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COMPLEX REGULATORY INTERACTIONS CONTROL CRH GENE EXPRESSION

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1. ABSTRACT

Glucocorticoids inhibit corticotrophin releasing hormone (CRH) production in the hypothalamus but stimulate production from the placenta. To identify key elements regulating the CRH gene, mouse pituitary tumor-derived cells (AtT20 cells) were used as a hypothalamic model in an analysis of the CRH promoter. Two cAMP responsive elements were identified: (I) a consensus cAMP response element (CRE) and (II) a previously unrecognized caudal-type homeobox response element (CDXRE). Glucocorticoids inhibit only the component of cAMP-stimulation occurring via the CRE through an action involving a negative glucocorticoid response element (nGRE). We also identified two regions that, in the absence of the nGRE, can be stimulated by glucocorticoids: (I) the CRE and (II) a region between -213 to -99bps. Electrophoretic mobility shift assays identified binding of the transcription factors CREB and Fos at the CRE in AtT20 cells, whereas CREB and cJun were detected in placental cells. In addition, a novel CRE-binding transcription factor has been identified that is expressed in the brain and in placenta. A model is presented whereby CRH gene regulation is mediated via tissue specific expression of transcription factors.

2. INTRODUCTION

Corticotropin-releasing hormone (CRH), a 41 amino acid neuropeptide, has a key role in integrating hormonal, autonomic and behavioral responses to stress (1-5). CRH is synthesized in the paraventricular nucleus (PVN) of the hypothalamus from where it is released into the hypophyseal portal circulation to orchestrate the pituitary-adrenal stress response, leading to production and release of glucocorticoids (1, 5). Glucocorticoids in turn inhibit hypothalamic paraventricular nucleus (PVN) production of CRH and pituitary production of ACTH, hence ensuring that serum glucocorticoid levels are appropriate to the stress experienced and that the system can be regulated by negative feedback (2, 6-7).

CRH has also been found in many extra-hypothalamic regions of the nervous system and in peripheral tissues. In the brain CRH has been found in the posterior pituitary, thalamus, cerebral cortex, cerebellum, pons, medulla oblongata and spinal cord where it acts as a neurotransmitter (8). In peripheral tissues CRH is produced in the adrenal medulla, ovary, testis, heart, lung, liver, stomach, duodenum, pancreas, T-lymphocytes and placenta (8-10).

Pathologically, CRH production in the CNS is increased in depression (11), and rheumatoid arthritis has been associated with polymorphisms of the CRH promoter (12) and CRH levels are increased in arthritis affected joints (13). Placental production of CRH is increased in pre-eclampsia, intra-uterine growth retardation, fetal asphyxia and during pregnancies that end in premature delivery (14-17).

In tissues outside the PVN the effect of glucocorticoids on CRH gene expression is different. In the central nucleus of the amygdala, glucocorticoids either increase or have no effect on CRH gene expression depending on the concentration of glucocorticoid used (18, 19). In the placenta and the bed nucleus of the stria terminalis, glucocorticoids stimulate CRH gene expression (20, 21). The mechanism by which glucocorticoids inhibit CRH gene expression in the PVN but stimulate CRH gene expression in other tissues is a major focus of our research.

The human genome contains one CRH gene, which is under the control of a single promoter region

