Evidence of direct estrogenic regulation of human corticotropin-releasing hormone gene expression. Potential implications for the sexual dimophism of the stress response and immune/inflammatory reaction.

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Research Article

Corticotropin-releasing hormone (CRH) plays major roles in coordination of the stress response and regulation of the immune/inflammatory reaction, two important functions associated with sexual dimorphism. Two overlapping segments of the 5' flanking region of the human (h) CRH gene, the proximal 0.9 kb (containing two perfect half-palindromic estrogen-responsive elements [EREs]) and the 2.4 kb (including the former and containing two additional perfect half-palindromic EREs), were examined for their ability to confer estrogen-mediated transcriptional enhancement to a homologous or heterologous promoter. The level of estrogen-induced transactivation by the 0.9- and 2.4-kb segments was determined by chloramphenicol acetyltransferase analysis in CV-1 cells cotransfected with estrogen receptor (ER) cDNA expression plasmids, and found to be respectively approximately 10% and 20% of that of the strongly estrogen-responsive Xenopus vitellogenin A2 enhancer. Gel retardation and immunoprecipitation demonstrated specific association between the perfect half-palindromic EREs of hCRH gene and the DNA binding domain of hER in vitro. These findings may constitute the basis of sexual dimorphism in the expression of the CRH gene in the central nervous system and periphery, and might shed light in existing gender differences in stress response and immune regulation.

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Evidence of Direct Estrogenic Regulation of Human Corticotropin-releasing Hormone Gene Expression

Potential Implications for the Sexual Dimophism of the Stress Response and Immune/Inflammatory Reaction

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Abstract

Corticotropin-releasing hormone (CRH) plays major roles in coordination of the stress response and regulation of the immune/inflammatory reaction, two important functions associated with sexual dimorphism. Two overlapping segments of the 5′ flanking region of the human (h) CRH gene, the proximal 0.9 kb (containing two perfect half-palindromic estrogen-responsive elements [EREs]) and the 2.4 kb (including the former and containing two additional perfect half-palindromic EREs), were examined for their ability to confer estrogen-mediated transcriptional enhancement to a homologous or heterologous promoter. The level of estrogen-induced transactivation by the 0.9- and 2.4-kb segments was determined by chloramphenicol acetyltransferase analysis in CV-1 cells cotransfected with estrogen receptor (ER) cDNA expression plasmids, and found to be respectively $\sim$ 10% and 20% of that of the strongly estrogen-responsive *Xenopus* vitellogenin A2 enhancer. Gel retardation and immunoprecipitation demonstrated specific association between the perfect half-palindromic EREs of hCRH gene and the DNA binding domain of hER in vitro. These findings may constitute the basis of sexual dimorphism in the expression of the CRH gene in the central nervous system and periphery, and might shed light in existing gender differences in stress response and immune regulation. (*J. Clin. Invest.* 1993. 92:1896–1902.) Key words: corticotropin-releasing hormone • gene regulation • receptors, estrogen • sex characteristics

Introduction

Corticotropin-releasing hormone (CRH),[1] a 41-amino acid peptide, plays a pivotal role in the coordination of the stress response and the regulation of the immune/inflammatory reaction (1–7). In the central nervous system (CNS), CRH modu-

1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyl transferase; CRH, corticotropin-releasing hormone; E_2, estradiol; ERE, estrogen-responsive element; GnRH, gonadotropin-releasing hormone; hER, human estrogen receptor; HPA, hypothalamic-pituitary-adrenal axis; HPG, hypothalamic-pituitary-gonadal axis; PVN, paraventricular nucleus; SERP, synthetic estrogen receptor peptide.

lates the synthesis and release of adrenocorticotropic hormone (ACTH) and mediates, together with norepinephrine, many hormonal, autonomic, behavioral, and immunological effects of stress (8–13). CNS CRH, thus, inhibits the reproductive and growth axes, stimulates the sympathetic system, causes enhanced arousal, and suppresses the immune system, the latter indirectly, through activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic system (3, 4, 7, 14, 15). Interestingly, CRH, designated immune CRH, is also secreted peripherally, in inflammatory sites, where it exerts direct proinflammatory actions (16, 17). The immune/inflammatory reaction appears, thus, as the outcome of two antithetical actions of CRH, one central, endocrine, which is inhibitory, and the other peripheral, auto/paracrine, which is stimulatory.

