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Structural organization of the 5' flanking region of the human corticotropin releasing hormone gene

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We have determined the nucleotide sequence of the proximal 3625 nucleotides 5' flanking the major mRNA start site of the human corticotropin releasing hormone gene (hCRH) and identified several putative regulatory elements. Interestingly, we did not detect any glucocorticoid responsive elements; we did however find five interspersed perfect half palindromic estrogen responsive elements, which might confer estrogen regulatability to the hCRH gene. We have identified a segment spanning from −2835 to −2972, which has about 72% homology to the 3' terminal half of the human Alu I family of highly repetitive elements, and another one, which spans from −2213 to −2580 and has greater than 80% homology to members of human type O family of repetitive elements. These elements may confer DNA fragility, since the loci for hCRH and the human fragile site FRA8F colocalize in human chromosome 8. The structural information reported represents a first step in the study of regulation of the hCRH gene at the molecular level.

KEY WORDS: Corticotropin releasing hormone, regulatory region, repetitive elements, regulatory elements.

ABBREVIATIONS: Corticotropin Releasing Hormone (CRH), Glucocorticoid Receptor (GR), Cyclic Adenosine Monophosphate Responsive Element (CREB), Glucocorticoid Responsive Element (GRE), Estrogen Responsive Element (ERE)

INTRODUCTION

Corticotropin releasing hormone (CRH), a 41 amino acid peptide, plays a pivotal role in the coordination of the stress response (Selye, 1946; Munck *et al.*, 1984; Swanson *et al.*, 1983). Thus, in addition to being a potent stimulator of synthesis and release of proopiomelanocortin-derived peptides from the anterior pituitary and the arcuate nucleus of the hypothalamus (Rivier and Plotsky, 1986; Vale *et al.*, 1981), CRH acts together with norepinephrine to mediate many hormonal, autonomic and behavioral effects of stress (DeSouza *et al.*, 1985; Chappell *et al.*, 1986; Butler *et al.*, 1990; Melia and Duman, 1991). Because of its overall physiological importance and its potential involvement in the pathogenesis of stress-related psychiatric disorders, CRH biosynthesis regulation and bioavailability have been subjects of intense investigation (Chrousos and Gold, 1992; Potter *et al.*, 1991).

The human (h) CRH gene is expressed in numerous central nervous system (CNS) and peripheral sites (Grino *et al.*, 1987; Plotsky and Sawchenko, 1987; Thompson *et al.*, 1987). The ~0.9 kb of the 5' regulatory region of the hCRH gene has been sequenced (Vamvakopoulos *et al.*, 1990) and several functional studies have been performed (Vamvakopoulos and Chrousos, 1993a, b). The transcriptional regulatory signals of the hCRH gene include two TATA boxes, a functional cyclic adenosine monophosphate responsive element (CREB) (Seaholtz *et al.*, 1988; Van *et al.*, 1990; Adler *et al.*, 1990), several apparently functional polyadenylation sites (Adler *et al.*, 1992) and other potentially functional regulatory elements, that have been evolutionarily preserved in several species (Thompson *et al.*, 1987; Vamvakopoulos *et al.*, 1990; Shibahara *et al.*, 1983; Roche *et al.*, 1988). Glucocorticoids negatively regulate the CRH gene in rat hypothalami *in vivo* and *in vitro* and stably transfected AtT-20 cells *in vitro* (Adler *et*

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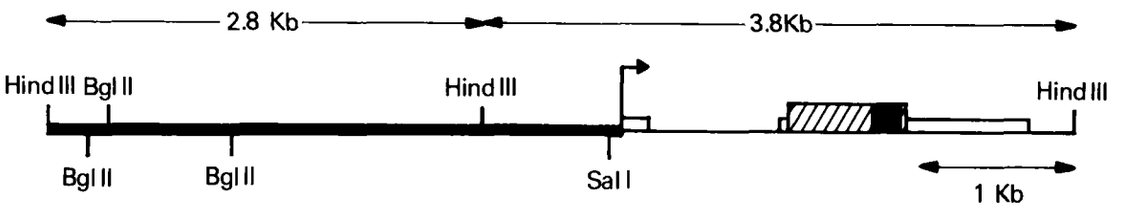