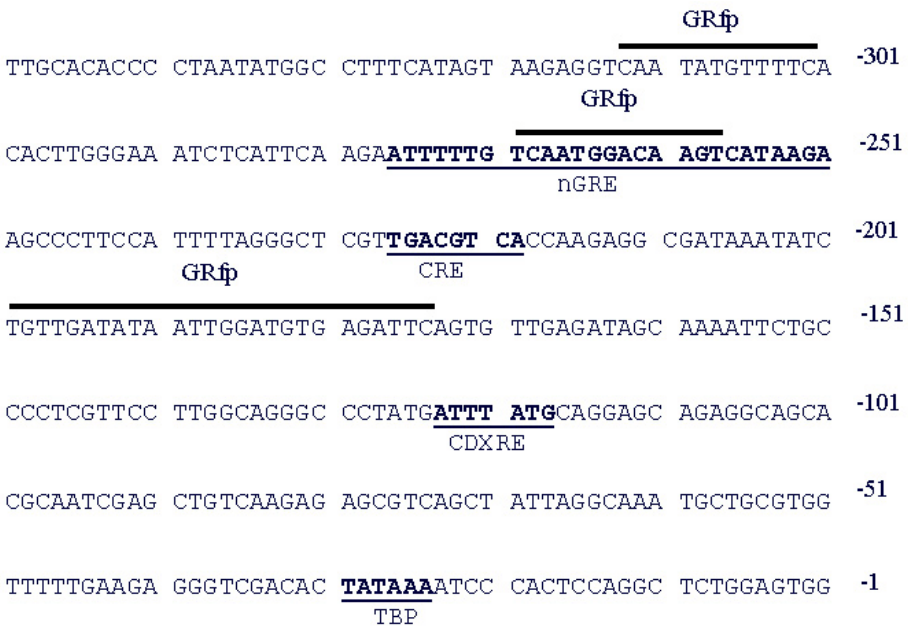

Figure 1. The Human CRH promoter DNA sequence and regulatory elements. The first 350 bp of the human CRH promoter is shown with several regulatory sites discussed in the text highlighted in bold-underlined font. The areas discussed in the text as GR-footprint (GRfp) regions on indicated by overlining the sequence.

(Figure 1) (22-24). The CRH promoter does not contain a consensus glucocorticoid regulatory element (GRE), but DNase I protection assays have identified a number of regions in the sequence where glucocorticoid receptors (GR) are able to bind (25). One overlaps a negative GRE (nGRE) that mediates, in part, the inhibition of CRH promoter activity by glucocorticoids (26).

In the placenta, glucocorticoids increase CRH promoter activity (21, 27) and we have demonstrated in the placenta that glucocorticoid-stimulation requires the CRE on the CRH promoter (27). Furthermore, the regions where the GR can bind the CRH promoter *in vitro* are not involved in this glucocorticoid-mediated stimulation (25, 27). We hypothesise that tissue specific expression of nuclear factors leads to glucocorticoid stimulation of the CRH promoter in some tissues but not in others.

To explore the interactions between cAMP and glucocorticoids in the regulation of hypothalamic CRH gene expression, we have used the mouse corticotroph cell line AtT20. The complexity of the neural pathways involved in CRH gene regulation and peptide release makes their study difficult and, therefore, AtT20 cells are a commonly used cell model of CRH gene regulation by glucocorticoids and cAMP in which inhibition of CRH release by glucocorticoids dominates as in the hypothalamus (25, 26, 28, 29).

3. CYCLIC AMP STIMULATES THE CRH PROMOTER AT TWO SITES

The consensus CRE of the CRH promoter has been identified as a site of action for cAMP mediated pathways in several cell systems (30, 31). We have shown that in AtT20 cells cAMP stimulates the CRH promoter through the CRE and an additional novel site in the region between –213 and –99 bps (32). Stimulation of the CRH promoter with cAMP resulted in a 14-fold induction of luciferase-reporter activity, while mutation of the CRE significantly decreased cAMP stimulated promoter activity, although it still remained at 3.5 fold above baseline (Figure 2). Deletion of the promoter to –213 bp, thereby removing the CRE, also reduced cAMP-stimulation to 3.5 fold above basal level. Further deletion leaving only the first 99 bp of the CRH promoter produced a reporter construct that showed no significant induction over basal levels after exposure of the cells to cAMP (Figure 2) confirming that there is a second region on the CRH promoter that confers responsiveness to cAMP.

Analysis of the CRH promoter by Transfac (33) identified a previously unrecognized caudal type homeobox response element (CDXRE) at –125 to –118 bps. Mutation of this CDXRE led to decreased cAMP stimulated CRH promoter activity in the AtT20 cells (Figure 2), but promoter activity 5.5 fold over basal activity still remained. The level to which cAMP stimulated activity of the CRH promoter containing a mutated CDXRE is significantly higher than with a mutated CRE, or when the promoter was deleted to -213 bp, indicating that the CRE is the major site for stimulation by cAMP.