Sex differences in the regulation of the stress response, in general, and the HPA axis, in particular, as well as sexual dimorphism of the immune/inflammatory reaction, including susceptibility to autoimmune disease, have been described in various species, including the mouse, rat, and human (18–22). These studies have suggested that gonadal steroids interact in a regulatory manner with CNS and peripheral substrates of the stress response, including the HPA axis, and the immune/inflammatory reaction. Estrogens and androgens have been implicated in these differences, however, whether their effects are direct or via estrogen- or androgen-dependent mediators is unclear. The exact site(s) of this interaction and its mechanism(s) is (are) not well understood, but the central role that CRH plays in these functions suggests that this hormone may be a common key point of such regulation.

To examine the potential sex steroid regulation of the human (h)CRH gene, we cloned and sequenced 3.7 kb of its 5′ flanking region (23; N. C. Vamvakopoulos and G. P. Chrousos, unpublished data; and Gene Bank Accession No. X 67661) and performed a computer search for the presence of consensus regulatory elements (24). We found five perfect half-palindromic estrogen-responsive element (ERE) motifs, but no discernible complete androgen or glucocorticoid-responsive elements (25). Given the availability of a highly sensitive, hormonally dependent, and estrogen receptor-mediated transactivation assay of ERE enhancer activity (26), we undertook to examine the possible estrogenic enhancement of hCRH 5′ flanking region-regulated chloramphenicol acetyltransferase (CAT) reporter constructs and to compare its effect to that of *Xenopus* vitellogenin A2 gene enhancer (27). We also tested the ability of a synthetic peptide spanning the DNA binding domain of the human estrogen receptor, to bind specifically to the two perfect half-palindromic ERE sequences proximal to the transcription start site of the hCRH gene, using gel retardation and immunoprecipitation assays.

Methods

Materials

Restriction enzymes and lipofectin were obtained from Bethesda Research Laboratories (Gaithersburg, MD); cell culture products from Biofluids Inc. (Rockville, MD); activated charcoal, 17-β estradiol (E_2), O-nitrophenyl-β-D-galactopyranoside, and β-galactosidase from Sigma Chemical Co. (St. Louis, MO); [α-^{32}P]dCTP and [^{14}C]-chloramphenicol from ICN (Costa Mesa, CA); TLC silica gel from J. T. Baker Chemical Co. (Phillipsburg, NJ); ethyl acetate from Pierce Chemical Co. (Rockford, IL); X-ray film from Eastman Kodac Co. (Rochester, NY); and, acetylCoA and poly (dIdC) from Pharmacia Inc. (Piscataway, NJ). The plasmids used were as follows: (a) the promoterless plasmid pUCCAT carrying the CAT reporter gene in a pUC vector and plasmid pA$_{10}$CAT carrying a minimal enhancerless SV40 promoter driving the CAT reporter gene cloned in pBR322 (a gift of Dr. G. Pavlakis of National Cancer Institute); (b) plasmids EREtk-CAT (VtERECAT) containing the hormone-responsive enhancer of Xenopus vitellogenin A2, which confers estrogen control to the heterologous thymidine kinase promoter driving the CAT reporter gene and its parent vector pBLCAT8+ (27, 28) and pSG5HEO (ERcDNA), containing the human estrogen receptor coding region and expressing functional receptor, and its parent eukaryotic expression vector pSG5 (ΔERcDNA) (29), donated by Dr. P. Chambon, INSERM, France; and (c) plasmid RAS-βgal expressing β-galactosidase, which was used to monitor transfection efficiency (donated by Dr. R. Evans of the Salk Institute). The Bluescript (+) plasmid was from Stratagene Inc. (La-Jolla, CA). The CV-1 cells were obtained from American Type Culture Collection (Rockville, MD).

Oligodeoxynucleotides were synthesized in a model 394 DNA/RNA synthesizer (Applied Biosystems Inc., Foster City, CA), resuspended in 0.5 ml of distilled, deionized water, and quantified by absorbance at 260 nm. To prepare double-stranded oligonucleotides, equimolar amounts of complementary single-stranded oligonucleotides were mixed, heat denatured, and slowly cooled to room temperature. These oligonucleotides were then labeled with [α-^{32}P]dCTP by the Klenow fragment of DNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN), diluted in Tris-EDTA, and stored at $-20°C$. The synthetic peptide, SERP, spanning the DNA-binding domain (amino acids 183–261) of the human estrogen receptor was chemically produced in a model 431A peptide synthesizer (Applied Biosystems Inc.). 10 mol zinc per mol SERP were detected by atomic emission spectroscopy of the peptide preparation. Immunoprecipitations were performed with a mouse anti-human estrogen receptor (anti-hER) monoclonal antibody (IgG1 type) produced and characterized by Traish et al. (30), which was obtained from Affinity BioReagents (Neshanic Station, NJ). Sheep anti-mouse IgG coupled to magnetic beads (Dynabeads M-450) was purchased from Dynal Inc. (Great Neck, NY).

