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Transcriptional regulation of proopiomelanocortin gene expression by corticotropin-releasing hormone

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Transcriptional Regulation of Proopiomelanocortin Gene Expression by Corticotropin-Releasing Hormone

by

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Chapter I.
Introduction and perspective

Overview of POMC system

1. POMC physiological roles

Functions of the mammalian body are finely regulated by the hormonal (endocrine) system. In general, the hormonal system is concerned principally with control of the different metabolic functions of the body, such as the rates of chemical reactions in cells or the transport of substances through cell membranes or other aspects of cellular metabolism like growth and secretion. By finely regulating these body functions, the endocrine system enables mammals to respond to a diverse environment.

A hormone is a chemical substance that is secreted into the body fluids by one cell or a group of cells and has a physiological control effect on other cells of the body. One of the most important hormones in the endocrine system, adrenocorticotropin (ACTH, see review, Antoni, 1986), is a 39 amino acid peptide secreted from the anterior pituitary and released to the general circulation, ultimately acts on the adrenal cortex stimulating synthesis and secretion of glucocorticoids. Almost any type of physical or even mental stress can lead within minutes to greatly enhanced secretion of ACTH and consequently of glucocorticoids as well. Glucocorticoids have important effects in regulating body metabolic functions. Their most significant effects are to increase blood glucose concentration, while at the same time, they can also play important roles in regulating both protein and fat metabolism (Guyton, 1991). Being the major stimulator for glucocorticoid secretion, ACTH is highly involved in the mammalian homeostatic response to stress.

2. POMC gene and its peptide

ACTH is derived from proopiomelanocortin (POMC), which is the precursor polypeptide for a variety of biologically important neuromodulators and endocrine hormones. Genomic DNA fragments containing POMC encoding sequences have been isolated and sequenced (Drouin and Goodman, 1980; Nakanishi et al. 1981; Uhler et al. 1983; Takahashi et al. 1983; Drouin et al. 1985). It was found that there is only one functional POMC gene present per haploid genome. After transcription, the POMC gene gives rise to a primary transcript with 3 exons and 2 introns. Exon 1 and exon 2 are relatively short (each of them spans only about 100 bases), while exon 3 contains all of the precursor protein coding sequence. The introns are much larger in comparison to the exons; intron 1 is about 3.5-4 kb, and intron 2 is about 2-3 kb. The POMC gene is highly transcribed in the pituitary, and low levels of transcript have also been detected in other tissues. In pituitary, the mature mRNA is detected as a transcript of approximately 1100 bases. It encodes a precursor peptide which is posttranslationally processed to different biologically active peptides, such as ACTH, β-endorphin, and β-lipotropin, which are the major products in the corticotrope; and α-MSH, corticotrophin-like intermediate lobe peptide, and acetyl-β-endorphin, which are produced in the melanotroph (see reviews, Eipper et al, 1980; Levin et al, 1991; Roberts et al, 1992).

3. Regulations of POMC secretion and expression

The secretion and expression of POMC peptide is regulated by a variety of hormones. In pituitary as well as in AtT20 cells (a clonal mouse anterior pituitary corticotrope-like tumor cell line), corticotropin-releasing hormone (CRF) and glucocorticoids are two major regulators. CRF can stimulate POMC release in corticotropes (Vale et al, 1983; Bilezikjian and Vale, 1983), and long term

treatment of anterior lobe cells in culture with CRF increases total content of ACTH relative to control cells, which is suggestive of increased synthesis of ACTH, along with increased secretion of POMC-derived peptides (Vale et al, 1983). Glucocorticoids, which is stimulated to release by ACTH, can feedback inhibit the expression of POMC peptide (reviewed by McEwen and Rostene, 1986).

The stimulation of secretion and expression of POMC peptide by CRF is accompanied by an increase of POMC mRNA level, and this regulation of POMC gene expression has been shown to happen at the transcriptional level (reviewed by Roberts et al. 1993). Two groups (Gagner and Drouin 1987; Eberwine et al. 1987) have shown a rapid stimulation by physiological concentrations of CRH on POMC gene transcription in primary cultures of anterior pituitary cells. Similar observations were also made in AtT20 cells (Roberts et al. 1987; Lorang et al. 1994).

4. Second messenger pathways involved in CRF regulation

Corticortropin releasing factor (CRF) is a 41 amino acid peptide secreted from the hypothalamus. After it arrives at the anterior pituitary, it can bind to its receptors on the plasma membrane of corticotropin cells (Perrin et al. 1986). Since the CRF receptor is coupled with a Gs protein, after CRF binding the adenylate cyclase will be turned on, and the cAMP level will be increased. This in turn will cause the activation of cAMP-dependent protein kinases and also the elevation of intracellular Ca^{2+} (Guild et al. 1986; Luini et al. 1985).

The stimulation of POMC gene transcription by CRF has been suggested to be elicited through these cAMP and Ca^{2+} second messenger pathways (Lorang et al. 1994). In vitro nuclear transcription run-on assays and solution hybridization/nuclease protection (SH/NP) assays both showed that CRF gave

time- and dose-dependent, 2-to 3-fold increases in POMC hnRNA relative to controls. Agents which increase intracellular cAMP levels were shown to be able to mimic these rapid effects of CRH on POMC gene expression. In SH/NP assays, treatment with 8-bromo-cAMP gave similar increases in POMC primary transcript; the adenylate cyclase-stimulating diterpene, forskolin (10 uM), induced hnRNA levels in AtT20 cells to similar magnitude and with a similar time-course as CRF. At the same time, it was shown that agents which increase intracellular Ca^{2+} levels were also able to mimic these CRF effects. Ionomycin or Bay K 8644 treatments increased POMC transcription level 2-3 fold in a time- and dose-dependent fashion. Combined CRH and Bay K treatment resulted in an additive induction (Lorang et al. 1994). The results from these studies as well as from others (Schoenenberg et al. 1987; Abou-Samara et al. 1987) with other various pharmacological agents which can change the intracellular cAMP and/or Ca^{2+} levels all verified the need of a rise in cAMP and intracellular Ca^{2+} for CRF induction of POMC transcription.

Transcriptional regulation of POMC gene expression

CRF stimulation of POMC gene expression has been shown to occur at the transcriptional level via the cAMP/Ca^{2+} second messenger pathways. So in order to understand the molecular mechanisms of this stimulation, we first need to understand the mechanisms of the regulation at the transcription stage, and then we can understand exactly how the cAMP/Ca^{2+} signals regulate these transcriptional events.

1. General introduction of regulation of gene transcription

Protein encoding gene transcription is carried out by RNA polymerase II,

which binds to the transcription initiation site and starts transcription. Close to the transcription initiation site is the DNA sequence called TATA box where the basal transcription factors bind. After these basal transcription factors bind to the TATA box, they cause a DNA configuration change around the transcription initiation site and at the same time recruit the RNA polymerase to this site, so the transcription may start.

Besides these basal transcription factors, there is another class of transcription factors called regulatory proteins. Regulatory proteins bind to cis-acting DNA elements in the promoter called cis-active elements. After binding to their cis-elements, these regulatory proteins can interact with the basal transcription factors and modulate their activities for binding to the TATA box as well as their abilities to recruit RNA polymerase, in this way, the gene transcription rate can be regulated.

So in order to understand the molecular mechanisms of regulation of gene transcription, we first need to identify the cis-elements which are responsible for this kind of this regulation, and at the same time, we also need to identify and characterize the regulatory protein(s) which bind to these cis-elements.

2. Cis-elements involved in POMC gene transcription

The enhancers and promoters of most genes contain a series of cis-active elements that mediate the temporal and spatial patterns of gene expression (see review of Maniatis et al. 1987). These cis-elements can be located either upstream or downstream of the transcription start site and function even at great distances. These combinations of cis-active elements specific to each gene are recognized by combinations of different DNA-binding proteins. The DNA-binding proteins positioned at a distance from the start site are likely to depend on DNA looping or other chromatin structures to interact with the basic transcriptional

machinery (Ptashne, 1988). For transcription factors that bind as dimers, the DNA cis-acting elements are usually variations of symmetrical core binding motifs (palindromes).

The rat POMC gene promoter has been extensively studied by several different groups, and multiple cis-elements within this promoter have been defined (see review, Roberts et al. 1993; Liu et al. 1992; Therrien et al. 1993). Initial studies by Jeannotte et al. (1987) showed that only 480 bases of the rat POMC promoter are necessary to drive high level expression of a reporter vector. Later, reports from Therrien and Drouin (1991) showed that when they divided the POMC promoter into three regions from -480 to -323, -323 to -166, -166 to -34, respectively, they identified a complex relationship between these elements. What they found is that the distal element had no basal activity but the central or proximal element alone maintained basal activity. When the distal and central elements were present together, basal activity was synergistically elevated and was equivalent to all three elements together, which suggests a complex interrelationship between different regions of the POMC gene necessary to maintain basal POMC transcription.

By DNAse I footprint analysis, Therrin and Drouin (1991) identified nine elements which were protected by AtT20 nuclear extracts, with four in the distal region (DE-1-4), two in the central region (CE-1 and CE-2), four in the proximal region (PE-1-4). In mutagenesis studies, they found that mutations in each of these nine elements had similar effects on promoter activity. Further studies by them (Therrin and Drouin, 1993) revealed that the DE-2 element conferred cell-specific activity on the POMC promoter.

Actually the PE-1 element overlaps with a POMC promoter element defined by Riegel and colleagues (1990) which they called PO-B. When this element was mutated, 75% of basal promoter activity was lost in AtT20 cells. The

sequence around the PE-3 element before has been identified as a putative negative glucocorticoid responsive element (nGRE, Drouin et al. 1989; Riegel et al. 1991).