Clearly, cAMP stimulates the CRH promoter through two separate response elements, a consensus CRE at -228 to -221 bps and a consensus CDXRE at –125 to –118 bps. The levels of induction by cAMP of the mutant CRE (3.5 fold induction) and mutant CDXRE (5.5 fold

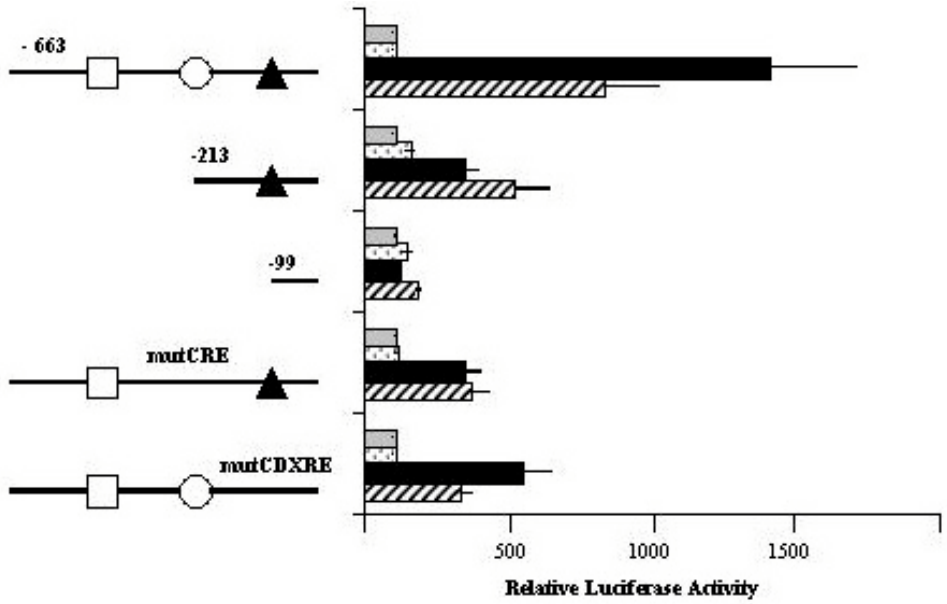

Figure 2. Effect of deletions and specific mutations of the CRH promoter in AtT20 cells. The CRH-663, CRH–213 and CRH–99 bp constructs containing the corresponding length of CRH promoter were made by serial 5' deletions of the CRH promoter. Mutation of the CRE or CDXRE was performed in the CRH-663 construct. The construct map on the left demonstrates the presence of response elements in each construct (square = nGRE, circle = CRE, triangle = CDXRE). AtT20 cells were transiently transfected with these constructs and treated with standard media (grey bars) or media with 0.1 μmol/L dex (speckled bars), with 3 mmol/L cAMP (black bars) or with 3 mmol/L cAMP and 0.1 μmol/L dex (striped bars). The luciferase activity was standardised with the basal construct activity in standard media set at 100.

induction) promoters, when added together, are less than the induction in the wild type construct (>14 fold induction). This suggests that although the CDXRE responds to cAMP independently of the CRE there may be a synergistic interaction between these regions. The CDX-homeobox proteins are expressed in the neuroectoderm during development of the embryo (34) and the gastrointestinal system (35). Whether CDX homeobox proteins interact with the CDXRE of the CRH promoter or if CRH producing cells in the gastrointestinal system contain CDX homeobox proteins is currently under investigation.

4. GLUCOCORTICOID INHIBITION OF THE CRH PROMOTER

A negative glucocorticoid response element (nGRE) in the CRH promoter has been reported, which mediates the inhibition of CRH gene expression in AtT20 cells stimulated by cAMP (26). We have found that the nGRE predominantly inhibits the component of cAMP activation occurring through the CRE, and not the component occurring through the CDXRE (32). Studies using progressive deletion of the CRH promoter showed that glucocorticoids inhibited cAMP stimulated CRH promoter activity until the deletion included the nGRE, located between –278 to –249bp (Figure 2). Moreover, in promoter constructs where the CRE was mutated, glucocorticoids did not inhibit the remaining cAMP stimulated promoter activity. In contrast, glucocorticoids did inhibit cAMP dependent promoter activity when the CDXRE was mutated (Figure 2). These data indicate that inhibition of cAMP stimulated CRH promoter activity by glucocorticoids requires the nGRE, and this inhibition affects the cAMP stimulated activity of the CRE but not that of the CDXRE.