Methods

Preparation of phCRH0.9CAT (hCRH construct 1) and phCRH2.4CAT (hCRH construct 2) constructs. The derivation of these constructs is shown in Fig. 1. The 881-bp-long SalI to HindIII hCRH DNA fragment (phCRH0.9) was enzymatically removed from a 3.8-kb HindIII fragment including the hCRH gene that had been subcloned in a Bluescript vector (23), purified after agarose gel electrophoresis by the geneclean method, and quantified as previously described (31). This DNA fragment was ligated at a ratio of 10:1 (insert to vector) to similarly di-
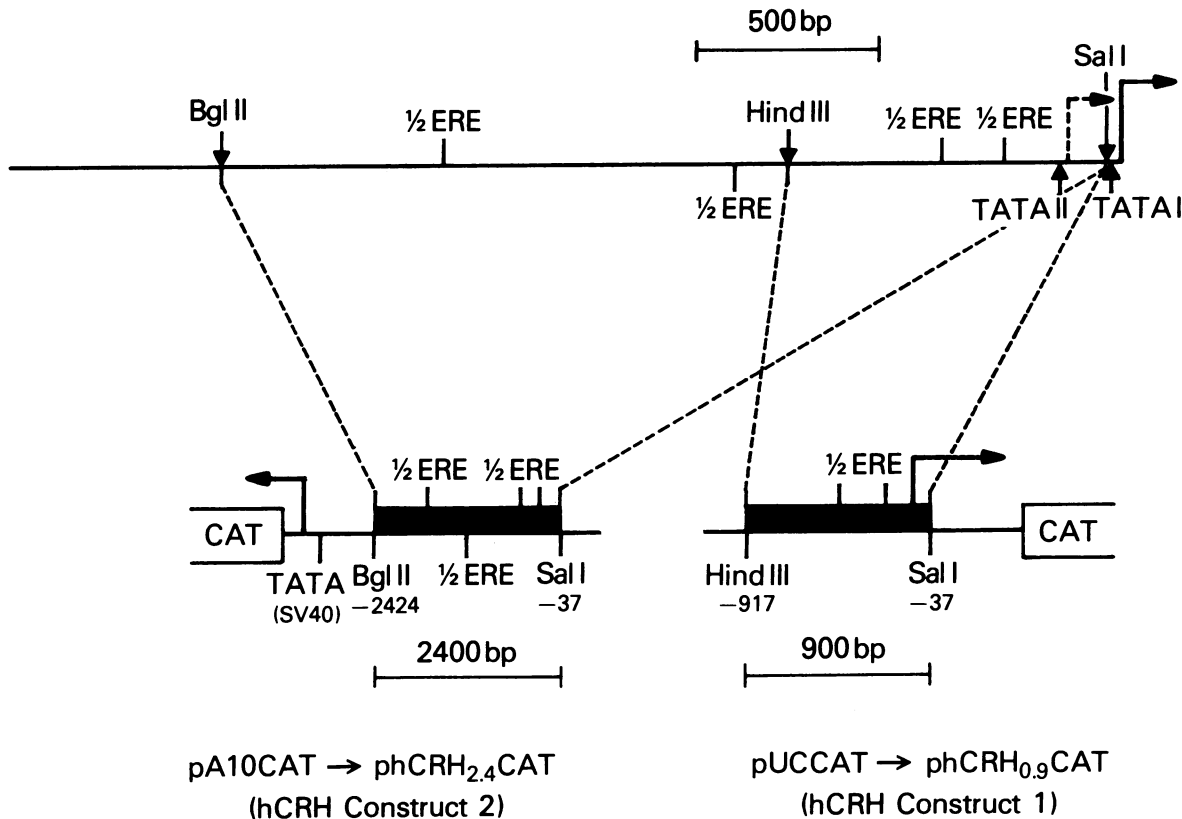
Figure 1. Derivation of hCRHCAT constructs. Shown on top is a portion of clone 11. The bent arrows, solid and dashed, point to the direction of TATA I– and TATA II–driven transcription, respectively. The half-palindromic EREs (*1/2 ERE*), shown above or below the midline, represent the two orientations of the element. The hCRH construct 1 uses the hCRH TATA II promoter and 0.9 kb of enhancer, while the hCRH construct 2 uses an SV40 promoter and 2.4 kb of hCRH enhancer, including that of hCRH construct 1 in inverted orientation.