Figure 1. Physical organization of a 6.6 kb DNA region containing the hCRH gene. The bent arrow marks the start and points towards the direction of transcription. The open boxes refer to transcribed and untranslated hCRH mRNA sequences, the striped box to the translated preprohCRH mRNA and the small black box to the segment coding for the active hCRH peptide that is derived from the precursor hCRH protein by posttranslational proteolytic cleavage. The 5' flanking region of the gene is indicated by a thick black line. The sequence of the 2.8 kb HindIII fragment was determined and is reported here together with the previously determined 0.9 kb segment proximal to the gene, encompassing a total of 3625 bp of the 5' flanking sequence.

al., 1988; Dorin *et al.*, 1989) and positively in dispersed human placental cells (Robinson *et al.*, 1988). The molecular mechanisms by which glucocorticoids exert this antithetical tissue-specific regulation on the CRH gene are not fully understood.

Since transcriptional regulatory elements are not exclusive to the 5' flanking region of a gene, it is possible that such elements may be present in the intron and other areas of the CRH gene. To understand further the hormonal regulation of the hCRH gene, we selected to study its 5' flanking region first, because of its high evolutionary conservation between the human, sheep and rat (Vamvakopoulos *et al.*, 1990). We have now sequenced further upstream and report the sequence of a total of 3625 bp 5' flanking this gene. Although several putative consensus regulatory elements were identified, no glucocorticoid response element consensus (Beato, 1989) was found. A repetitive element with homology to human O family members (Paulson *et al.*, 1985) and a 137 bp segment homologous to a partial Alu I family member (Schmid and Jelinek, 1982) were noted at positions −2213 and −2835, respectively. The data presented will hopefully aid the study of regulation and dysregulation of the hCRH gene.

RESULTS

The physical localization of the 2.8 kb HindIII fragment, whose sequence is reported in this communication, is shown in Fig. 1. The sequence of the entire 3625 bp 5' flanking region of the hCRH gene is shown in Fig. 2. This was examined for the presence of common consensus regulatory elements by a published computer program and the key findings are summarized *in Table 1. The* relative distribution of these regulatory elements is summarized in Fig. 3. Only the elements that have been evolutionarily preserved in CRH genes from other species have been included, with the exception of distal consensus regulatory elements for TATA and CAAT boxes, AP1, half palindromic EREs and half GREs, which were also included, because they were present in the sequence several times.

Comparison of the 5' flanking region of the hCRH to non-CRH sequences in the GenBank, revealed two segments, underlined in Fig. 2, homologous to human repetitive DNA elements. The rest of this portion represented a unique and novel sequence, suggested by the lack of homology to any other known sequence. The segment underlined by the solid line in Fig. 2, is homologous to the human O family of moderately repetitive elements and its sites of insertion are consistent with consensus insertion sites for these "mobile" sequences (Paulson *et al.*, 1985). The segment underlined by the broken line in Fig. 2, has homology, up to 72%, to a portion of the highly repetitive human Alu I family members (Schmid and Jelinek, 1982) (Fig. 4A and B). Shown in Fig. 4B, is a comparison of the hCRH partial Alu I sequence with the Alu I repeat (HTPA-12) present in the

Figure 2. The nucleotide sequence of 3625 bp 5' flanking the hCRH gene, that is represented by the thick black line in Fig. 1. Shown for orientation purposes are the restriction sites for Sal I, Hind III and Bgl II. The segment underlined with the solid line is homologous to members of human type O family of repetitive elements. The segment underlined with the broken line has limited homology to the 3' terminal half of a member of hAlu I family of highly repetitive elements. Gene Bank accession no X67661.