Recently, Liu et al. (1992) reported that, in transgenic mouse experiments using the POMC promoter with the TK-K1Tag reporter gene, they found the POMC promoter region -323/-34 is sufficient for tissue specific expression and hormonal regulation in transgenic mice. They also identified three elements in this region which were bound by AtT20 nuclear extracts. These sites were -107 to -160, -182 to -218 and -249 to -281, which overlap with PE-4, CE-1 and CE-2, respectively.

Although multiple elements in the POMC promoter have been identified, none of them have been examined for their role in CRF regulation of POMC gene transcription. Roberts et al. (1987) identified a region within the rat POMC promoter which is responsible for CRF inducibility of a POMC-CAT fusion construct transfected into AtT20 cells. They found that POMC promoter sequence -236 to -133 confers both CRF and forskolin inducibility when it was tested in a TK-CAT vector. Recently, Boutillier et al. (1994) have shown that the AP-1 like sequence in the POMC first exon is involved in CRF regulation of POMC gene transcription.

3. Trans-acting factors involved in POMC gene transcription

Cis-elements interact with trans-acting DNA binding proteins. Virtually all transcription factors can be considered to contain multiple, occasionally overlapping functional domains, including an obligatory DNA-binding domain and a transcriptional activation (or repression) domain. According to DNA binding motifs, transcription factors can be divided into at least 12 classes. These 12 DNA-binding motifs are: the homeodomain (reviewed in Scott et al. 1989), the

POU domain (Herr et al. 1988), the paired box (reviewed in Kessel and Gruss, 1990), the nuclear receptor-type Zinc finger (reviewed in Evans, 1988), TFIIIA-type Zinc finger (Miller et al. 1985), Leucine Zipper (Landschulz et al. 1988), helix-loop-helix (Murre et al. 1989), "ets homology" (Karim et al. 1990), "rel-NF-kB-dorsal homology" (Steward, 1987), "SRF-MCM homology" (Norman et al. 1988), "fork head-NF3a homology" (Weigel and Jackle, 1990), and "HMG homology" (Jantzen et al. 1990). Although the DNA-binding domains vary structurally among classes, they are likely to contain helical structures in the core DNA-binding region and clusters of basic amino acids (Otting et al, 1990). Many proteins bind to DNA as homo- or heterodimers via bipartite DNA-binding structures, with a core region for directly contacting DNA and an adjacent or overlapping region for protein dimerization.

The biochemical nature of transcriptional activation domains is not well understood, although activating regions often share structural features such as acid-rich (Ma and Ptashne, 1987), proline-rich (Mermod et al. 1989), glutamine-rich (Courey and Tjian, 1988), or serine- and threonine-rich amino acid sequences (Ingraham et al. 1990). While some transcription factors may exert constitutive effects, many are modulated by a variety of extra- or intracellular signals, such as binding of hormone to the ligand-binding domain, phosphorylation or dephosphorylation of either DNA-binding domain, transactivating domain or even nuclear translocation domain, etc.

Although multiple cis-elements have been defined for the POMC gene promoter, none of these DNA binding proteins have been cloned. Riegel and colleagues (1990) identified a protein they called PO-B, which binds to an element between the TATA box and the transcription start site (-15/-3). PO-B is believed to be responsible for the basal expression of POMC gene transcription. After purification, PO-B was found to have two components, with molecular

weights of 54 and 56 kD. Their DNA binding abilities were induced approximately 30-fold by dephosphorylation with acid phosphatase (Wellstein et al. 1991). Besides PO-B, another factor has been characterized by Therrien and Drouin (1993), which they called CUTE (for corticotrope upstream transcription element-binding) protein. CUTE was found to bind to a CANNTG motif in the DE-2 element, and was characterized as a helix-loop-helix factor which was suggested to be responsible for the tissue specific expression of POMC in pituitary. Recently, Liu et al. (1993) reported that, by southwestern experiments, there are at least three proteins with molecular weights of 60 kD, 40 kD, and 38 kD, that bind to the -107/-160 element specifically.

To date, no transcription factor has been identified to be responsible for the transcriptional regulation of POMC gene expression. Since CRF regulates POMC gene transcription via the cAMP/Ca^{2+} second messenger pathways, the transcription factor (s) mediating this regulation must be able to respond to cAMP and/or Ca^{2+} signals. Upon stimulation by these signals, these transcription factors should be able to be modified and activated to regulate POMC gene transcription.

Molecular mechanisms of transcriptional regulation of POMC expression by CRF

1. Transcriptional regulation of POMC gene expression through cAMP second messenger pathway.

The cAMP second messenger pathway has been studied extensively during the last decade (see reviews, McKnight, 1991; Lee, 1991; Roesler et al. 1988; Karin, 1991). This pathway has been found to be involved in many biological activities. In most of these cases, protein kinase A plays a significant role in the cAMP regulatory cascade (McKnight et al. 1988). Protein kinase A is composed

of four subunits, two regulatory (R) and two catalytic (C). cAMP activates PKA in the cytoplasm by binding to the R subunits thus liberating active C units. The active C units are able to phosphorylate other proteins, and alter their biological activity (Mellon et al. 1989).

The requirement for PKA in mediating the effects of CRF stimulation on POMC gene expression has been demonstrated (Reisine et al. 1985). It was shown that when a PKA inhibitor (PKI) was incorporated into AtT20 cells by liposome fusion, it blocked the stimulation of POMC gene expression by CRF or 8-bromo-cAMP.

a. Classical cAMP pathway - CRE/CREB.

While the molecular mechanism of how CRF treatment induces POMC gene transcription via the cAMP/PKA pathway is still obscure, there are many reports on the mechanisms of other genes being regulated by this pathway (see reviews, McKnight, 1991; Ziff, 1990). It was found that in the promoters of most of these genes responding to cAMP, there are consensus nucleotide sequences termed CRE (cAMP Response Element) present in their promoter which is the 8-bp palindromic sequence TGACGTCA. A family of transcription factors termed CREB (CRE Binding factor) were found to be able to bind to this consensus sequence as a homodimer. After cAMP level goes up and PKA is activated, CREB is phosphorylated at Ser 133 and its transcriptional activating activity will be turned on (see reviews, Lee, 1991; Gonzalez et al. 1989).

The gene encoding CREB has been cloned (Berkowitz et al. 1990; Meinkoth et al. 1991). Structure-function analyses of CREB indicates that it contains a carboxyl-terminal basic domain and a leucine zipper, which are involved in dimerization and DNA binding, and it also contains an amino-terminal domain which can be phosphorylated by PKA and is required for transcriptional activation. CREB protein appears to be expressed at similar levels in a range of

tissues, and it was also reported that in some cases CREB can form heterodimers with other leucine zipper proteins, implying a possible interaction between transcription factor members in this family.

Although CRE/CREB are involved in most cases of cAMP-regulated genes, there are no consensus CRE and no CREB binding sites in the POMC promoter. Thus, POMC gene regulation by CRF is most likely mediated by other cAMP dependent mechanisms.

b. Non-classical cAMP pathway

C-Fos

Recently, Dr. Loeffler's lab reported that c-Fos is involved in POMC gene expression induced by CRF (Boutillier et al. 1991). They showed that CRF induces a transient increase in c-Fos mRNA levels, and this induction occurs via PKA and the Ca^{2+}/calmodulin kinase. The elevation of c-fos protein in turn stimulates POMC gene transcription. These results were obtained by cotransfection experiments with the POMC promoter linked to a CAT reporter gene along with an expression vector coding for c-fos.

C-Fos belongs to the early immediate early gene family. This gene can be rapidly induced by a variety of extracellular signals such as growth factors, neurotransmitters, and depolarizing agents, and it is under the control of the cAMP and Ca^{2+} second messenger pathways (Greenberg et al. 1985; Morggon et al. 1986; Bartel et al. 1989). C-Fos protein contains a leucine zipper motif and therefore can form heterodimer with other leuzine zipper family members such as c-jun, JunB, ect (Chiu et al. 1988; Sonnenberg et al. 1989; Busch et al. 1990). The heterodimer of c-Fos/c-Jun binds to a consensus sequence motif TGAGTCA (AP-1 site), and exerts transcriptional activation of gene expression.

In the POMC promoter, there is an AP-1 site in the first exon which has been characterized as functional by Loeffeller and Roberts' group (Boutillier et al.

1993). They demonstrated that in vitro translated cFos/c-jun protein binds this AP-1 site efficiently, and when this AP-1 site was put in front of the CAT reporter gene, it responds to both CRF treatment and overexpression of c-fos protein.

But since the POMC major CRF responding sequence is in the -236/-133 region, there must be transcription factors other than c-Fos which are involved in regulating POMC gene transcription responding to CRF induction. Sequence analysis of the -236/-133 region revealed that there are actually several known consensus elements present in this region. Among these, the most notable ones are the AP-2 site located at -144 to -133, and the NF-kB site located at -152 to -140.

AP-2

Activating protein 2 (AP-2) binding sites were originally shown to be basal transcription enhancers present in the SV40 and human metallothoinein IA promoters (Mitchell et al. 1987). Later, it was found that the AP-2 sequence could also be responsible for induction by either phorbol esters, which activate protein kinase C, or by forskolin, which raises the concentration of cAMP (Imagawa et al. 1987). The human AP-2 gene was cloned by Tjian' s group (Williams et al. 1988). It encodes a 52 kD protein. AP-2 protein sequence doesn't contain a domain resembling any previously identified DNA binding motif, although it contains a proline and glutamine clustered region which has been found within the activation domains of other transcription factors. AP-2 protein has been found to be able to interact with other proteins (such as T-antigen), and besides its ability to mediate cAMP and phorbol ester induction of gene transcription, AP-2 was also suggested to be involved in the control of developmentally regulated gene expression (Mitchell et al. 1991). But so far, the mechanisms of how AP-2 protein mediates these transcriptional regulation function are still not clear.