gested and purified pUCCAT vector, and the product was used to transform competent *Escherichia coli* DH5α cells. Transformants were grown in a small volume of Luria-Bertani broth with ampicillin overnight and miniprep DNA was fractionated in agarose gel to screen for phCRH0.9 incorporation. The identity of the candidate clone was further verified by double digestion and migration relative to 881 SalI/ HindIII fragment and plasmid DNA from hCRH construct 1 was prepared for transfection studies. The 2.4-kb BglII to SalI hCRH DNA fragment was removed from clone 11. This is a subclone of 6.6-kb genomic hCRH containing phage DNA in a bluescript vector generated by partial HindIII digestion. Clone 11 contains a 3.8-kb HindIII fragment including the hCRH gene and a 2.8-kb HindIII segment extending into the 5' upstream area of the gene. The 2.4-kb BglII to SalI fragment was purified and ligated to similarly digested and purified pA10CAT vector, as described for the hCRH construct 1, generating the hCRH construct 2.

Cell culture and DNA transfection. Cells were maintained in 90% DME supplemented with 10% charcoal-treated FCS (31), glutamine (286 mg/liter), penicillin (100 μg/ml), streptomycin (100 μg/ml), and kanamycin (50 μg/ml). They were cultured in 6-mm petri dishes at 37°C under 95% O_2 – 5% CO_2 until 70% confluent, and washed with DME before transfection with DNA–lipofectin complex, prepared as described by the supplier. The DNAs transfected were phCRH0.9CAT (15 μg), phCRH2.4CAT (15 μg), VtERECAT (15 μg), ERcDNA (4 μg), Δ(ER)cDNA (4 μg), and RAS-βgal (1 μg) per plate. The latter was used to control for efficiency of transfection. A total of 20 μgg of DNA per transfection was used. The DNA was dissolved in 50 μl of water and mixed with an equal volume of lipofectin (25 μg) diluted 1:1 in water. The 100-μl transfection mixture was delivered to properly prepared CV-1 cells, under constant agitation in an horizontal shaker over 6 h. Cell growth was resumed in 5% charcoal-treated FCS serum supplemented media in the presence or absence of 10^{-7} M E_2. Cells were harvested 48 h after transfection.

Assay for CAT activity. CAT activity was determined as previously described (32). Quantitation of the conversion achieved was performed in a betascope radioactive counting device (Betascope 603 blot analyzer, Betagen Co., Waltham, MA). The degree of acetylation depended linearly on the amount of added protein. Assay for β-galactosidase was done as previously described (33).

Gel retardation. Complex formation between SERP and the various test oligomeric DNAs was first assessed by gel retardation. The binding reaction was started by adding 1 μl (about 0.5 pmol) of the appropriate ^{32}P-3'-end-labeled synthetic oligonucleotide to 9 μl of binding assay buffer containing 5 μg of SERP and 1 μg of poly(dIdC), as previously described (34), and allowed to proceed at room temperature for 30 min. Samples were then run on a 8% polyacrylamide gel (30:1 acrylamide to bisacrylamide) that had been pre-electrophoresed at 30 mA in high salt buffer (35) at room temperature.

Immunoprecipitation. Complex formation between SERP and the various test oligomeric DNAs was also assessed by immunoprecipitation with a mouse anti-hER monoclonal antibody followed by a 12% SDS-PAGE. Complexes prepared with or without SERP, as described above, were further incubated at 4°C overnight, after addition of 10 μl of anti-hER antibody. At that time, 250 μl of PBS containing 0.05% Tween-20 and 50 μl of a suspension of magnetic beads coated with sheep anti–mouse IgG (Dynabeads M-450), were added. The tubes were then rotated at 4°C for 30 min and the precipitates were washed five times with 500 μl of PBS containing 0.05% Tween-20. The complexes were then eluted from the beads by heating to 95°C in 100 μl of SDS sample buffer and analyzed on a 12% SDS-polyacrylamide gel, as previously described (36).