5. GLUCOCORTICOID STIMULATION OF THE CRH PROMOTER

We have observed that in the absence of the nGRE glucocorticoids can stimulate the CRH promoter in AtT20 cells. The possible role of the CRE in this response was explored by isolating this element from other regulatory elements in the CRH promoter (32). A Transfac search of the CRH promoter-99 to 0 bps did not identify any mammalian response elements (33) and this region of the promoter had no significant response to glucocorticoids or cAMP. When a CRE was inserted upstream of this minimal CRH promoter the unstimulated promoter activity was increased by 5 fold, and this activity was further increased almost 2 fold by glucocorticoids and nearly 3 fold by cAMP (Figure 3). The combined effect of glucocorticoids and cAMP on this promoter construct was additive.

To further examine the role of the CRE in glucocorticoid mediated induction we used a minimal rabbit beta-globin promoter (36), which did not respond to cAMP or glucocorticoids, to which a 38 bp fragment of the CRH promoter (the CRE with 20bps upstream and 10 bps downstream) was inserted 5' to the beta-globin-promoter (27, 30, 32). The basal activity observed with this promoter construct was 3 fold higher than with the minimal globin

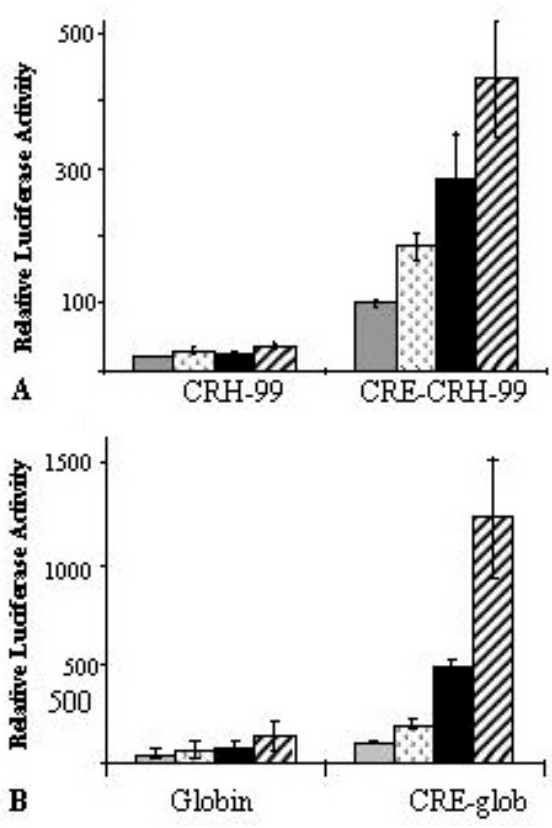

Figure 3. The CRE confers glucocorticoid mediated stimulation of expression. The CRE was inserted upstream of the minimal CRH promoter (CRH-99) to produce CRE-CRH-99 (A), and upstream of a minimal rabbit beta-globin promoter (Globin) to produce CRE-Globin (B). Following transient transfection into AtT20 cells the cells were exposed to standard media (grey bars), or 0.1 μmol/L dex (speckled bars), or 3 mmol/L cAMP (black bars), or both dex and cAMP (striped bars). The luciferase activity was standardised so that the basal activity of the construct containing the CRE equalled 100 (error bars representing +/- 95 % confidence intervals). **A.** CRH-99 activity was not significantly affected by dex or cAMP. CRE-CRH-99 was stimulated by dex and cAMP and together their effect was additive. **B.** Globin activity was not significantly affected by dex or cAMP. CRE-Globin was stimulated by dex and cAMP and together their effect was synergistic.