NF-κB

Another notable consensus sequence within the -236/-133 region, NFκB site (see review, Leonard et al. 1989), was first identified as an enhancer sequence in the immunoglobin κB gene (Sen and Baltimore, 1986). NFκB is not only a regulator of gene action during development but is also involved in the inducible expression of a large number of genes in different cell types, such as major histocompatibility complex (MHC) class I, cytokines, and viruses. NFκB activity is inducible by cellular-activating stimuli such as antigens for lymphocytes, cytokine stimulation, or virus infection (Baeuele et al. 1991; Griffin et al. 1989; Osborn et al. 1989). NFκB protein contains a 50 kD peptide (P50) and a 65 kD peptide (P65), which are encoded by two different genes. Mouse p50 and p65 genes have been cloned (Ghosh et al. 1990; Kieran et al. 1990). Sequence analysis revealed that p50 peptide is derived from a 105 kD precursor peptide, and that its amino terminus shares high homology with P65 as well as with several rel-related oncogene proteins (Ruben et al. 1991; Hannink et al. 1989; Bull et al. 1990). Therefore NFκB proteins are also considered as rel family members. p50 and p65 are present in cytoplasm as heterodimers, and p65 is believed to be bound by another protein, IκB (Baeuerle et al. 1989; Ghosh et al. 1990), which upon binding to p65, can prevent NFκB protein from entering nucleus. After stimulation, IκB is phosphorylated by activated cellular protein kinases, and dissociates from p65, so NFκB protein can translocate into the nucleus and bind to ispecific sites in the promoter to activate gene transcription.

Interaction between multiple elements

Although AP-2 and NFκB could be good candidates for the major CRH induced regulator of POMC gene transcription, the mechanism of how the -236/-133 region can be the main CRF responsive region won't be clear until we can identify which particular element is actually involved in this regulation. It is even

possible that none of these individual elements in the -236/-133 region can fully substitute the whole piece, and only when several elements work together, can the full responsiveness to CRF be achieved.

This kind of stimulation has been reported before (Albanese et al. 1991). Albanese et al. (1991) characterized the human chorionic gonadotropin β-subunit gene (CGβ). They found that in the CGb gene promoter, there was a cAMP responsive region, which is located from -311 to -202 bp. When this region is fused to a heterologous promoter (-99aLUC), it has the same cAMP responsiveness as the full length promoter does. But when this region was dissected into five elements, none of these elements had any cAMP responsive ability. They concluded was that several elements within the -311 to -202 region must cooperate with each other to get the full cAMP responsiveness.

Tissue specific cAMP responsiveness

cAMP-inducible genes can also be activated in a tissue-specific manner (Lefevre et al. 1987; Horiuchi et al. 1991; Scott et al. 1992; Keech et al. 1992). In the case of the growth-hormone gene (Lefevre et al. 1987), its cAMP-responsive region in the promoter is recognized by GHF-1, a pituitary-specific transcription factor that belongs to the homeodomain family. cAMP can induce the expression of GHF-1 factor, and this in turn increases the expression of the growth-hormone gene. In this case, induction of a responsive gene by cAMP is mediated via an indirect mechanism that involves increased production of a cell-type-specific transcription factor. Recently, the rat prolactin gene was also reported to be regulated in a tissue specific manner by cAMP (Keech et al. 1992). But in this case, cAMP acts via a posttranslational modification of a preexisting transcription factor (Pit-1/GHF-1) instead of synthesis of more protein, and it was found that elements in the prolactin promoter which exert tissue-specific response to cAMP are redundant.

c. Conclusions

The cAMP second messenger pathway has been shown to be involved in CRF regulation of POMC gene transcription, and the POMC promoter region -236/-133 has been found to be the major CRF responsive region. Thus, there should be cis-element(s) in this region which are bound by transcription factors and which respond to the cAMP signal. CREB is not a good candidate, since there are no consensus CRE sites in this region. The consensus elements AP-2 and NFκB are possible candidates, but whether one of them (or even another element), can by itself be responsible for the full CRF responsiveness of the -236/-133 region, or whether several of these elements function cooperatively to achieve the full CRF responsiveness remains to be elucidated.

2. Transcriptional regulation of POMC gene expression through a Ca^{2+} second messenger pathway.

Besides the cAMP second messenger pathway, a Ca^{2+} second messenger pathway has also been suggested to be involved in transcriptional regulation of POMC gene expression by CRF. Since there are many other genes, including immediate early genes and those encoding neuropeptides and neurotransmitter biosynthetic enzymes, which have also been shown to be regulated by Ca^{2+} signals (Greenberg et al. 1986; Morgan and Curran, 1986; Bartel et al. 1989; Sheng et al. 1990), the molecular mechanisms involved in this kind of regulation have received a great deal of attention in the last few years. Some of these characterized biochemical pathways used by Ca^{2+} signals to regulate gene transcription are listed below.

a. phosphorylation of transcription factors by CaM kinases

CaM kinases are Ca^{2+}-calmodulin-dependent protein kinases. After Ca^{2+}-calmodulins bind to their auto inhibitory domains, their kinase domains are

activated leading to phosphorylation of the protein substrates at Ser or Thr sites. CaM kinases have been suggested to respond to Ca^{2+} signals for regulation of gene transcription by phosphorylating transcription factors such as CREB and C/EBPb.

Sheng et al (1990) found that Ca^{2+} signals can induce c-fos transcription via phosphorylation of CREB. Further studies revealed that CREB can be phosphorylated by CaM kinase at the same Ser 133 site as PKA. After phosphorylation by CaM kinases, CREB was activated and able to stimulate c-fos gene transcription via the same sequence element as CRE (Sheng et al. 1991).

Studies by Rosenfeld's group (Wegner et al. 1992) showed that, in pituitary cells, another transcription factor C/EBPb was phosphorylated in response to increased intracellular calcium concentrations as a consequence of the activation of a CaM kinase. Phosphorylation of serine at position 276 within the leucine zipper of C/EBPb by CaM kinase appeared to confer Ca^{2+} regulated transcriptional stimulation of a promoter that contained binding sites for C/EBPb.

Besides these two transcription factors, it is believed that there must be other factors which are phosphorylated by CaM kinases in response to Ca^{2+} signals. Besides CaM kinases, other kinases such as PKC and even PKA can also be involved in regulating gene transcription in response to Ca^{2+} signal (Ginty et al. 1992; Bachs et al. 1992).

b. Dephosphorylation of transcription factors by calcineurin

Calcineurin is a calcium-calmodulin-dependent serine phosphatase. It plays an important role in regulation of IL-2 gene expression responding to Ca^{2+} signal (Clipstone and Crabtree, 1992). It was found that in the IL-2 promoter, there is a cis-element called the NF-AT binding site where the transcription factor NF-AT binds and exerts its transcriptional activity. NF-AT consists of cyclosporin A

(CsA)-sensitive and CsA-insensitive components. Induction of NF-AT involves a calcium-dependent step that is sensitive to immunosuppressive drugs like CsA. Overexpression of wild-type calcineurin renders this transcriptional response more resistant to the drug; in addition, its calcium-dependence can be overcome by the expression of a deregulated subunit of calcineurin, which suggests that the phosphatase activity of calcineurin directly or indirectly contributes to the activation of NF-AT (O'Keefe et al. 1992).

The CsA-sensitive component of NF-AT resides as a preformed cytoplasmic protein that is translocated to the nucleus upon Ca^{2+} signal activation. Recent studies indicate that the cytoplasmic form of NF-AT is a phosphoprotein of 110-140 kD, while the nuclear form is considerably smaller, possibly as a consequence of the phosphatase activity of cytoplasmic calcineurin (Jian et al. 1993).

c. Ca^{2+}-dependent block of transcriptional elongation

Gene transcription rate can be regulated at the transcription initiation step as well as at the transcriptional elongation step. Studies using macrophage and fibroblast cell lines have shown that c-Fos transcription elongation is blocked within the first intron of the c-Fos gene, and relief of this elongation block requires Ca^{2+} and can be achieved by many different stimulatory agents including activators of PKC and a Ca^{2+} ionophore (Coliart et al. 1991; Mechti et al. 1991). But so far the exact molecular mechanism of this Ca^{2+} dependent transcription elongation block is still not clear.

d. Convergence on cAMP/PKA pathway

Besides the pathways listed in the previous paragraphs, Ca^{2+} signals can also use pathways which converge on the cAMP/PKA pathway to regulate gene expression. For example, a Ca^{2+}-dependent protease, calpain, was found to be able to degrade the regulatory subunit of PKA and thereby lead to increased

levels of its catalytic subunit (Aszodi et al. 1991). In certain neurons, such as pyramidal and granule cells of the hippocampus, a Ca^{2+} activated adenylate cyclase is expressed at high levels (Xia et al. 1991). In these cases, intracellular Ca^{2+} levels can modulate cAMP levels/PKA activities, via which it can regulate gene expression.

e. Molecular mechanisms of regulation of POMC gene transcription by Ca^{2+} signals

Although Ca^{2+} signals have been shown to be able to regulate POMC gene transcription (Lorang et al. 1994), the molecular mechanism of this regulation is still not clear. No group has yet reported the role CaM kinases play in regulating POMC gene expression. Within the POMC promoter, there is no consensus NF-AT binding element sequence, no consensus CRE and no consensus C/EBPb either. Thus, Ca^{2+} regulation of POMC gene transcription may work through other cis-element(s).