Results

Two hCRH 5' flanking region-driven CAT constructs were prepared and transiently expressed in CV-1 cells, supplemented with a cotransfected ER cDNA expression plasmid.
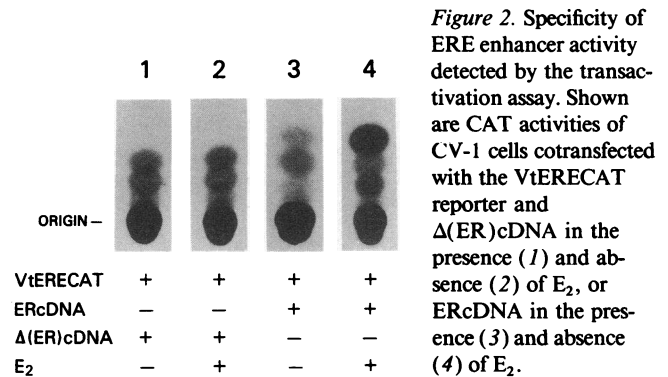
Figure 2. Specificity of ERE enhancer activity detected by the transactivation assay. Shown are CAT activities of CV-1 cells cotransfected with the VtERECAT reporter and Δ(ER)cDNA in the presence (1) and absence (2) of E_2, or ERcDNA in the presence (3) and absence (4) of E_2.

	1	2	3	4
VtERECAT	+	+	+	+
ERcDNA	−	−	+	+
Δ(ER)cDNA	+	+	−	−
E_2	−	+	−	+

Fig. 1 shows the derivation of these constructs. Construct 1 uses a homologous promoter to drive CAT activity (37) and contains two perfect half-palindromic EREs within the 0.9 kb of hCRH 5' flanking sequence included. Construct 2 is driven by a heterologous promoter, regulated by a 2.4-kb 5' flanking segment of the hCRH gene, which includes the hCRH segment of construct 1. The CRH enhancer of construct 2 is positioned in an opposite orientation relative to that in the hCRH gene and contains a total of four perfect half-palindromic EREs. A *Xenopus* vitellogenin A2 CAT construct was used as the natural model of a biologically potent ERE for comparisons.

Fig. 2 shows that the transfection assay employed depended directly on the presence of all the components of the estrogen transduction pathway examined, including a functional ERE-containing CAT construct, functional ER, and its ligand. Thus, CAT activity in the absence of added functional ER was minimal and independent of the hormone (lanes 1 and 2), and hormonally dependent CAT activity was mediated by exogenously added ER, since endogenous cellular ER levels were not sufficient to elicit a CAT effect (lanes 3 and 4). Table I summarizes the results of hCRH ERE activity expressed relative to that of *Xenopus* vitellogenin A2 enhancer. Construct 2, having four perfect half-palindromic EREs, gave a more potent estrogenic effect than construct 1, which had only two of these elements. These effects, were 10–20% of that obtained with the *Xenopus* vitellogenin CAT construct. The three different CAT constructs, had comparable baseline activity in transfections with Δ(ER)cDNA, in the presence or absence of E_2, and with ERcDNA, in the absence of estradiol.

The stimulation of transcription of the two hCRH promoter-driven CAT constructs by E_2 indicated that the ER may bind to the perfect half-palindromic ERE sequences present in the promoter region of the hCRH gene. Analogous binding of the ER has been demonstrated in the ovalbumin gene (34). To test this hypothesis, we synthesized 35-mer oligonucleotides containing the two perfect half-palindromic EREs present in common in both hCRH constructs examined (hCRH-1, hCRH-2), a perfect ERE, a mutated half-palindromic ERE (hCRH-2 Mut), and a fragment lacking an ERE but containing a TRE consensus (Fig. 3 A), and examined their ability to bind to a 79-amino acid synthetic peptide spanning the DNA binding domain of the human ER (SERP) (Fig. 3 B). Gel retardation analysis revealed characteristic complexes migrating as double bands between SERP and either perfect ERE, hCRH-1, or hCRH-2 (Fig. 3 C, lanes 1–3), but not between SERP and hCRH-2 Mut or TRE (Fig. 3 C, lanes 4 and 5). Immunoprecipitation analysis revealed, as expected, no im-

Table I. Relative ERE Enhancer Activity of the CAT Constructs Examined

DNAs transfected	Percent CAT activity*		Percent CAT conversion per hour*	Relative ERE strength[‡]
	$-E_2$	$+E_2$		
1. hCRH construct1 + ERcDNA	1.02	2.94	0.16	11.4
2. hCRH construct1 + Δ(ER) cDNA	1.05	1.10	—	—
3. hCRH construct2 + ERcDNA	2.6	5.7	0.26	18.6
4. hCRH construct2 + Δ(ER) cDNA	2.5	2.6	—	—
5. VtERECAT + ERcDNA	1.86	4.67	1.4	100
6. VtERECAT + Δ(ER) cDNA	1.99	2.04	—	—

