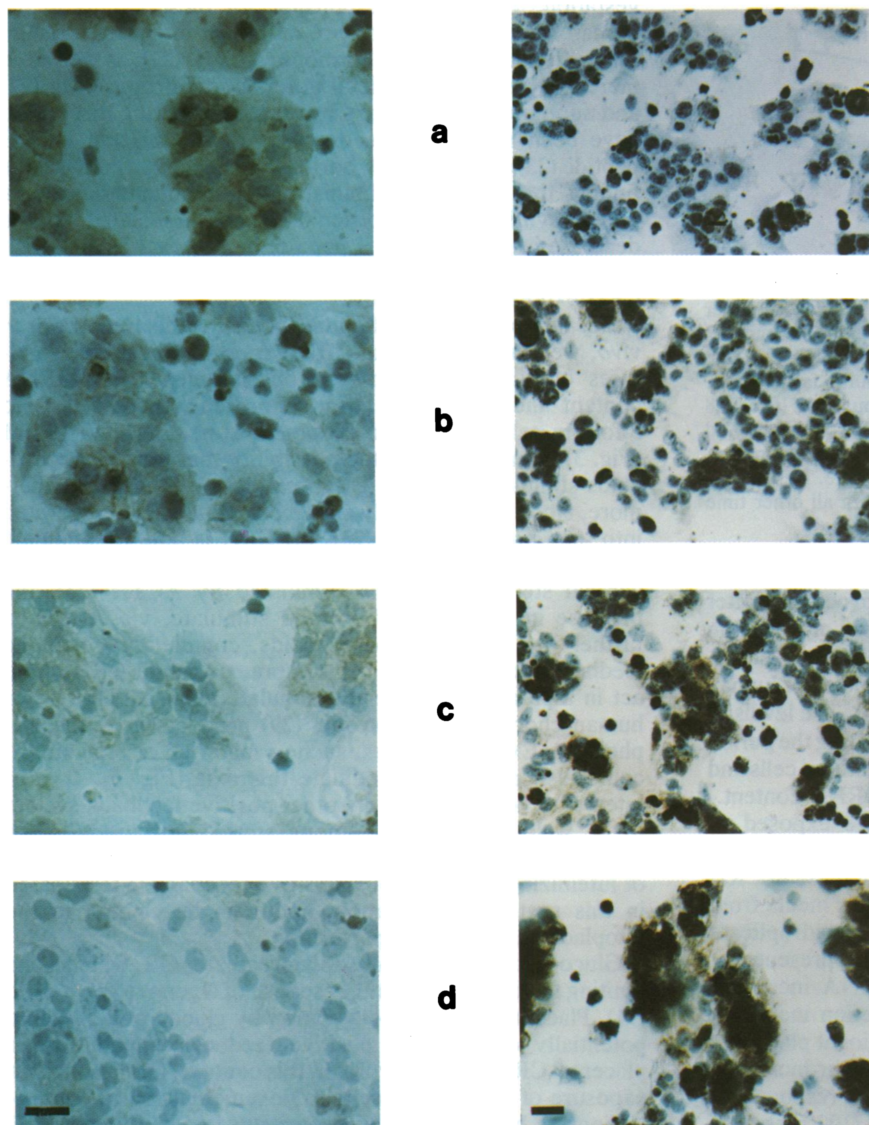

FIG. 1. Immunocytochemical staining of trophoblast cultured for 16 hr (*a*), 24 hr (*b*), 48 hr (*c*), and 72 hr (*d*). (*Left*) Cells were stained with the monoclonal antibody 18B/A5, which is predominantly localized to the cytotrophoblast cells (6). (*Right*) Cells were stained with a polyclonal antiserum to placental alkaline phosphatase, which is localized to syncytiotrophoblast cells (16). (Bars = 30 μm.)