It is interesting to note that within the region from -236 to -133, there is an almost perfect palindromic sequence located at -171 to -160, and the core sequence of this palindromic element, CTGTGC, is also present in -215 to -190 region. Computer sequence analysis revealed that the palindromic core sequence CTGTGC doesn't belong to any known consensus sequence, and that between -171 and -160 there is a consensus MRE (metal regulatory element) sequence (TGCGCG).

MRE consensus sequences were originally defined by Hamer's group in the mouse metallothoinein-I gene (Seguin et al. 1987; Culotta et al. 1989). Metallothoinein, a small metal-binding protein, can be induced by various heavy metal ions such as cadmium, zinc, and copper, at the transcriptional level. MRE elements have been shown to be involved in responding to these heavy metal ions. It was shown that heavy metal ions can induce a specific gel-shifted band

when mouse nuclear extracts were incubated with MRE probe in the reactions. In the transfection experiments, the MRE-TKCAT reporter genes were also induced by heavy metal treatments. It was believed that there must be specific MRE binding proteins participating in these events. Several nuclear proteins have been reported to bind the mouse MRE element; they varied both in molecular weights and the sensitivities to heavy metal ions (Imbert et al. 1989; Labbe et al. 1991; Foster et al. 1991; Furst et al. 1988; Saguin, 1991). One of them, which was characterized by Otsuka's group (Koizumi et al.1992), was even suggested to be a negative regulator, because its binding to MRE was inhibited by both Cadmium and zinc ions. But so far, none of these proteins have been cloned, and the mechanism of how they interact with heavy metal ions to regulate MT gene expression is still obscure.

So far, no report has shown any relations between MRE and Ca^{2+} regulation of gene expression, and no transcription factors have been shown to be bound and regulated by Ca^{2+} ions directly. Whether in the POMC promoter the MRE works as a Ca^{2+} responsive element and whether some kind of transcription factor(s) can bind to this element and respond to Ca^{2+} signals awaits to be elucidated.

Summary and Significance

The proopiomelanocortin gene encodes a precursor polypeptide for a variety of peptide hormones, such as ACTH and β-endorphin. It is predominantly expressed in the anterior pituitary, and this anterior pituitary POMC system is a major component in the physiological response to stress. The POMC gene expression in the corticotrope cells is under multi-hormone control. The major regulators are CRF and glucocorticoid. CRF positively regulates POMC peptide secretion as well as its gene transcription, while glucocorticoid exerts a negative

feedback effect.

The POMC promoter has been characterized by several groups. The promoter region from -480 to the first exon has the full promoter activity in terms of basal expression as well as hormonal regulation. Multiple elements in the POMC promoter have been shown to be bound by nuclear proteins, but none of them have been examined for a possible role in CRF regulation of POMC gene transcription. Previous studies from the Roberts' lab have mapped the major CRF responsive region to be POMC promoter region -236 to -133.

Up-regulation of POMC gene expression by CRF has been suggested to be mediated by the cAMP as well as Ca^{2+} second messenger pathways. But sequence analysis of the promoter region from -236 to -133 has shown that there is no classical CRE sequence in this region. So the mechanism of CRF and cAMP/Ca^{2+} stimulation of POMC gene expression is probably beyond the well-known CREB effect.

The POMC gene is a good model for examining the mechanism of cAMP/Ca^{2+} regulation of gene transcription. The POMC protein contains several important peptides with different biological functions (see review, Eipper et al. 1980). The regulation of the production of these peptides must be able to meet their different physiological needs. CRF, working together with other POMC regulating hormones, is probably able to regulate the POMC gene expression in a manner which can distinguish these different physiological needs. But so far little is known about this mechanism. In order to be able to get a clear understanding of this mechanism, it is important to identify which elements in the POMC promoter are responsible for this regulation, and then to find out what protein factors bind to these elements.

The ability to respond to extracellular signals is essential for the development, survival, and the potential of all living organisms to adapt to

changing and adverse environmental conditions. Signal transduction pathways which regulate gene expression is a common response to extracellular signals. For the POMC gene, its expression is under the control of many signals. By studying its major regulator, CRF, and elucidating the molecular mechanism of how this signal brings a cascade of changes through the cAMP/Ca^{2+} second messenger pathways and induces the transcription of the POMC gene, we will understand more of this basic biological question of how the gene is regulated to respond to outside signals.

Chapter II.
Identification and characterization of the POMC CRH-Response Element (PCRH-RE).

Introduction

The ACTH encoding gene, proopiomelanocortin (POMC), is the precursor polypeptide for a variety of biologically important neuromodulators and endocrine hormones (Reviewed in Eipper et al. 1980). The POMC gene is predominantly expressed in the anterior pituitary and its expression in the corticotrope cells is under multi-hormonal control. Corticotrophin releasing hormone (CRH) is the main positive physiological regulator of POMC gene transcription in anterior pituitary corticotrophs (Giguere et al. 1982; Eberwine et al. 1987). In these cells, CRH not only increases cAMP levels, but also intracellular free Ca^{2+} levels, most likely as a result of an increase in cAMP. These two second messengers in turn have been shown to be potent stimulators of POMC gene expression (reviewed in Roberts et al. 1993). While the initial part of this regulatory cascade (membrane and cytoplasmic) by which CRH stimulates POMC transcription is well understood (Perrin et al. 1986; Guild et al. 1986; Luini et al. 1985), less is known about the nuclear mechanisms involved in this regulation. Usually cAMP and Ca^{2+} signals activate transcription through short DNA elements (cAMP or Ca^{2+} responsive elements, CRE or CaRE, respectively) located in the 5' promoter regulatory region of many genes, which are variants of the basic palindromic TGACGTCA motif (for review, Lee, 1991). There is a family of transcription factors, which includes the CREB (CRE binding protein, Kara et al. 1990; Berkowitz et al. 1991; Karpinski et al. 1992) that binds to this element. Upon cAMP and Ca^{2+} signal stimulation, the transcriptional activities of CREB

can be enhanced through phosphorylation by either PKA (reviewed in McKnight, 1991) or CaM kinase (Sheng et al. 1991). However, in the rat POMC promoter region, there are no elements highly homologous to this CRE motif, which suggests that other sequences and corresponding transacting factors mediate the transcriptional response to changes in cAMP and Ca^{2+} levels.

The rat POMC promoter has been characterized by several groups (Reviewed by Roberts et al. 1993). Although multiple elements have been found to be bound by nuclear proteins, none of these elements has been shown to be able to mediate the transcriptional response to CRF induction. Recent work from our laboratories has shown that CRH/cAMP-dependent activation of the Fos/Jun complex, distinct from the CRE/CREB complex, can stimulate POMC transcription from the AP1 element in the first exon (Boutillier et al. in revision). Our previous work (Roberts et al. 1987), however, showed that a major CRH/cAMP responsive element, resided in the -236/-133 region of the POMC promoter.

While studies by two groups have examined the entire rat POMC promoter by footprint analysis (Riegel et al. 1990; Therrien et al. 1991), these studies did not focus on the major CRH responsive region between -236 and -133. So a detailed footprint analysis of the rat POMC -236/-133 region with AtT20 whole cell extracts was performed at higher resolution by Dr. Stephen Salton in our lab, and it revealed multiple elements whose DNase sensitivity was protected or enhanced by bound nuclear proteins: -230/-215, -213/-184, -171/-160, -152/-144, and -138/-123 (figure 2-1).

Sequence analysis of this -236/-133 region showed multiple DNA elements that are homologous to several consensus sites. These are bound by known factors such as NFκB (reviewed in Lenard et al. 1989), AP2 (Williams et al. 1988; Mitchell et al. 1991), Sp1 (Briggs et al. 1986) and metal-responsive

element binding protein (MREB, Segiun et al. 1987; Culotta et al. 1989). Only the AP2 element has been found to regulate gene transcription responding to cAMP signals (Imagawa et al. 1987; Hyman et al. 1988), but the exact mechanism is still obscure. In order to find out whether any of these elements are responsible for the CRH responsiveness of the -236/-133 region, we dissected this region into smaller elements and characterized their individual properties. Here we demonstrate that multiple elements in this region were bound by nuclear proteins and that the binding of nuclear proteins to only one of these elements was induced by CRH stimulation. When this same element was tested in TK-CAT vector in transfection experiments, it showed the most CRF inducibility comparing to the other elements, although the induction was still less than the whole -236/-133 region.

Results

Delineation of a functional CRH responsive element within the -236/-133 region of POMC promoter.

Previous studies in our lab have identified a major CRH responsive region in the POMC promoter, which is the promoter flanking sequence -236 to -133 (Roberts et al. 1993). The sequence of the -236/-133 region is shown in figure 2-2. The elements bound by AtT20 cell proteins which were identified in DNase-footprint experiment are underlined. No consensus CRE element was found within this one hundred base region by sequence analysis using the GCG program (TFII sites); several elements were found to be homologous to known transcription factor binding sites, including AP2, NFκB and MRE (figure 2-2).