* The extracts were heat-inactivated and incubated at 37°C for 12 h, except for reactions 5 and 6, that were incubated at 37°C for 2 h. Transfections and subsequent analyses were performed in triplicate, and efficiencies were internally monitored by cotransfecting small amounts of a β-galactosidase producing plasmid. ‡ The CAT conversion rates per hour calculated from 2-h incubations of reactions 1 and 3 was 0.1% and 0.14%, respectively, with corresponding ERE strengths of 7.1% and 10% relative to VtERECAT for CRH1CAT and CRH2CAT DNAs, respectively.

munoprecipitates in the absence of SERP (Fig. 3 *D*, lanes *1, 3, 5, 7,* and *9*). Prominent immunoprecipitates were observed between SERP and ERE, hCRH-1 and hCRH-2, while very weak or nonexistent immunoprecipitates were seen between SERP and hCRH-2 Mut or TRE (Fig. 3 *D*, lanes *2, 4, 6, 8,* and *10,* respectively).

Discussion

Perfect half-palindromic EREs in the 5' flanking region of the chicken ovalbumin gene were recently shown to confer estrogenic regulation to an adjacent reporter CAT gene in a transient expression assay (34), indicating that they may have a role in estrogen-induced enhancement, in addition to other potential roles as regulatory elements interacting with "orphan" receptors (41, 42). Our transfection studies provide evidence for direct estrogenic transcriptional regulation of the hCRH gene and suggest that, as with the chicken ovalbumin gene, its perfect half-palindromic EREs may be of functional significance. This is supported by the fact that transcriptional enhancement was dependent on the presence of estrogen receptor and estradiol and by our in vitro studies, demonstrating specific association between the two TGACC motifs proximal to the transcription start site of the hCRH gene, present in both enhancer segments studied, and the 79-amino acid synthetic peptide spanning the DNA binding domain of the human estrogen receptor. The latter contained excess zinc, presumably required for the formation of zinc fingers and binding to DNA. A similar 71-residue synthetic peptide spanning the DNA binding domain of hER (amino acids 180–250), has been shown to bind zinc and fold in its proper DNA-binding fingerlike conformation (43).
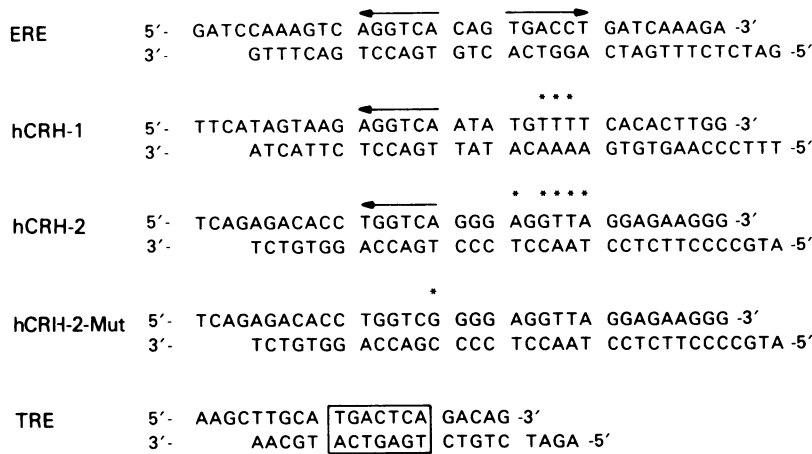
The estrogenic enhancement observed was weak compared to that observed with the *Xenopus* vitellogenin A2 enhancer. It was, however, specific and reproducible. This regulatory property of the human gene may explain the high level of CRH expression seen in the human placenta (44) and is further supported by our observation of hCRH gene expression in the estrogen receptor positive human breast cell line MCF-7 (N. C. Vamvakopoulos, unpublished observations) and rat and human ovaries (45). hCRH gene expression is stimulated by glucocorticoids in the placenta (46), and the mechanism of this is unclear, in light of the lack of a discernible complete glucocor-