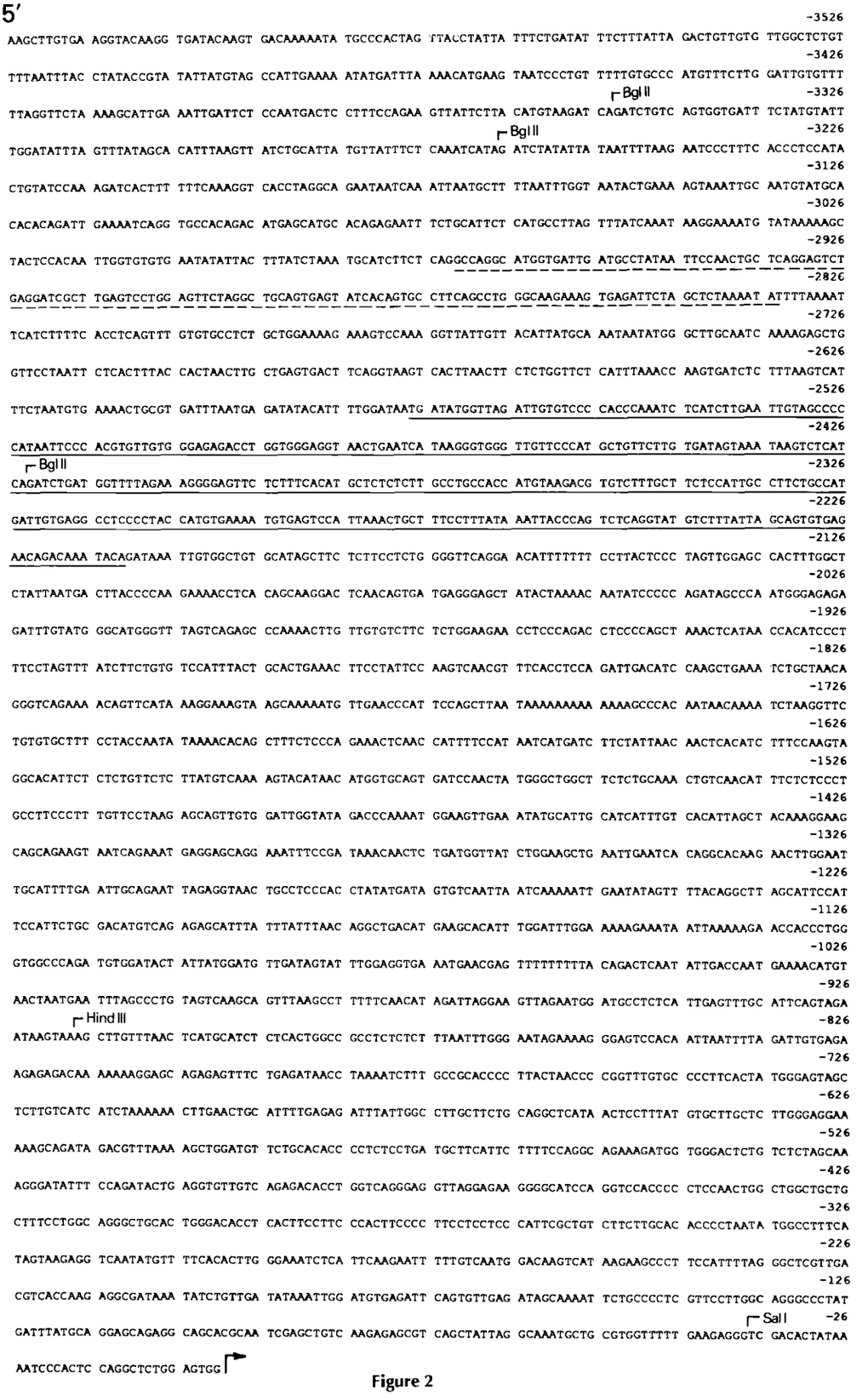

Figure 2

Table 1 Potential regulatory elements in the 5'-flanking region of hCRH gene

Element	*Consensus*	*Location (nucleotides)*	*No. of sites*
TATA box 1	5'TATAAA(+) 5'TTTATA(–)	–30(+),–195(+),–1707(+) –2266(–),–2269(+),–3035(+) –3309(–)	
TATA box 2	5'TATAAT(+) 5'ATTATA(–)	–2950(+),–3254(–),–3257(+)	10
CAAT box	5'CCAAT(+) 5'ATTGG(–)	–186(–),–677(–),–1040(+) –1490(–),–1711(+),–2038(+), –3012(–),–3395(+)	8
CREB	5'ACGTCA(+) 5'TGACGT(–)	–223(–),–228(+)	
	5'TGACGTCA(+) 5'AGACGTCA(–)	–221(–),–228(+)	1
AP-1	5'TGAATCA(+) 5'TGATTCA(–)	–1262(–),–1346(–),–1352(+) –2000(–),–2476(–),–2482(+)	
	5'TGAGACTT(+) 5'AAGTCTCA(–)	–2427(–)	
	5'TGACTAA(+) 5'TGAGTCA(–)	–2000(–)	
	5'TTAGTCAG(+) 5'CTGACTAA(–)	–2006(+)	
	5'CTGACTAA(+) 5'TTAGTCAG(–)	–1999(–)	8
Pu box	5'GAGGAA(+) 5'TTCCTC(–)	–370(–),–631(+),–2178(–)	3
OCTA	5'ATTTGCAT(+) 5'ATGCAAAT(–)	–2753(–)	1
ApoCIII	5'GGTGACCTT(+) 5'AAGGTCACC(–)	–1392(–)	1
Palindromic half-ER site	5'GGTCA(+) 5'TGACC(–)	–317(+),–485(+),–1039(–) –1824(+),–3198(+)	5
Second half GR site	5'TGTTCT(+) 5'AGAACA(–)	–598(+),–1612(+),–2222(–) –2453(+)	4

(+)/(–), represents the consensus element at two different orientations.

human tissue plasminogen activator (hTPA) gene (Friezner Degen *et al.*, 1986), with which the highest degree of homology (71.74%) was found. The HTPA-12 Alu I repeat is 290 bp long, spans from position 10066 to 10355 of the human gene and its overlap to hCRH partial Alu I begins from position 137 of the complete Alu I located at 10202 in the hTPA gene and ends at position 289 of the plate Alu I, which corresponds to position 10354 of this gene (Fig. 4A). The size of the gaps in the partial Alu I sequence of the hCRH gene found from alignment to its most homologous complete human Alu I (HTPA-12) sequence, increases towards the missing first half end of this element (Fig. 4A).

DISCUSSION

In addition to the previously identified cAMP response element (CREB) (Lee *et al.*, 1987) and the two TATA boxes (Bucher and Trifonov, 1986; Siegele *et al.*, 1989), the hCRH gene has a large number of AP1 sites (Lee *et al.*, 1987), compatible with its regulation by phorbol esters (TPA) and epi-