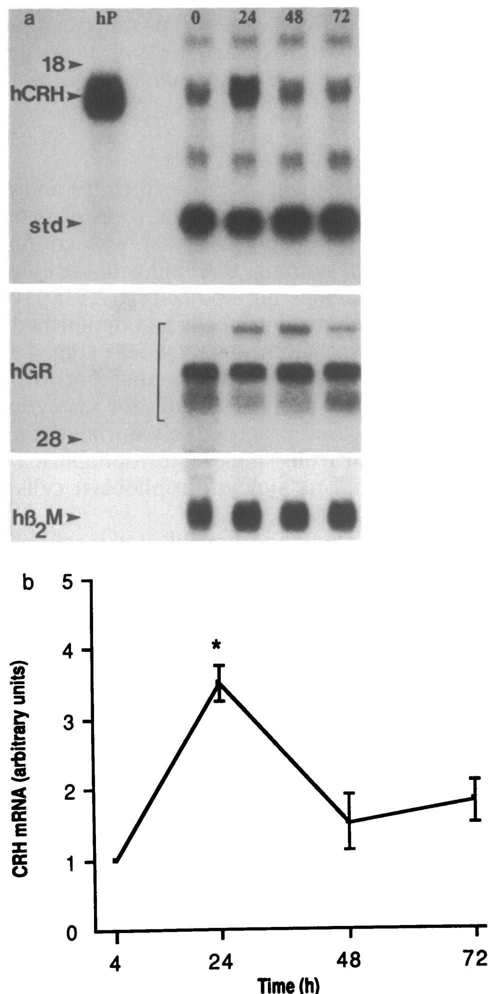

FIG. 2. Time course of mRNA expression in cultured trophoblast. (*a*) Blot hybridization analysis using 20 μg of total RNA per lane. (*Top*) Human (h) CRH mRNA (1.7 kb); (*Middle*) human GR mRNA (7.1, 6.1, and 5.6 kb); (*Bottom*) human β_2m mRNA (0.8 kb). Human placental (hp) RNA (1 μg) was analyzed in the far left lane. Hours in culture are indicated at the tops of the four right lanes; 28 and 18 denote the positions of 28S and 18S rRNA, respectively; and std denotes the position of the 400-nucleotide CRH cRNA standard. (*b*) Quantitation of trophoblast CRH mRNA over time. Results are mean ± SEM. * Denotes $P < 0.005$ compared with all other time points.

ent in both cytotrophoblast and syncytiotrophoblast; peak levels (3.5 ± 0.3-fold above baseline, $P < 0.005$) occurred after 24 hr in culture (Fig. 2), during the major period of transition from cytotrophoblast to syncytiotrophoblast. These observations are similar to those made in previous studies of human chorionic gonadotropin mRNA expression (18). The levels of GR and β_2m mRNA (Fig. 2) remained stable during the period of culture, indicating both the viability of the cultured cells and the specificity of the observed rise in CRH mRNA content.

After various times in culture, cells were exposed to dexamethasone, 1 μM, for 24 hr (Fig. 3). Dexamethasone treatment was associated with a 2- to 5-fold increase in CRH mRNA content and CRH peptide secretion into media from cells cultured for 24–72 hr (Fig. 3 *a* and *b*). In quadruplicate preparations of cells cultured for 24 hr in the presence of dexamethasone (Fig. 3 *c* and *d*), CRH mRNA increased 3.7-fold ($P < 0.001$) and CRH peptide secretion increased 2.3-fold ($P < 0.001$). In cultures of four additional placentae (each assayed in duplicate), CRH mRNA at 24 hr increased 3.7 ± 1.1-fold above baseline ($P < 0.05$) in response to dexamethasone. A dose of dexamethasone 1/100th as high (10 nM) also stimulated CRH mRNA by 4.6-fold (mean of duplicate determinations) in cells cultured for 24 hr. GR mRNA levels did not change significantly in response to dexamethasone at 1 μM or 10 nM (Fig. 3 and data not shown).

The transcriptional initiation site utilized by the CRH gene in these trophoblast cultures was identified by a technique employing oligonucleotide-directed RNase H cleavage (Fig. 4). The major CRH mRNA transcriptional initiation site [nucleotide 334 of the human CRH gene (13)] was 30 nucleotides downstream from a TATAAA sequence (Fig. 4*a*), as determined from identification of a major 5′ CRH mRNA fragment, 136 nucleotides long (Fig. 4*b*, − lane, position of lower asterisk), after RNase H treatment. Dexamethasone stimulated the accumulation of CRH mRNA transcribed from this same site (Fig. 4*b*, + lane). A minor 5′ CRH mRNA fragment approximately 255 nucleotides long (Fig. 4*b*, upper asterisk) indicated an additional site of CRH mRNA transcriptional initiation approximately 120 nucleotides upstream from the major initiation site. Both of these initiation sites have also been identified by primer extension analysis of term placental human CRH mRNA (ref. 4 and D.M.F., unpublished observations).