promoter alone. This CRE-globin promoter construct was stimulated by almost 2 fold by glucocorticoids alone, stimulated >5 fold by cAMP alone, and the combination of glucocorticoids with cAMP was synergistic (>13 fold induction) (Figure 3). The reason that the CRE-globin response to the combination of glucocorticoids with cAMP appears to be synergistic while the CRE-CRH-99 response appears to be additive is unclear since neither the globin, nor the CRH–99 appear to contain response elements. However, the globin promoter does not contain a TATA box, hence the initiation complex may be different to that formed on the CRH TATA box. The activating complex that forms on the CRE in response to glucocorticoids and cAMP may interact differently with the initiation complex. Nevertheless, these results indicate that in AtT20 cells, glucocorticoids are capable of stimulating CRH promoter activity through the CRE, in the absence of inhibitory upstream sequences. The GR does not bind directly to the CRE (Figure 4) suggesting that GRs stimulate the CRE indirectly either by affecting nuclear factors before they interact with the CRE or through protein/protein interactions with nuclear factors binding to the CRE.

We have also demonstrated that the region between –213 and –99 is responsive to stimulation by glucocorticoids in AtT20 cells (Figure 2). The exact site of stimulation is presently unknown, however, Guardiola-Diaz *et al*, using a DNase 1 protection assay demonstrated a potential GR binding site within this region, at –202 to –175 bps (see Figure 1) (25).

There is evidence that during periods of stress, glucocorticoids may augment PVN CRH gene expression. In adrenalectomised rats, low dose glucocorticoids increase resting and hypotensive CRH levels in the hypophysial-portal vessels (37). CRH mRNA levels fall when adrenalectomised rats are stressed by hypovolaemia, but the mRNA is restored to normal stress levels by glucocorticoids (38). The CRE or the region –213 to –99 bps could potentially be sites where glucocorticoids act to augment PVN CRH gene expression during periods of hypovolaemic stress. These regions of the CRH promoter may also be involved in glucocorticoid stimulation of CRH production in tissues outside the PVN (20, 21).

6. NUCLEAR PROTEIN COMPLEX ANALYSES

We propose that tissue specific expression of nuclear factors acting at the CRE determine whether inhibition occurs in response to glucocrticoids. When we compared nuclear factors acting at the CRE in AtT20 cells with nuclear factors from placental cells, we found that a number of the factors were different (Figure 4). The nuclear proteins from AtT20 cells formed three different protein complexes on the CRE, while placental nuclear proteins formed two protein complexes. The three bands observed with AtT20 nuclear proteins have different binding characteristics as they were differentially competed away by unlabeled CRE oligonucleotides containing different point mutations (32). This indicates that each band is a unique protein complex and not just monomers and dimers of the same protein.

A super-shifted band was detectable with AtT20 cell nuclear proteins and anti-CREB or anti-Fos antibodies, while placental nuclear proteins were super-shifted with anti-CREB and anti-cJun antibodies (Figure 4). The response of promoters containing AP-1-like elements to glucocorticoids has been shown to depend on the composition of AP-1 binding at the site. When cJun-cJun homodimers bind at such sites glucocorticoids cause stimulation, but when cJun-cFos heterodimers bind at the same site glucocorticoids cause repression (39). Our data support the concept that this mechanism determines the behaviour of the CRH promoter in different tissues.

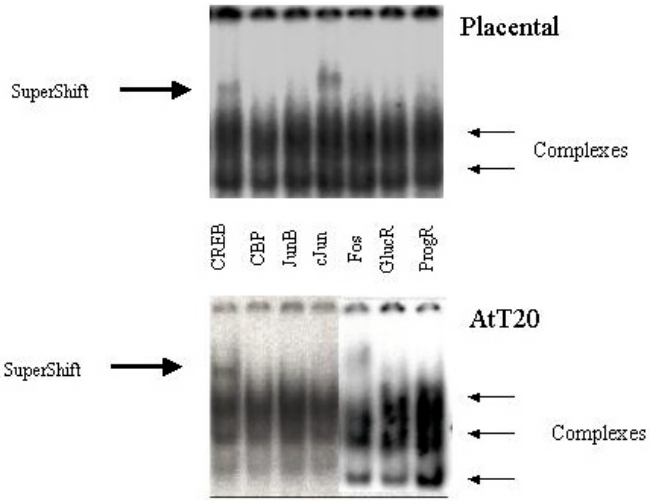

Figure 4. Identification of CRE binding factors by super-shift-EMSA. Placental and AtT20 cell nuclear proteins binding to the CRE by EMSA were exposed to commercial anti bodies against specific nuclear factors prior to electrophoresis. CREB = anti-CREB antibodies, CBP = anti-CREB binding protein antibodies, JunB = anti-JunB antibodies, cJun = anti-cJun antibodies, Fos = anti-Fos antibodies, GlucR = anti-glucocorticoid receptor antibodies and ProgR = anti-progesterone receptor antibodies.