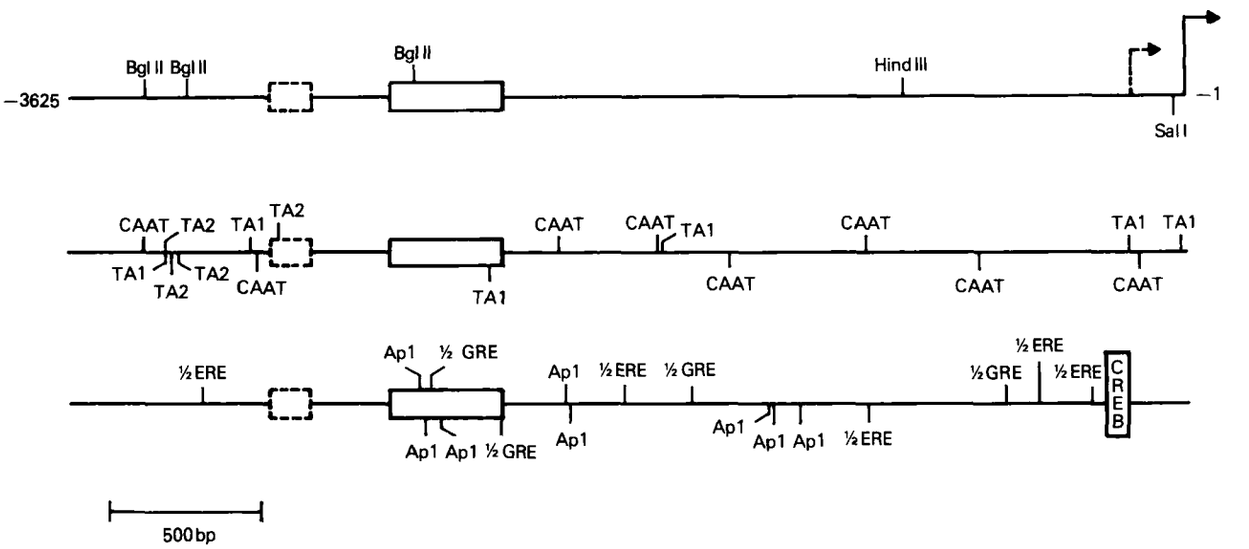

Figure 3 Physical organization of the combined structural and putative regulatory features identified in the 3725 bp 5' flanking the hCRH gene. Shown are: *Top,* landmark restriction sites, the two initiation sites indicated by solid and broken bent arrows pointing towards the direction of transcription, the hCRH type O member of repetitive elements represented by the box in solid line and the hCRH partial Alu I family member shown by the box in broken line. *Middle:* locations of TATA box 1 (TA1), TATA box 2 (TA2) and CAAT box consensus elements. *In (+) orientation the arrow points above and (−) below the midline. Bottom:* locations of CREB, AP1, half palindromic ER site (1/2 ERE) and second half GR site (1/2 GRE) consensus elements in (+) and (−) orientation, above and below the midline, respectively.

dermal growth factor (EGF) (Luger *et al.*, 1987; Emanuel *et al.*, 1990). The CREB site at −220 also includes a sequence necessary for basal expression and induction by EGF and TPA (Fisch *et al.*, 1987). Eight additional TATA boxes, some properly located downstream from six additional CAAT boxes (Chodash *et al.*, 1988), were found in the 2.8 kb HindIII fragment, and some of them may possess genuine promoter activity and direct the transcription of yet unidentified genes within this sequence. Indeed, novel peptide sequences corresponded to the open reading frames (ORFs) present in this area, with the two longest ORFs detected, encoding 100 and 95 amino acids, respectively. Using an improved computer method for the identification of protein-coding exons of new genes in DNA sequences, also known as GRAIL (Uberbacher and Mural, 1991), however, we did not detect any potential exons in the 3625 bp long 5' flanking region of the hCRH gene. Alternatively, the eight clustered TATAs present at −1.7 to −3.3 kb of the hCRH gene, may bind the tumor suppressor gene product p53, and play a role as silencers of hCRH gene expression (Mack *et al.*, 1993).

The program detected a variety of additional elements, such as Pu box (Klemsz *et al.*, 1990), octamer (Singh *et al.*, 1986) and apoCIII (Reue *et al.*, 1988), shown in Table 1, as well as others, most of them present once or twice in the sequence, which might or might not contribute significantly to the regulation of hCRH gene. Their preservation in the 5' flanking region of the CRH gene from other species will be indicative of a possible functional role in CRH regulation. No functional glucocorticoid response element (GRE) consensus sequence (Beato, 1989) was found in the 3625 kb of hCRH 5' flanking region analyzed. This is a 15-nucleotide sequence with partial dyad symmetry: 5'GGTACANNNTGTTCT (Beato, 1989). The hexamer 5'TGTTCT or half GRE, which appears to be invariant within this sequence, is, however, present four times in the hCRH promoter. Half-GREs have been proposed not to mediate GR induction, probably because the GR acts as a dimer at its consensus GRE site (Beato, 1989); however, recent studies have suggested that mild and delayed glucocorticoid effects may be mediated by such sequences (Chan *et al.*, 1991). The recently demonstrated interaction of the DNA binding domain of GR to a single site as a monomer in the mouse α-amylase 2 promoter (Slater *et al.*, 1993), further supports the functionality of 1/2 GREs in transcriptional enhancement. Our results on the