DISCUSSION

Production of fetal adrenal glucocorticoids, principally cortisol sulfate (19), and the size of the fetal zone of the adrenal cortex (20) increase markedly in the last 5 weeks of human gestation. The factors responsible for these events in the human have remained obscure, although in sheep, a rise in fetal adrenocorticotropic hormone (ACTH) (21), as well as an increased sensitivity of fetal adrenocorticotropic cells (22) and adrenocortical cells to trophic stimuli during this period have been reported. The dramatic rise in placental CRH peptide (23) and mRNA (4) during this time, together with the finding that maternal administration of betamethasone, a potent synthetic glucocorticoid, is not associated with a decrease in placental CRH peptide in maternal plasma (39), suggested that glucocorticoids might stimulate placental CRH expression in humans.

Because of obvious difficulties in testing this hypothesis *in vivo*, we studied placental CRH expression in primary cultures of purified human trophoblast cells. CRH mRNA content and peptide secretion into media increased 2- to 5-fold in cells exposed to dexamethasone, 1 μM to 10 nM (Fig. 3), consistent with the possibility that fetal glucocorticoids stimulate placental CRH expression *in vivo*. Furthermore, since (*i*) biologically active placental CRH is secreted into the fetal circulation (23), (*ii*) CRH is able to stimulate ACTH release from human fetal pituitary (24), and (*iii*) fetal adrenal steroidogenesis is stimulated by ACTH (25), the increase in placental CRH could stimulate, via ACTH, a further rise in fetal glucocorticoids, completing a positive feedback loop (Fig. 5). Furthermore, placental CRH could act in a paracrine manner to stimulate local ACTH (28) or human chorionic gonadotropin (29) production by the trophoblast. Extrinsic factors, such as maternal or fetal stress, could also potentially modulate this axis (Fig. 5). It is of interest to note that the only other positive feedback system to be well characterized among steroid hormones is the stimulatory effect of ovarian estrogens on the midcycle surge of luteinizing hormone (30). Positive feedback is terminated in this system by ovulation, and it is terminated in the fetoplacental system by parturition.

Glucocorticoid classically down-regulates GR, which is the limiting factor governing the response of a gene to the steroid (31). Placental GR down-regulation by glucocorticoid could potentially dampen the positive feedback stimulation of placental CRH by the steroid. In this context, our finding that exposure of trophoblast cells to dexamethasone in concentrations as high as 1 μM did not result in a decrease in GR