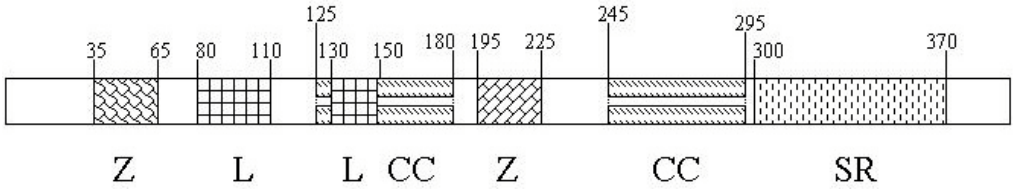

Figure 5. A schematic diagram of the CREAP1 protein. The locations of the proposed functional domains in the CREAP1 protein are shown as described in the text. The amino acid numeric location of domain boundaries is shown at top. Z = zinc-finger motif, L = leucine zipper motif, CC = potential coiled-coil domain, SR = the SR-rich domain.

Using EMSA we demonstrated that glucocorticoids affect nuclear factors before they interact with the CRE (32). Three different protein-DNA complexes bound at the CRE and the binding of these complexes increased following exposure of the AtT20 cells to cAMP and when glucocorticoids were added the binding of two complexes decreased and binding of the third complex increased. Glucocorticoids have been shown to inhibit CREB and cFos activity in the PVN and this has been proposed as a mechanism for glucocorticoid mediated inhibition of CRH production (40, 41). A reduction in binding of these nuclear factors at the CRE does not necessarily decrease CRH promoter activity since glucocorticoids can still stimulate activity through the CRE, presumably through interactions with other transcription factor (32). This highlights the importance of the interaction between the nGRE and CRE in the inhibition of CRH promoter activity.

7. DISCOVERY OF A NOVEL CRE BINDING PROTEIN

Using a yeast one hybrid system a novel transcription factor was identified which functionally bound to the CRE, and is highly expressed in several fetal tissues and in several regions of the adult brain. This protein has been named CREAP1, for CRE-Associated-Protein (Figure 5) (Shipman K, Robinson P, King B, Smith R, Nicholson RC, manuscript in preparation). The CREAP1 protein contains two leucine-zipper-like domains (42), a zinc finger-like domain with DNA binding potential (43), another zinc finger-like domain with RNA binding potential (44), and two coiled-coil domains with potential for protein-protein interactions (45). The CREAP1 protein also has a SR-rich domain indicative of proteins involved in RNA splicing (46). Thus CREAP1 seems to be what has been referred to recently as a multifunctional regulatory protein (47, 48), which are proteins with the ability to perform different functions in the nucleus or cytoplasm, or with the ability to bind both DNA and RNA. The high level of expression of CREAP in fetal tissues and in brain suggest important roles in development and in neurobiology.

8. A MODEL OF THE CRH PROMOTER REGULATION

Using the AtT20 cell model of glucocorticoid mediated negative regulation of the CRH promoter we have demonstrated that cAMP stimulation occurs through the CRE and CDXRE. While glucocorticoids inhibit cAMP stimulated CRH promoter activity through interactions of