In order to characterize their functional activities and identify which of these elements can confer CRH responsiveness, oligonucleotides covering each of these footprints (-240/-210, -210/-180, -180/-150, -160/-130, -140/-105) were made and cloned 5' to the TK promoter driving a chloramphenical acetyl transferase (CAT) reporter gene (figure 2-3). The orientation and copy number of the inserts were checked by DNA sequencing. DNA from these constructs was transiently transfected into AtT20 cells, and CAT activities in extracts of untreated and 10 nM CRH-treated cells were measured. Variable induction of the construct with TK promoter alone was noted (up to two fold). Figure 2-3 shows the transcriptional activities of these reporter vectors in which the various oligonucleotides have been inserted. Expression of the construct containing the -180/-150 oligonucleotide was inducible five to seven fold, while expression of all the other constructs was inducible only two to four fold. Although reproducible, the CRH induction of the -180/-150 construct remained of smaller amplitude than that obtained with the complete -236/-133 CRH inducible region. Since only the -180/-150 construct showed a major response to CRH, we will subsequently refer

to the footprinted element in this region (-171/-160) as the POMC-CRH Regulatory Element (PCRH-RE).

As the TK-CAT vector itself is CRH inducible, probably caused by the presence of an AP-1 element within its plasmid sequence (Kushner et al. 1994), we reconstructed this vector by deleting the plasmid sequence between restriction site EcoR 109 and Nde I in order to eliminate this AP-1 element, and the resulting reconstructed vector is called ΔTK-CAT. Figure 2-4 shows the result of the transfection and CAT assay experiment in which the ΔTK-CAT constructs containing POMC promoter region -236/-133 and -180/-150 were tested. As we can see from this result, the ΔTK-CAT was not CRH inducible anymore; the -180/-150 construct and -236/-133 construct remained to be CRH inducible, although the induction fold of each of them was less than the ones in TK-CAT vector. Again, the fold induction of -180/-150 construct remained smaller than the one of -236/-133 construct.

A CRH inducible factor(s) binds to the PCRH-RE:

Nuclear extracts from control and CRH treated (10 nM, 60 min) AtT20 $_{D16-16}$ cells were prepared exactly as described (Dignam et al. 1983) and shown to bind efficiently to a smaller oligonucleotide (-175/-156) containing the rat PCRH-RE element in a gel shift assay. Greater amounts of the shifted complex were detected in assays that contained nuclear extracts from AtT20 cells treated with CRH for 1 hour, compared to those containing extracts from untreated cells (figure 2-5). No enhancement in the shift was seen with CRH treated AtT20 extracts for the other four oligonucleotides studied above (data not shown). This correlates with the increase in reporter gene transcription conferred by the PCRH-RE shown in figure 2-3. The oligonucleotide containing the same region of PCRH-RE in the mouse POMC promoter shows greater binding for factors

present in AtT20 extracts, and both rat or mouse elements can compete for binding of either (figure 2-5, left and middle panels). However, a mutant PCRH-RE (Δ rP) oligonucleotide with three nucleotides changed did not compete the binding (figure 2-5, right panel), suggesting that this region in the PCRH-RE is important for the binding specificity.

The binding specificity of the PCRH-RE was further analyzed by competition experiments with a series of oligonucleotides. Figure 2-6 shows that PCRH-RE binding is efficiently competed by the MRE from the mouse metallothionein promoter (MREd, sequence as in figure 2-7; Culotta et al. 1989), suggesting that the two sequences may be functionally related. In order to verify binding specificity with the POMC promoter, PCRH-RE binding was analyzed in the presence of the set of oligonucleotides covering the whole CRH (-236 to -133) inducible sequence. As shown in figure 2-6, PCRH-RE binding is efficiently competed not only by the larger sequence covering this element (-180 to -150), but also by a more upstream sequence (-210 to -180), which does not present obvious homology to the MRE (figure 2-7), yet does show homology to the PCRH-RE. There is also partial competition from the -240/-210 oligonucleotide which does share MRE homology (figure 2-7).

PCRH-RE binding activity is modulated by divalent cations:

Sequence comparison in figure 2-7 shows that there is a MRE conserved core sequence present in several of those oligonucleotides used in the competition experiment shown in figure 2-6, and in figure 2-6, it has been shown that these oligonucleotides competed in some degree with PCRH-RE in gel shift assay. To test whether PCRH-RE displays binding characteristics similar to that previously reported for the MRE (Imbert et al. 1989; Labbe et al. 1991; Foster et al. 1991; Furst et al. 1988; Saguin, 1991; Koizumi et al. 1992), gel shift analyses

of AtT20 nuclear extracts were performed in the presence of several divalent cations. As shown in figure 2-8, Cu^{2+} and Cd^{2+} (100 μM) abolished PCRH-RE binding. Interestingly, Zn^{2+} which has been reported to display properties similar to Cu^{2+} and Cd^{2+} when analyzed in the context of metallothionein gene regulation (Koizumi et al. 1992), was without detectable effect on PCRH-RE nucleoprotein complex formation, which suggested that the protein that binds to the PCRH-RE is probably not any of the MRE binding proteins which have previous been defined.

Comparison of the binding of nuclear proteins to -180/-150 region with the one to PCRH-RE:

In order to see whether it is the same nuclear protein complex binding to -180/-150 as to PCRH-RE, gel shift was performed using the POMC promoter element -180/-150 as probe. As we can see in figure 2-9, the gel-shifted band formed between the AtT20 nuclear extracts and -180/-150 element appeared to migrate slower than the one formed with PCRH-RE, suggesting that a larger nuclear protein complex is binding to -180/-150 element than the one binding to PCRH-RE, since the migration rate of gel-shifted band is decided dominantly by the size of the proteins binding to the probe rather than the size of the probe.

While a three base mutation in PCRH-RE abolishes its ability to bind nuclear proteins (figure 2-5), the same three base mutation in the -180/-150 element appeared to have no effect on nuclear protein binding. As shown in figure 2-9, the oligonucleotides containing three base mutation in -180/-150 element was still able to compete the binding of nuclear protein to -180/-150 probe, suggesting a complex protein-protein and a protein-DNA interaction is probably involved in -180/-150 and PCRH-RE binding.

Discussion

Studies from several laboratories have shown the transcriptional regulation of the POMC gene to be complex with multiple elements involved in basal expression and in the negative regulation by glucocorticoids (Roberts et al. 1987; Jeannotte et al. 1987; Drouin et al. 1989; Riegel et al. 1990; Riegel et al. 1991; Therrien et al. 1991). CRH, the primary stimulator for ACTH peptide secretion, has also been shown to be the major positive regulator of the POMC transcriptional response, although much less is known about the mechanism by which CRH mediates this positive transcriptional response. Like basal promoter expression and glucocorticoid negative regulation, it appears that there are multiple elements in the POMC promoter responsible for this positive CRH response (Roberts et al. 1987; Liu et al. 1992; Boutillier et al. 1992). However, none of them appear to be the classical CRE element, TGACGTCA, which will bind CREB-like transcription factors. Even the most minimal promoter construct, deleted to -31/+63 but still containing the functional AP1 site of the first exon, is capable of responding strongly to a CRH challenge to the transfected AtT20 cell (Boutillier et al. 1992). Possibly, the redundancy and complexity of POMC promoter elements has resulted from a need to respond to different hormonal and environmental signals in various tissues, including the pituitary and hypothalamus, and throughout the immune system.

In this study, we have extended our earlier observation that a region from -236/-133 of the rat POMC promoter gave the most induction in response to CRH in a heterologous TK promoter CAT expression vector (Roberts et al. 1987). Sequence analysis of this region revealed the presence of multiple known transcription factor binding elements. While all of these "identified" DNA elements showed the ability to bind proteins in extracts from AtT20 cells, and showed the ability to stimulate transcriptional activity of a heterologous promoter

in response to CRH-treatment, only the -180/-150 element showed the most CRH inducibility compared to the other four elements in the -236/-133 region. However, stimulated expression of the -180/-150 heterologous promoter following CRH-treatment was always approximately half of the entire -236/-133 heterologous promoter. Thus, it appears that the transcriptional enhancer activity of the -236/-133 region resides in the -180/-150 element, but requires some interaction with its surrounding DNA in order to give full CRH responsiveness.

Our study showed PCRH-RE to bind one or more proteins present in a nuclear AtT20 cell extract and binding was inducible by CRH treatment, while no enhancement in the shift was seen with CRH treated AtT20 extracts for the other four oligonucleotides. These results correlate with the observation we made in the study shown in figure 2-3, that PCRH-RE appeared as the major CRH responsive element in the -236/-133 promoter region. Increase of binding of protein(s) to PCRH-RE by CRH induction can be caused by two possible mechanisms. One is that CRH treatment can induce the production of one or several proteins which bind to PCRH-RE specifically. Since CRH treatment for 60 minutes is enough for this gel-shift induction, the protein(s) whose production is induced by CRH should belong to the immediate early gene families like fos or jun. The second mechanism is that CRH treatment may lead to modificatcn of the PCRH-RE binding protein(s), such as phosphorylation or dephosphorylation, and the modifications could increase the DNA binding ability of this protein(s) or the ability of another protein to bind to the PCRH-RE. Through either of these two mechanisms, the total transcriptional activity conferred by the PCRH-RE binding factor(s) to the general transcriptional machinery will be increased by CRH treatment, so the POMC gene transcription rate will be increased.