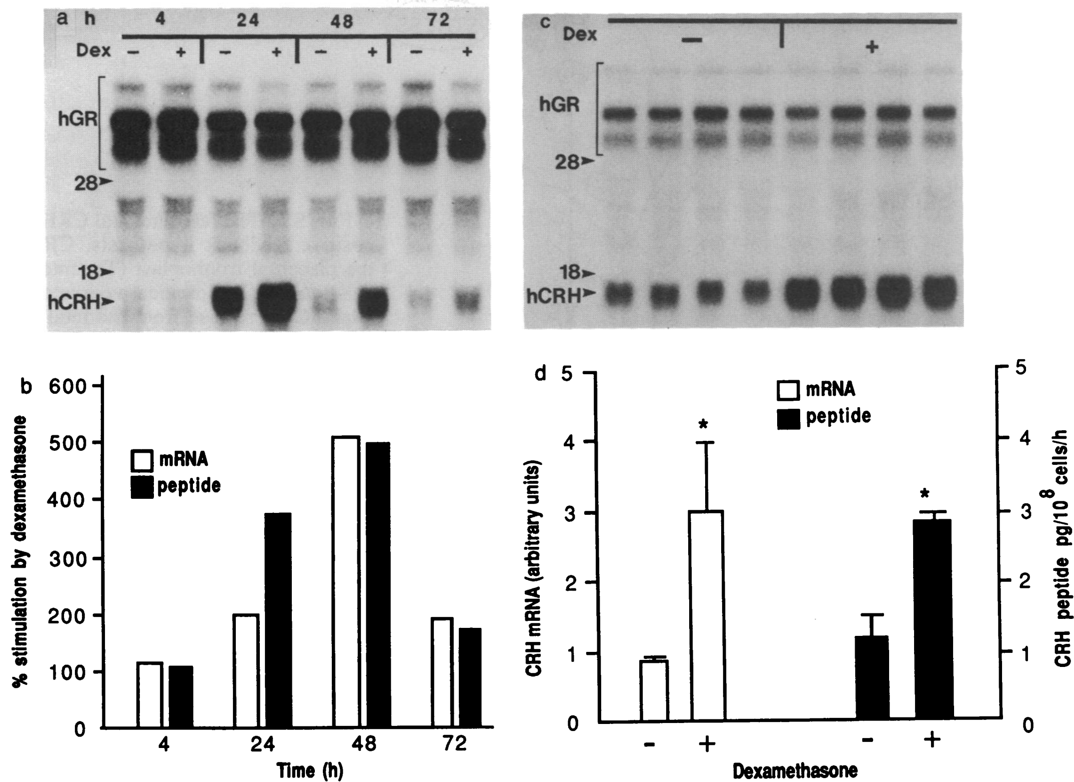

FIG. 3. Effect of dexamethasone on cultured trophoblast CRH and GR mRNA. (*a* and *b*) Time course of the effect of 1 μM dexamethasone on cultured trophoblast. (*a*) Blot hybridization analysis of CRH and GR mRNAs isolated from cells grown in the presence (Dex +) or absence (Dex −) of dexamethasone. Each lane contained 10 μg of total RNA (abbreviations as in Fig. 2). (*b*) Quantitation of CRH mRNA displayed in *a* together with CRH peptide levels in media at times shown. Histogram values are expressed as the percent stimulation in the presence versus absence of dexamethasone. In the absence of dexamethasone, basal levels of CRH mRNA were 1.0, 15.0, 1.8, and 1.7 arbitrary units, and levels of CRH peptide secretion were 0.3, 0.8, 2.4, and 1.3 pg per 10^8 cells per hr at 4, 24, 48, and 72 hr of culture, respectively. (*c* and *d*) Effect of 1 μM dexamethasone on CRH mRNA and CRH peptide levels in media after 24 hr in quadruplicate cultures. (*c*) Blot hybridization analysis of CRH and GR mRNAs, using 10 μg of total RNA per lane. (*d*) Quantitation of CRH mRNA displayed in *c* together with CRH peptide levels in media in the presence (+) and absence (−) of dexamethasone. *, $P < 0.001$ compared with − dexamethasone.

mRNA content is consistent with the presence of a CRH–glucocorticoid positive feedback loop in the fetoplacental unit. This response is very different from the fall in GR mRNA caused by dexamethasone in a variety of rat tissues (32) and mouse anterior pituitary cells (G. Adler and J.A.M., unpublished observations). Because progesterone is known to bind to GR (33), and its production by the trophoblast rises progressively during gestation (34), we examined the effect of progesterone treatment on the levels of GR and CRH mRNA in cultured trophoblast cells. Progesterone at 1–100 nM had no effect on the levels of these mRNAs in this system (B.G.R., unpublished observations).

Glucocorticoid has opposite effects on the regulation of CRH in placenta and hypothalamus. In both sites, CRH is a product of the same gene, located on the long arm of chromosome eight (35). In placental cultures, glucocorticoid stimulates an increase in CRH mRNA transcribed from an initiation site 30 nucleotides downstream from a consensus promoter element (Fig. 4). It will be of interest to determine whether this same promoter mediates the negative regulation