ticoid responsive element in the 3625-bp 5' flanking the gene. Synergism of estrogens and glucocorticoids, however, has been noted in the chicken ovalbumin gene (47), suggestive of some interaction between the two pathways, which might explain this phenomenon.
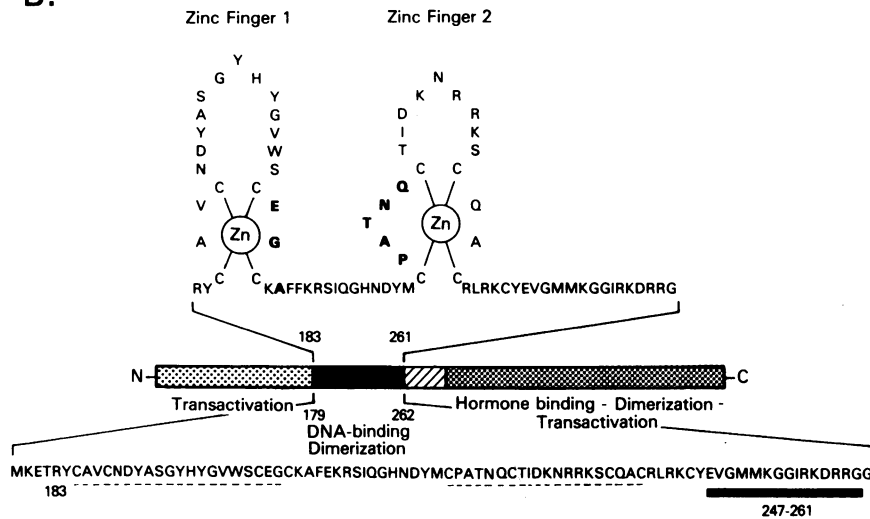
Estrogen receptors are widely distributed in the brain, including the limbic system and the hypothalamus (48). Our findings implicate the CRH gene and, therefore, the HPA axis, as a potentially important target of ovarian steroids and a potential mediator of gender related differences in the stress response and HPA axis activity. Data obtained in rats, demonstrating that chronic estradiol treatment of ovariectomized animals stimulates paraventricular nucleus (PVN) CRH mRNA levels (49) and increases ACTH and corticosterone secretion basally and in response to stress (50), and the demonstration of elevated PVN CRH mRNA in the afternoon of proestrous, at the approximate time of the estrogen-induced preovulatory surge of luteinizing hormone (51), support this concept. These effects of estrogens on the CRH neuron suggest that the hypothalamic-pituitary-gonadal (HPG) axis, which is known to be inhibited by hormones of the HPA axis at the hypothalamic, pituitary, gonadal, and sex steroid target tissue levels during stress (14, 15), appears to be also influencing the latter in a positive fashion, by slightly enhancing CRH gene transcription. Thus, these data support a mutual, bidirectional interaction between the HPG and HPA axes. In addition to explaining the slightly increased, basal and stress-stimulated HPA axis function in the female gender, the estrogen-induced enhancement of the CRH neuron may also help explain the paradox of negative estrogen feedback effect on the hypothalamic gonadotropin-releasing hormone (GnRH) neuron (52), which, unlike the PVN, lacks estrogen receptors (53). The negative estrogen feedback might, thus, be exerted indirectly, via a subgroup of CRH neurons. CRH has been reported to suppress GnRH secretion through a direct and an indirect, arcuate nucleus proopiomelanocortin/β-endorphin–mediated path (15, 54).

The immune/inflammatory reaction is greater in female than in male animals and humans, and in keeping with this, autoimmune inflammatory disease has significantly higher prevalence in the female than the male sex of several species (55). Markedly elevated secretion of immune CRH in various inflammatory sites has been demonstrated in the Lewis rat (16, 17, 56), an animal model of increased susceptibility to autoim-
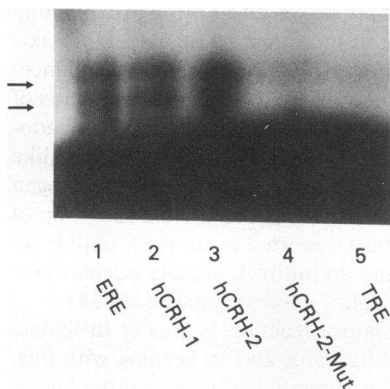
A.