In gel-shift assays, rat PCRH-RE and mouse PCRH-RE showed a similar binding pattern (figure 2-5), and they were able to cross-compete with each other

for protein binding; so we assume that it was the same protein(s) binding to these two oligonucleotides. Sequence comparison of these two oligonucleotides showed that there are 5 out of 19 bases mismatched between them (figure 2-7), which may explain why mouse PCRH-RE showed much more binding activity than rat PCRH-RE; since AtT20 cell is mouse cell line, the protein factor must be able to bind to its own corresponding element better than to a slightly different element. Although the 5 bases mismatch makes the rat PCRH-RE a less efficient protein binding element, these 5 bases are not as important for PCRH-RE binding specificity as those three bases which are mutated in the Δrp element, as seen in figure 2-4, the three bases mutation in Δrp made it completely lose the competing ability for PCRH-RE binding.

Further analysis of the binding of this factor to various oligonucleotides showed it to be related in some fashion to the MRE conserved core sequence of the mouse metallothionein gene (figure 2-7). While the binding to this PCRH-RE element was clearly inhibited by the MRE sequence, the protein that was binding and causing the gel shift did not appear to have the same properties as those that had been previously described for binding to the MRE (Furst et al. 1988; Imbert et al. 1989; Labbe et al. 1991; Foster et al. 1991; Saguin, 1991; Koizumi et al. 1992). In the classically described MRE, the binding of various MRE-binding proteins so far defined were either enhanced by a variety of heavy metal ions like zinc, cadmium and copper, or like the MRE-binding protein defined by Koizumi et al (1992), binding was inhibited by zinc as well as cadmium. Interestingly, with the PCRH-RE Cu^{2+} and Cd^{2+} inhibit the binding in the gel shift analysis, while Zn^{2+} is completely without effect. Thus it appears that the protein(s) which binds the PCRH-RE is not exactly the same as those previously described for the metallothionein gene MRE, although it may be related, since its binding is both sensitive to heavy metal ions and competed by the MRE.

The comparison of nuclear proteins binding to -180/-150 element with the one to PCRH-RE revealed that a larger nuclear protein complex is binding to the -180/-150 region. Although the -180/-150 element contains PCRH-RE, it includes 6 bases more on each side than PCRH-RE. The sequences adjacent to PCRH-RE might allow additional protein to bind to the 180/-150 element. In the competition experiment of the -180/-150 element gel shift assay, it was unexpected to see that the same three-base mutation in -180/-150 element as the ones in PCRH-RE was able to compete for -180/-150 binding, since the same three-base mutation totally abolished the PCRH-RE binding ability in gel shift assay (figure 2-9). In figure 2-10, we propose a model, trying to explain this phenomenon. Presumably, in addition to protein(s) binding to PCRH-RE (protein A, figure 2-10), there are proteins binding to the rest of the -180/-150 element (protein B, C, figure 2-10), and these proteins are interacting with each other. So when PCRH-RE was used in gel shift assay, only protein A was able to bind to it, and when the three bases which are important for its binding were mutated, it would not be able to bind this mutated element any more. But when the -180/-150 element, which is larger than PCRH-RE, was used in gel shift assay, protein A, protein B and C were also binding to it, which would form a larger complex. Since protein B and C were able to interact with protein A, the binding of protein B and C to the -180/-150 would allow protein A also to be included in the whole binding complex, although the three bases for binding of protein A were mutated. Probably that is the reason that the mutated -180/-150 oligonucleotides appeared not to have effect in competition assay (figure 2-9). Results from these studies suggested a complex mechanism is possibly involved in the CRH responsiveness of this element.

In this study, we identified a major POMC CRH responsive element

(PCRH-RE) in the -236/-133 region. AtT20 nuclear protein(s) binding to this element was induced by CRH treatment. The binding is related in some fashion to the MRE of the mouse metallothionein gene, although the results from the binding assay with divalent cations suggested that the factor binding to PCRH-RE was not like any of those MRE binding proteins. Molecular cloning of the gene encoding this PCRH-RE binding factor will be necessary to identify exactly what kind of protein this binding factor is and what kind of mechanism it uses to elicite the CRH transcriptional response in the -236/-133 region.

Material and Methods

Cell Culture: Mouse AtT-20 D16/16 tumor cells were cultured in Dulbecco's modified Eagle medium (DME, Gibco) supplemented with 10% fetal calf serum in a humidified 5% CO2 incubator. Cells for transfection were cultured in 6-well dishes (Falcon) to 60% confluency and switched to serum-free medium (DMEM) for 24 hours before experiments. Cells for preparation of nuclear extracts were plated in 15 cm dishes.

Plasmid DNAs: POMC reporter genes: pJL 169 has been described (Roberts et al. 1987) and contains the POMC promoter sequence (-236 to -133 base pairs relative to the transcription initiation start site) cloned in front of the -109/+56 promoter of the thymidine kinase gene of the Herpes Simplex virus and the CAT reporter gene (TK-CAT vector, Roberts et al. 1987). Oligonucleotides spanning PCRH-RE or additional putative regulatory elements (see figure 2-3) were also cloned in the TK-CAT vector. ΔTK-CAT vector was constructed by digestion of the TK-CAT vector with Nde I and Eco109 and religation of the remaining TK-CAT vector.

Nuclear extracts: AtT 20 cells were grown until 70-80% confluency and serum deprived 24 hours prior to a 1 hour treatment with or without 10 nM CRH (pennesula labs). Cells were then harvested in cold phosphate-buffered saline (PBS). Nuclear extracts were prepared as described (19). Final concentrations were typically 2 to 3 μg/μl as determined by the Biorad protein assay (FRG, Germany).

Gel shift assays: All oligonucleotides were synthesized by the MSSM DNA

synthesis facility. Rat PCRH-RE (5'-TCGACCTGCTGTGCGCGCAGCCCG-3'), mouse PCRH-RE (5'-TCGACGTGCCTTGCGCTCAGCCAG-3'), MRE (CTCTGCACTCCGCCCGA). Five pmoles of double stranded oligonucleotide were end-labeled with (γ-^{32}P) ATP (3000 Ci/m mole, NEN) using polynucleotide kinase, resolved on a 10% acrylamide gel and eluted from the gel at 4° C in TE buffer (10 mM TRIS-HCl, pH 8, 1 mM EDTA) or dH_2O (when used for testing effects of divalent cations on gel shift). Approximately 0.2 ng of labeled DNA (15,000 to 20,000 cpm) were added to the pre-incubated nuclear extracts.

5-15 µg of proteins were pre-incubated 10 min at room temperature in 12 µl of binding buffer with 1 µg of poly (dI-dC) and 1 mg of nonspecific single stranded DNA. 0.2 ng of ^{32}P-labeled probe was added and the binding reaction was left at room temperature for 15 min. In competition experiments, 50-fold molar excess of unlabelled competitor oligonucleotides were added in the pre-incubation reaction. In experiments testing effects of divalent cations, different divalent cations were added in the pre-incubation reactions. The protein-DNA complexes were resolved on a 4% polyacrylamide gel in 0.5X TBE. The gels were dried and autoradiographed with intensifying screens at -70 C with Kodak X-OMAT AR films.

Transfection and Chloramphenicol Acetyl-transferase Measurement: Transfection experiments were performed using a lipopolyamine-based method (TRANSFECTAM ™, Promega). AtT20 cells were plated in 2 cm 6-well plates (Corning) two days prior to transfection. Before transfection, cells were incubated in serum-free DME medium for two hours. For each well, 2 µg of DNA and 4 ml of DOG will be first diluted in 50 ml 150 mM NaCl solution individually, then they were mixed and incubated at room temperature for 15 min and added to 1 ml of DME before being applied to the plates. Cells were incubated in this

transfection medium for 6-8 hours, the medium was removed and cells were incubated in fresh serum-free medium overnight. They were left either in this medium only, or with 10 uM CRF for another 24 hours before being harvested. Cell extracts for CAT Assay were prepared by three rounds of freeze-thaw lysis in 0.25 M Tris-HCl pH 7.5 solution. Aliquots of extract for CAT assay were heated at 65°C for 10 minutes to inactivate a heat-labile inhibitory thioesterase activity present in AtT20 cells. CAT activity was determined by ^{14}C-chloramphenicol method (Gorman et al. 1982). After autoradiography, the different forms of chloramphenicol were located, cut out of the silicate gel and counted.

DNase Footprint: DNase footprint analysis was carried out essentially as described by Angel et al (50). AtT20 cells were grown in monolayer culture and harvested at 80-90% confluence, scraped off the plate with a rubber policeman into ice-cold PBS and the cell pellets frozen at -80° C. Cell pellets representing approximately 10^9 cells were used for each preparation. Extracts were prepared from a whole cell lysate of AtT20 cells by the method of Manly et al (51), were desalted by G-25 column chromatography equilibrated in TM buffer (50 mM Tris-HCl pH 7.9, 1 mM EDTA, 1 mM DTT, 12.5 mM $MgCl_2$, 20% glycerol, 0.1 M KCl, 10 mM Na_2MoO_4 and 0.1 mM PMSF) and fractionated by heparin-agarose affinity chromatography. The extract was loaded on a heparin-agarose column equilibrated in TM buffer, washed with TM and then step-wise eluted with TM buffer containing 0.5 M KCl, and the enriched extract aliquoted and stored at -70° C.