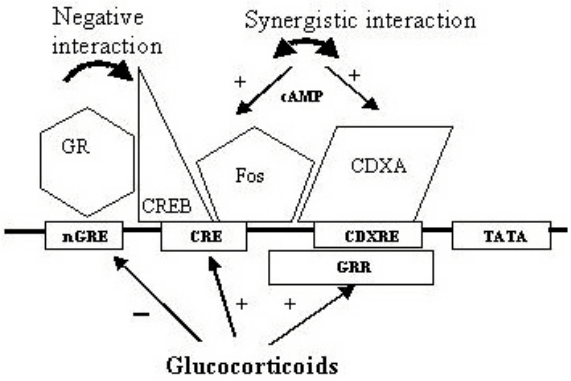

Figure 6. A schematic model of CRH promoter regulation in the hypothalamus. The nGRE is a negative glucocorticoid regulatory element, CRE is the cAMP regulatory element, GRR represents the region located between –213 to –99 bps that is stimulated by glucocorticoids, CDXRE is caudal type homeobox response element and TATA is the TATA box. Stimulatory (+) and inhibitory (-) regulatory effects by cAMP and glucocorticoids through the different elements (thin arrows), negative (thick arrow) and synergistic stimulatory (double headed arrow) interactions between sites is shown.

the CRE and a nGRE, we have shown that they can also stimulate promoter activity through the CRE and a region between –213 and –99 bps, in the absence of negative factors.

From the four regulatory regions described here and information about their function from this and other studies (26, 27, 30), it is possible to predict ways in which the CRE promoter could mount different responses to apparently identical messengers in different cell types (Figure 6).

The effect of cAMP on CRH gene expression appears to be stimulatory in all cells that produce CRH. On the other hand, the effects of glucocorticoids on CRH gene expression are diverse depending on the tissue and the level of gene expression when the glucocorticoids affect the cell. The possible effects of glucocorticoids on CRH gene expression fall broadly into three responses: (I) inhibition of promoter activity; (II) no effect on promoter activity; and (III) stimulation of promoter activity. The CRH promoter can respond in any one of these three ways depending on the environmental context of the CRE and the CDXRE with other regions of the promoter.

In AtT20 cells, glucocorticoids inhibit CRH promoter activity when the nGRE is functional and when the promoter is stimulated through the CRE. Glucocorticoids had no inhibitory effect on CRH promoter activity when the nGRE was deleted or when cAMP mediated stimulation occurred through the CDXRE (Figure 2).

Although glucocorticoids have been defined as negative regulators of the CRH promoter in AtT20 cells, we show that the CRH promoter can be stimulated by glucocorticoids when the CRE is isolated from the elements upstream of –213 bps (Figure 3) or when all the elements 5' to –213 bps were deleted (Figure 2). In placental syncytiotrophoblast cells, the CRE is a major functional element in the regulation of CRH promoter activity, whereas other sites including the nGRE, CDXRE and the glucocorticoid responsive region between –213 to –99 bps have minimal effect on this response (27, 30).

The role played by different nuclear factors in each of these patterns of glucocorticoid mediated CRH promoter response has not been defined. We know that glucocorticoids can decrease nuclear protein binding on the CRE (32), consistent with the findings of others (40, 41). Whether this glucocorticoid mediated decrease in nuclear factor binding to the CRE leads to inhibition of CRH promoter activity is controversial. We have shown that even though some nuclear factors were inhibited, the CRE could still be stimulated by glucocorticoids, probably as a result of the increase in binding of other factors. If a cellular signal only stimulated c-Fos and/or CREB then inhibition of these nuclear factors by glucocorticoids could lead to inhibition of CRH promoter activity. Alternatively, inhibition of the CRE by the nGRE may require binding of a specific nuclear factor or co-factor at the CRE, thereby allowing inhibitory interactions to occur between these sites. This would suggest that nuclear factors acting at the CRE that did not interact with factors at the nGRE would continue to function. This could explain why the CRH promoter activity was only suppressed to ~50% activity by glucocorticoids in our and other's studies (26). In the placental syncytiotrophoblast cells of the placenta, the nuclear factor involved in this cross talk may not be present, hence the nGRE would not interfere with activation of the promoter through the CRE.

9. PERSPECTIVE

Integration of internal and external environmental signals of threats to homeostasis or stress occurs through central production of the peptide CRH. Glucocorticoids are a major modulator of CRH expression. Our studies have begun to elucidate the complex interactions between glucocorticoids and other factors that determine the production of this key peptide hormone that forms the biological basis of an individual's response to stress.

10. ACKNOWLEDGMENTS

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