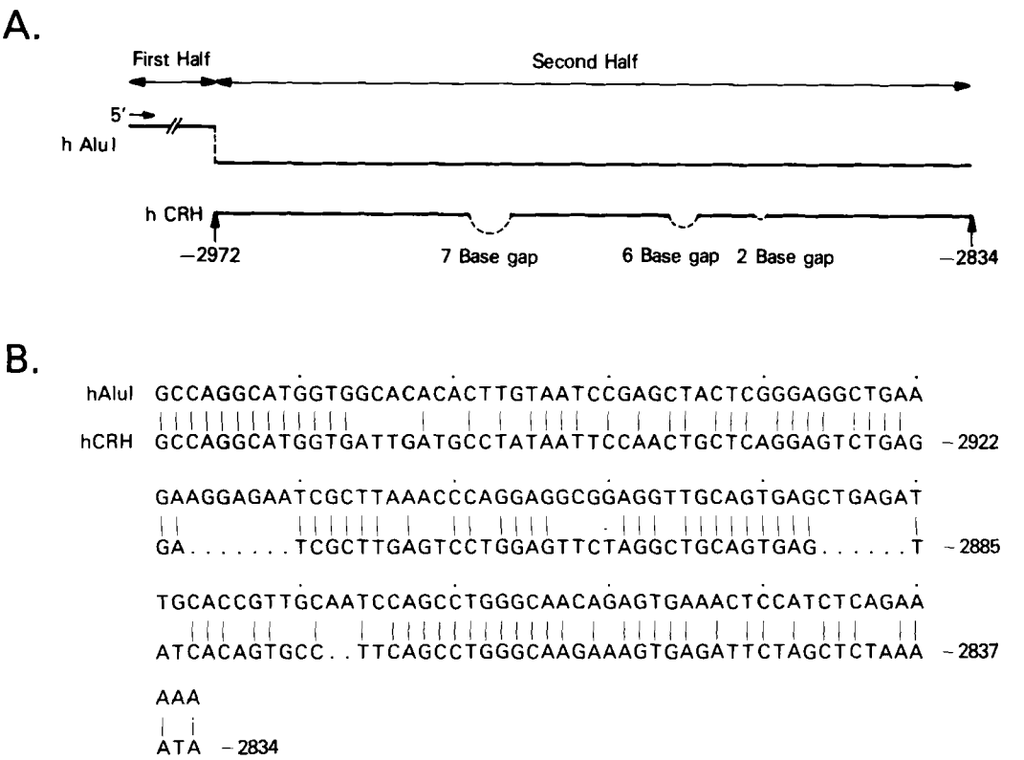

Figure 4 Comparison of hAlu I and hCRH homologous segments. A. Schematic representation of the area of overlap between hAlu I (HTPA-12) and hCRH homologous segments illustrating also the position of gaps in the hCRH partial Alu I fragment. B. Alignment of 3' terminal half of the human Alu I repetitive element (HTPA-12) present at position 10066 of the human tissue plasminogen activator gene to the partial Alu I segment spanning from −2972 to −2834 of the hCRH gene.

transient expression of the hCRH promoter-driven CAT reporter support the possibility that the 1/2 GRE present at position −598 of the hCRH gene, may be functional (Vamvakopoulos and Chrousos, 1993a). Although GREs may account for positive and negative glucocorticoid effects on gene expression (Diamond *et al.*, 1990), a consensus sequence mediating uniquely negative glucocorticoid effects has been identified at position −63 in the promoter of the proopiomelanocortin gene (Drouin *et al.*, 1989). This negative GRE sequence 5'CGTCCA was absent from the hCRH gene promoter.