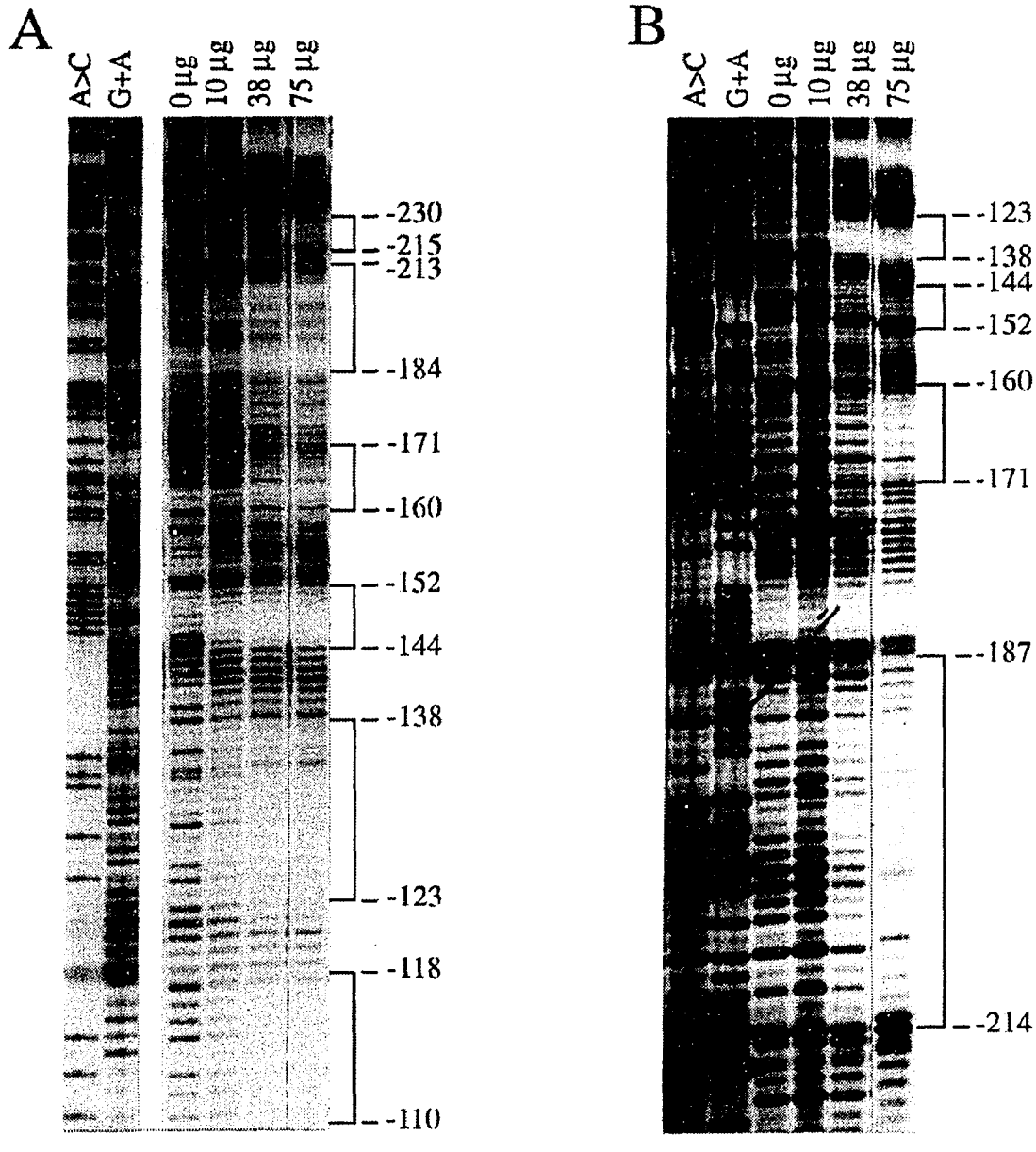
For DNase footprinting, DNA fragments were labeled at the 3' end using α-^{32}P-dNTP's and Klenow enzyme. End-labeled fragments were then purified by preparative agarose gel electrophoresis and electroelution. Extract (0-75µg) was incubated for 20 minutes with 0.5-2 ng of ^{32}P-labeled DNA in 25 mM Tris-HCl pH

7.9, 6.25 mM MgCl2, 50 mM KCl, 1 mM EDTA, 0.5 mM DTT, 1% polyvinylalcohol, 10% glycerol with 1 ug p(dI-dC)/20 µg protein (as nonspecific competitor DNA) at 4° C. Samples were warmed to 20^0 C for 5 minutes, and briefly treated with DNase I (Worthington), titrated to give a partial digestion pattern. Digestion was terminated by the addition of EDTA and treatment with proteinase K. DNA was extracted with phenol:$CHCl_3$ and ethanol precipitated. Reaction products were then analyzed on standard 6% polyacrylmide-8M urea sequencing gels next to a sequencing ladder generated from the same end-labeled DNA fragment to precisely identify the borders of DNase protection. Footprinting was performed on both strands of DNA.

Figure 2-1

DNase Footprinting Analysis of the POMC Promoter Region:

Upper (A) and lower (B) strands 3' ^{32}P-labeled at position -463 and +63, respectively, were subjected to DNase footprint analysis in the presence of various amounts of AtT20 cell extract as indicated (0-75 mg). A Maxam/Gilbert sequencing ladder of the same end labeled fragment are shown to identify the exact footprints. Positions of each footprint are shown relative to the start site of transcription.


A
A>C
G+A
0 µg
10 µg
38 µg
75 µg
-230
-215
-213
-184
-171
-160
-152
-144
-138
-123
-118
-110
B
A>C
G+A
0 µg
10 µg
38 µg
75 µg
-123
-138
-144
-152
-160
-171
-187
-214

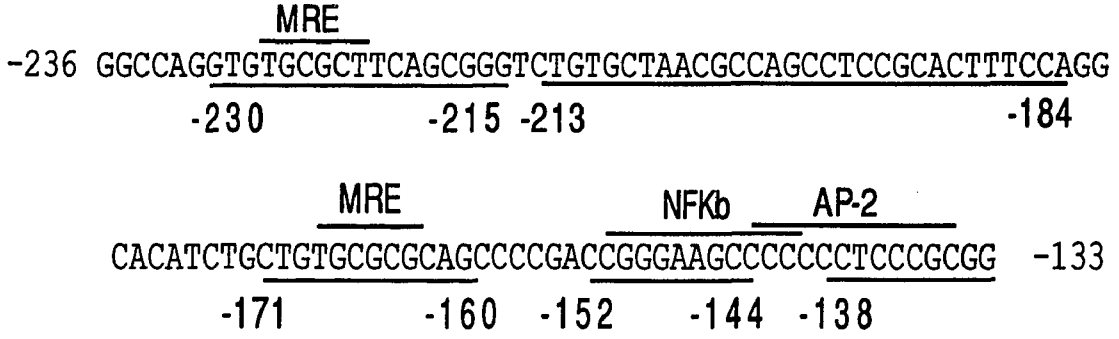
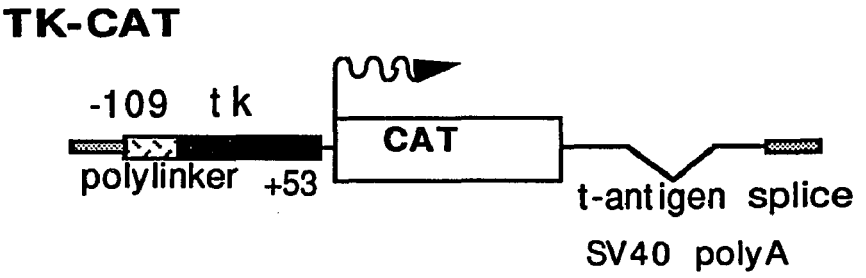


Figure 2-2
Sequence Analysis of POMC Promoter Region -236/-133.
Nucleotide sequence of the POMC promoter region -236/-133 is shown. Several transcription factor consensus elements which were revealed by computer program search (GCG) are indicated above the sequence. Different promoter regions which were protected in footprint experiments (figure 1) are underlined.

Figure 2-3

Major CRH Responsive Elements in POMC Promoter

Upper panel: Diagram of TK-CAT vector. Lower panel: Summary of CAT assay results. Vector alone (TK-CAT), constructs with the entire CRH responsive region (-236/-133) or various fragments of this region subcloned into the TK-CAT vector were analyzed in the presence or absence of 10 nM CRH after transfection into triplicate AtT20 cell cultures. The fold induction of CAT activity by CRH is shown.