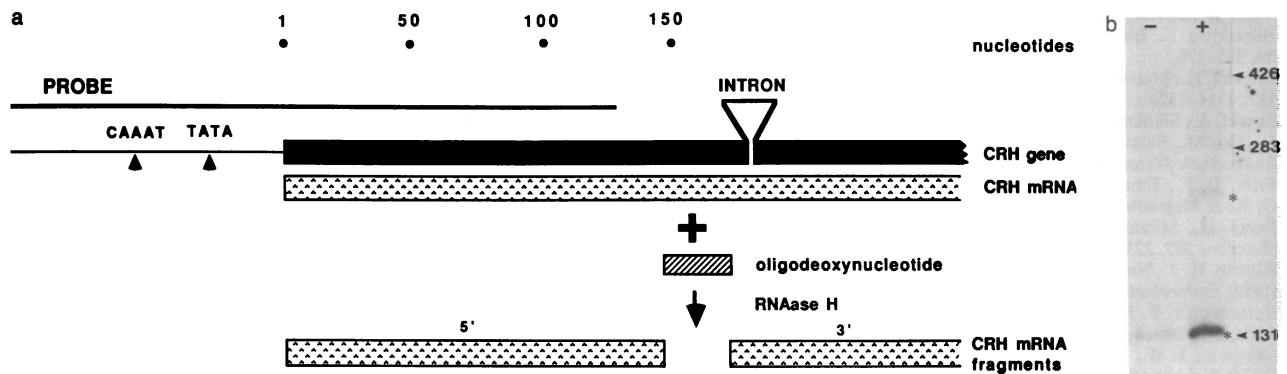

FIG. 4. Identification of CRH mRNA transcription initiation site in cultured trophoblast. (*a*) Schematic representation of transcription site mapping by using RNase H. See text for details. (*b*) RNA blot hybridization analysis of CRH mRNA treated with RNase H after isolation from trophoblasts cultured for 24 hr in the absence (−) or presence (+) of dexamethasone (1 μM). Upper and lower asterisks denote the positions of the minor and major 5′ CRH mRNA fragments, which indicate minor and major transcription initiation sites, respectively. Positions of RNA size markers (in nucleotides) are indicated.

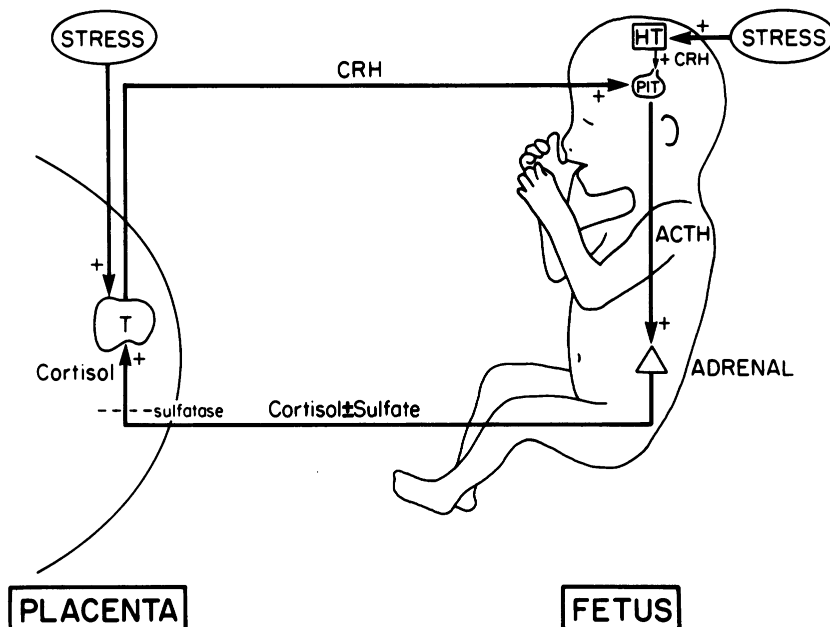

FIG. 5. The fetoplacental CRH-glucocorticoid positive feedback hypothesis. CRH, secreted by the placental trophoblast (T), enters the fetal circulation via the umbilical vein and stimulates (+) fetal ACTH release from the fetal pituitary (PIT). Fetal ACTH stimulates secretion of fetal adrenal cortisol sulfate, which enters the placental circulation via the umbilical artery. Cortisol sulfate may be converted to cortisol by placental sulfatase (26), and cortisol stimulates further placental CRH secretion, thereby completing the positive feedback loop. Fetal CRH, secreted from the fetal hypothalamus (HT), may independently stimulate fetal ACTH release, and placental and fetal hypothalamic CRH may be directly stimulated by environmental stresses. In addition, placental ACTH (27) may stimulate the fetal adrenal directly.

of hypothalamic CRH mRNA by glucocorticoid (2). Another example of a similarly regulated gene is the type IIA myosin heavy chain gene, which is regulated oppositely by thyroid hormone in different muscle types (36).

The role played by fetal adrenal steroidogenesis in human parturition is unknown. Studies of fetal anencephaly in humans (37) suggest that the proper timing of parturition depends upon the presence of an intact fetal hypothalamic-pituitary-adrenal axis. Glucocorticoids play an important role in the maturation of many fetal organ systems (38). An attractive feature of the fetoplacental CRH-glucocorticoid hypothesis is that fetal maturation and timing of parturition would, under most circumstances, be coupled. Such a relationship would be advantageous for neonatal survival. Further studies are necessary to determine the *in vivo* characteristics of this positive feedback loop and whether it has a physiological role in fetal maturation or parturition.

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