There was no perfect palindromic ERE (5'GGTCA NNNTGACC) sequence consensus (Beato, 1989) in the hCRH promoter region. However, five perfect half-palindromic EREs were found. In contrast to half GREs, half EREs appear to play a role in transcriptional regulation (Kato *et al.*, 1992; Wilson *et al.*, 1992). Thus, half EREs were recently shown to possess genuine enhancer activity with orphan receptors (Wilson *et al.*, 1992). They also bind weakly to estrogen receptor, however, when present in pairs; binding to the receptor increases, and is associated with major increase of estrogenic effects in gene expression, presumably because estrogen receptors act as dimers (Kato *et al.*, 1992). Nevertheless, the half-EREs present in the hCRH promoter might confer estrogenic effects on the hCRH gene, by analogy to their actions in the chicken ovalbumin gene (Kato *et al.*, 1992). Evidence of direct estrogenic regulation of the hCRH gene has been obtained (Vamvakopoulos and Chrousos, 1993b), explaining in part the paradoxical estrogen-induced gonadotropin suppression via the hypothalamic gonadotropin releasing hormone neuron (Yen and Lein, 1976) which lacks estrogen receptors (Shivers *et al.*, 1983). This suggests that estrogen negative feedback may be mediated through the CRH-POMC pathway, which is inhibitory to the gonadotropin releasing hormone neuron

(Ferrin *et al.*, 1984; Chrousos and Gold, 1992). The regulatability of the hCRH gene by estrogen, may also constitute the basis of sexual dimorphism in the stress response and the immune/inflammatory reaction (Vamvakopoulos and Chrousos, 1993b).

The extended 5' region of the hCRH gene examined lacked a GC box (SP1 site) (Briggs *et al.*, 1986). These sites have been associated with "house keeping" genes and the presence of multiple start sites (Zong *et al.*, 1990). Their absence suggests that the hCRH gene must have only one or few minor transcription initiation sites, appropriately located downstream from its two proximal TATA boxes. Available experimental findings are consistent with this prediction (Handanos and Dorin, 1993; Vamvakopoulos and Chrousos, 1993a).

An interesting regulatory peculiarity of the hCRH gene appears to be its inhibition by glucocorticoids in the hypothalamus, as suggested by clinical evaluations of the activity of the hypothalamic-pituitary-adrenal axis (Chrousos and Gold, 1992), and its stimulation by the same hormones in the placenta (Robinson *et al.*, 1988). Glucocorticoid receptor-mediated modulation of AP1 activity by differential concentration of receptor or ligand in these tissues, could provide an explanation for this phenomenon (Diamond *et al.*, 1990). Indeed, we have demonstrated that a hCRH-CAT construct can elicit antithetical glucocorticoid effects in transient expression assays, depending on the coexpression of GRcDNA, and proposed that the half GRE of the hCRH promoter construct used in this assay conferred the observed stimulatory effect (Vamvakopoulos and Chrousos, 1993a). The presence of half EREs might also be related to the positive regulation of hCRH gene by glucocorticoids in the placenta considering that by analogy to the chicken ovalbumin gene, estrogens and glucocorticoids may elicit synergistic effects when present at high levels (Otten *et al.*, 1988).