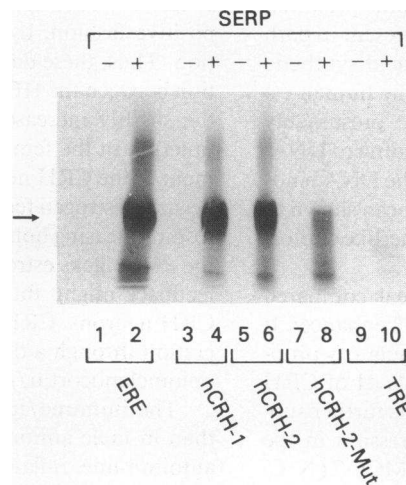|							
ERE	5'-	GATCCAAAGTC	AGGTCA	CAG	TGACCT	GATCAAAGA -3'	
	3'-	GTTTCAG	TCCAGT	GTC	ACTGGA	CTAGTTTCTCTAG -5'	
hCRH-1	5'-	TTCATAGTAAG	AGGTCA	ATA	TGTTTT	CACACTTGG -3'	
	3'-	ATCATTC	TCCAGT	TAT	ACAAAA	GTGTGAACCCTTT -5'	
hCRH-2	5'-	TCAGAGACACC	TGGTCA	GGG	AGGTTA	GGAGAAGGG -3'	
	3'-	TCTGTGG	ACCAGT	CCC	TCCAAT	CCTCTTCCCCGTA -5'	
hCRH-2-Mut	5'-	TCAGAGACACC	TGGTCG	GGG	AGGTTA	GGAGAAGGG -3'	
	3'-	TCTGTGG	ACCAGC	CCC	TCCAAT	CCTCTTCCCCGTA -5'	
TRE	5'-	AAGCTTGCA	TGACTCA	GACAG -3'			
	3'-	AACGT	ACTGAGT	CTGTC TAGA -5'			

B.

C.

D.

Figure 3. Formation of perfect half palindromic CRH enhancer ERE-ER complexes in vitro. (*A*) Sequences of the synthetic 35-mer oligonucleotides used in gel retardation and immunoprecipitation experiments. Half-palindromic motifs are overlined with an arrow, indicating their direction. The top strand of the hCRH-1 element spans from nucleotide position −329 to −295 and the top strand of hCRH-2 from −497 to −463, relative to the transcription start site of the hCRH gene promoter (Gene Bank Accession No X67661). The stars over oligonucleotides hCRH-1 and hCRH-2 indicate the nucleotide changes compared with the complete perfect palindromic ERE (34). The star in oligonucleotide hCRH-2-Mut indicates the nucleotide change compared to hCRH-2. In oligonucleotide TRE (AP-1 factor binding TPA-responsive element [38]), the TPA-responsive element is boxed. (*B*) Domain structure of the hER (39, 40). The primary and secondary structures of SERP, which spans amino acids 183–261 of the DNA binding domain of human ER are shown. The synthetic peptide 247–261, underlined by a continuous line, was used as the antigen in the generation of the anti-hER monoclonal antibody employed (30). The segments of the DNA-binding domain of the ER that participate in the formation of zinc fingers are underlined by an dashed line. The amino acid residues involved in the discrimination of DNA binding sites are shown in boldface type in the secondary structural model. (*C*) Gel retardation of complexes formed between SERP and oligonucleotides containing complete perfect, perfect half-palindromic, mutant half-palindromic and non-ERE controls (see *A*). The oligonucleotides were 3'-^{32}P-labeled and complex formation was assayed as described in Methods. Double-electrophoretic bands were seen between ERE, hCRH-1, or hCRH-2 and SERP (lanes *1-3, arrows*) but not between hCRH-2-Mut or TRE and SERP (lanes *4* and *5*). (*D*) Immunoprecipitation analysis of complexes formed between SERP and oligonucleotides containing complete perfect, perfect half-palindromic, mutant half-palindromic and non-ERE controls (see *A*). 3'-^{32}P-labeled oligonucleotides were incubated in the absence (−) or presence (+) of SERP, the complex was immunoprecipitated, and the oligonucleotides bound were analyzed on a 12% SDS-PAGE, as described in Methods. Only the complete perfect and perfect half-palindromic ERE-containing oligonucleotides were immunoprecipitated (lanes *2, 4,* and *6, arrow*).

mune inflammatory disease, in which we have demonstrated also decreased hypothalamic CRH secretion and, hence, diminished glucocorticoid production and defective suppression of inflammation (56–58). Although the decreased production of CNS CRH and increased secretion of immune CRH are associated with the high susceptibility of this animal to autoimmune inflammatory disease in both sexes, both the susceptibility and the actual inflammatory responses, including expression of immune CRH in peripheral inflammatory sites, are greater in the female than the male (59, 60). Estrogen-mediated enhancement of immune CRH secretion might be an explanation for this sexual dimorphism in the Lewis rat, as well as, albeit to a lesser extent, in other rat strains or animal species (61). These findings suggest that homeostatic regulation involves complex mutual interactions between the reproductive axis, HPA axis and the immune system, in which estrogens and CRH may be playing central roles. Certainly, other molecules involved in the regulation of these axes, such as several neurotransmitters, cytokines, and lipid mediators, may also participate in the above interactions and may contribute to their sexual dimorphism.

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