Type O repetitive DNA elements have been implicated in DNA mobility/fragility (Paulson *et al.*, 1985). An element with nearly perfect (greater than 80%) homology to the human type O consensus spans from position −2213 to −2580 of the hCRH gene and may be related to mobility/fragility of this gene. Since usually these elements exist in pairs, it is possible that an additional type O element is present upstream from the *HindIII* site located at −3625 bp. Seven of the eight TATA boxes identified in addition to those of the hCRH promoter are located upstream of the type O element, in the area that may potentially be subject to rearrangement. The sequences contained within the type O element(s) may influence hCRH gene transcription (i.e. bind p53, modulate or include tissue specific elements or other), and may be related to the strong placental expression of the hCRH gene. Structural analysis of the corresponding segment of the rat CRH gene will be necessary to identify structural features potentially responsible for the markedly different control of placental CRH gene expression between human and rat (Grino *et al.*, 1987; Robinson *et al.*, 1989). A segment with reduced homology (less than 72%) to the 3' terminal half of a human Alu I repeat is also located further upstream, from position −2835 to −2972 of the hCRH gene, and this may represent a fragment of an authentic Alu I element (Schmid and Jelinek, 1982). Alignment of the partial hCRH Alu I with the most homologous HTPA-12 complete Alu I revealed gaps in the partial Alu I which increased in size towards its missing first half end (Fig. 4A). This may pertain to the mechanism of this rearrangement. Of considerable interest in the chromosomal proximity of the hCRH partial Alu I (position 8q13) to the HTPA-12 complete Alu I of the human tissue plasminogen activator gene (position 8p12-q11.2) (O'Brien, 1990), with which it shares the highest degree of homology (>10% compared to other human Alu I family members). These structural features are compatible with the colocalization of the hCRH gene with chromosomal fragility determinants on human chromosome position 8q13 (O'Brien, 1990), and may contribute to the regulatory arsenal of the hCRH gene, by their ability to mobilize and rearrange genetic material. Such rearrangements might account in part for differences in the tissue-specific regulation of the CRH gene among different species.

MATERIALS AND METHODS

DNA Sequencing Strategy

Partial HindIII digestion of genomic hCRH containing phage DNA was used to generate a 6.6 kb fragment consisting of a 3.8 kb *HindIII* segment including the hCRH gene and 0.9 kb of its 5' flanking region and a 2.8 kb HindIII segment extending into the 5' upstream area of the gene. This fragment was subcloned in the HindIII site of a pBluescript II SK+ (Stratagene) vector to generate clone 11. Clone 11 containing two HindIII fragments from the hCRH gene of about 2.8 and 3.8 kb in length, was digested with HindIII. The 2.8 kb *HindIII* fragment was gel-purified and subcloned into the HindIII site

of pBluescript II SK+. Nested deletions were generated using a modified procedure based on the exonuclease III/S1 nuclease method described by Henikoff (Henikoff, 1984 and 1987). Exonuclease III-susceptible and -resistant ends were produced at the T7 end by digestion with Cla I and Apa I, respectively. Exonuclease II-susceptible and -resistant ends were produced at the T3 end by digestion with EcoRV and SacI, respectively. T7 and T3 deletion clones were picked and plasmid DNA was prepared using a modified alkaline lysis procedure. Plasmid DNA from individual deletion clones was digested with PvuI and analyzed by electrophoresis on 1% agarose gels. Deletions were sized and clones with deletions distributed every 150–200 bases were selected for sequencing. A total of 38, 19 T7 and 19 T3, deletion clones were used for sequencing. DNA sequencing reactions were carried out using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) as modified for the use of [^{35}S]ATP as the label (Biggin *et al.*, 1983) and T7 DNA polymerase (Tabor and Richardson, 1987). All reactions contained 7-deaza dGTP to reduce the effects of GC band compressions. Sequencing reactions were analyzed on 6% polyacrylamide wedge gels containing 8M urea. Internal sequencing primers were synthesized as necessary. The orientation of the two *HindIII hCRH* fragments was established by sequencing clone 11 across the internal HindIII junction. The sequence can be found in the Gene Bank under accession no x67661.

Computer Analysis

This included a Gene Bank search for sequence homologies, sequence alignments and search for open reading frames in the hCRH promoter region. Except of the two repetitive DNA elements found, the remaining hCRH 5' flanking sequence was unique. Search for consensus signal sequences was made by a published computer program (Ghosh, 1990). Screening for protein-coding exons, was performed by the Gene Recognition Analysis Intenet Link (GRAIL) computer program (Ubenbacher and Mural, 1991).

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