TK-CAT
-109 tk
CAT
polylinker +53
t-antigen splice
SV40 polyA

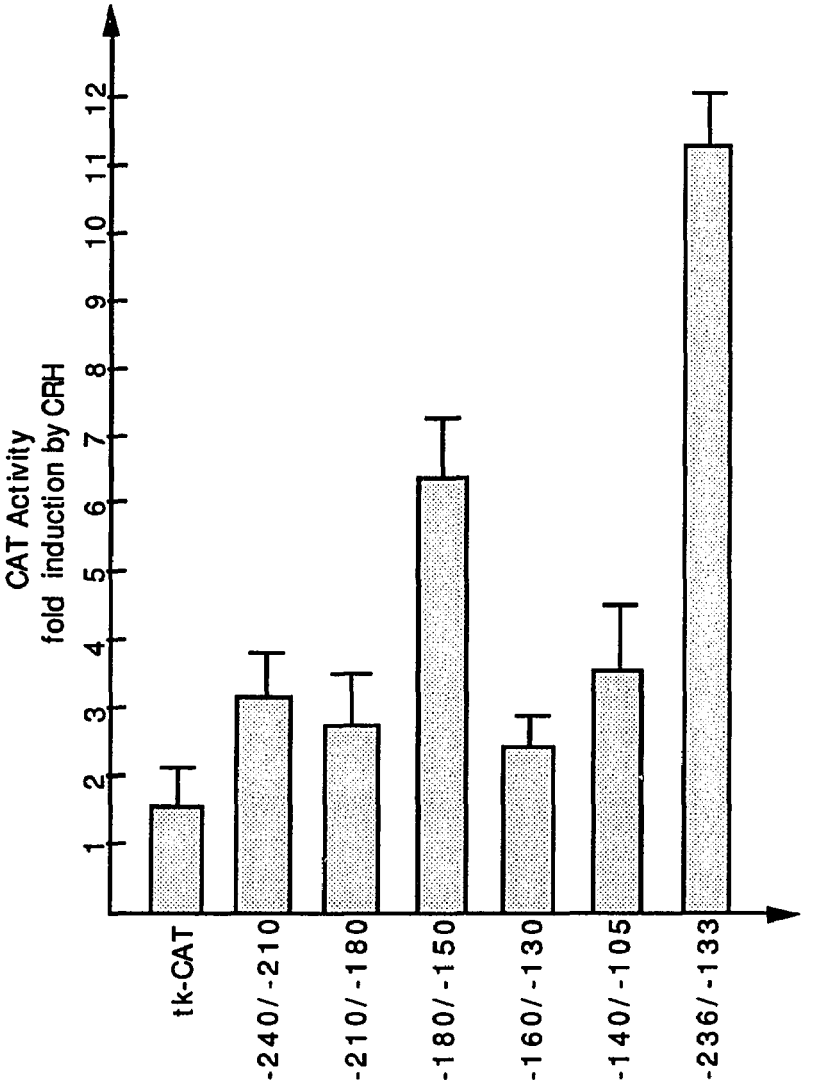
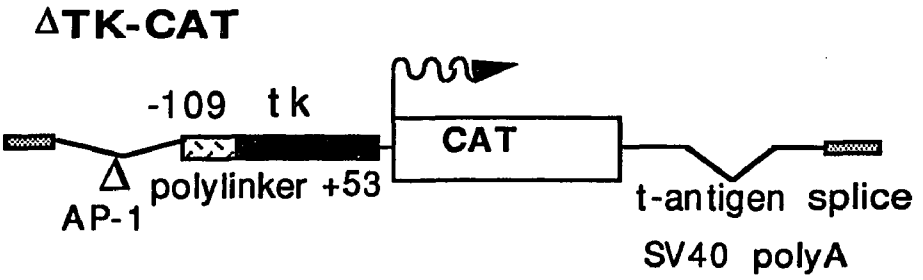

CAT Activity
fold induction by CRH
1
2
3
4
5
6
7
8
9
10
11
12
tk-CAT
-240/-210
-210/-180
-180/-150
-160/-130
-140/-105
-236/-133

Figure 2-4

CAT Assay Results with Mutant TK-CAT Vectors

Upper panel: Diagram of ΔTK-CAT vector. Lower panel: Summary of CAT assay results. Vector alone (ΔTK-CAT), constructs with the entire CRH responsive region (-236/-133) and element -180/-150 subcloned into the ΔTK-CAT vector were analyzed in the presence or absence of 10 nM CRH after transfection into triplicate AtT20 cell cultures. The fold induction of CAT activity by CRH is shown.


ΔTK-CAT
-109 tk
CAT
Δ
AP-1
polylinker +53
t-antigen splice
SV40 polyA

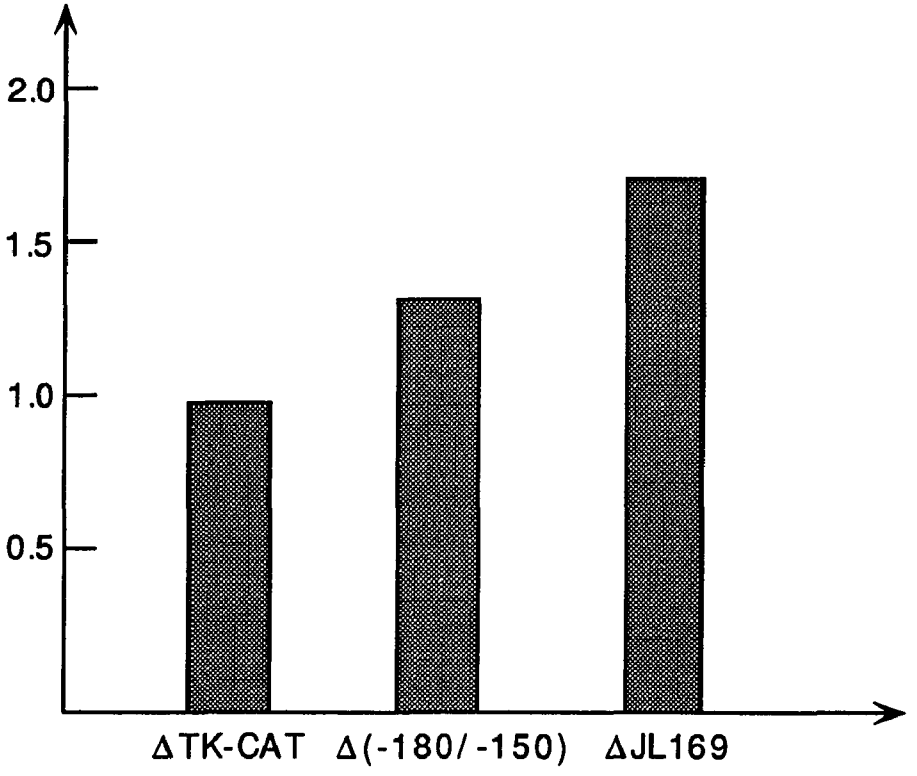

2.0
1.5
1.0
0.5
ΔTK-CAT
Δ(-180/-150)
ΔJL169

Figure 2-5

Nuclear Proteins Bind to Rat and Mouse PCRH-RE

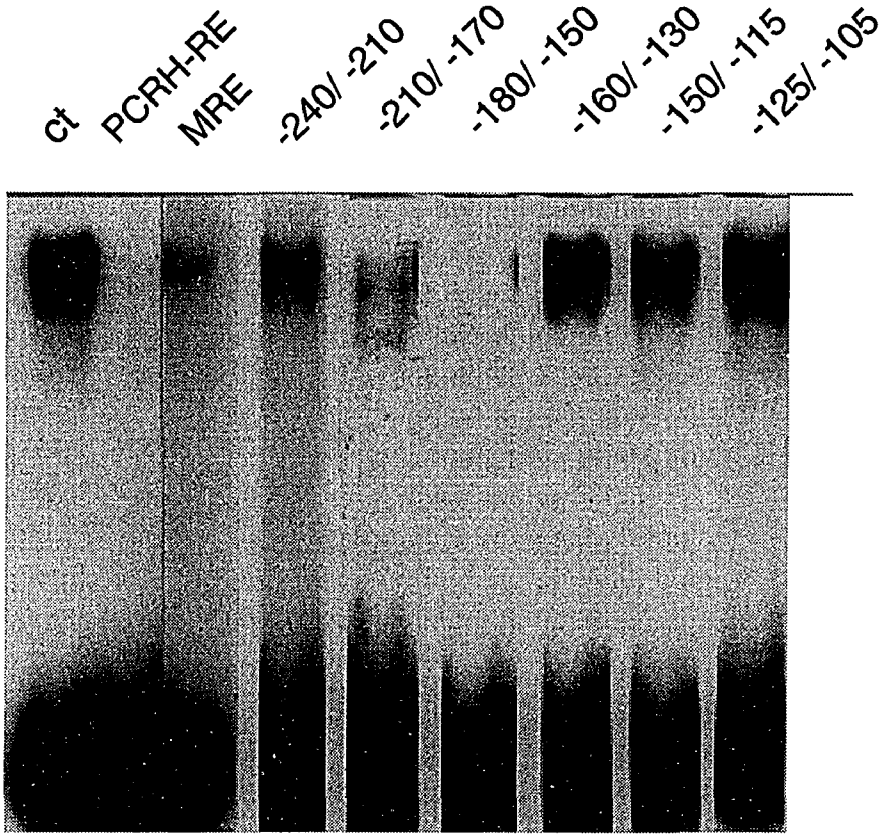
Gel shift analyses with nuclear extracts (15 μg) from untreated (ct) or 10 nM CRH-treated (CRH) AtT20 cells. Left: ^{32}P labeled rat PCRH-RE was used as probe with no competitor (0) or 50-fold cold rat PCRH-RE (rP) and mouse PCRH-RE (mP) used as competitors. Middle: ^{32}P-labeled mouse PCRH-RE used as probe with the same set of cold oligonucleotide competitors. Right: ^{32}P labeled rat PCRH-RE was used as probe with no competitor (0) or 50-fold cold rat PCRH-RE (rP), mutant rat PCRH-RE (ΔrP) and a sequence unrelated oligonucleotide (N.Sp1) as competitors. The sequence of ΔrP is 5'-TCTGCTGTAAACGCAGCCCCG-3'. N.Sp1 is 5'-AGCTTCGGCAGGACAATTATTCGA-3'.

Labeled Oligo
Rat-PCRH-RE
Mouse-PCRH-RE
Rat-CRH-RE
Extract
ct
CRH
Competing Oligo
0
0
mP
rP
ct
CRH
0
0
mP
rP
CRH
0
rP
DrP
NSpl

Figure 2-6

Competition on Mouse PCRH-RE Gel-shift with Nuclear Proteins

Gel shift analysis with nuclear extracts (15 µg) from 10 nM CRH-treated AtT20 cells, using mouse PCRH-RE as probe and various different cold oligonucleotides as competitor (50-fold). Lane 1: control (ct), no competitor. Lane 2: mouse PCRH-RE competitor. Lane 3: Metallothionein MRE. Lane 4-10: competing oligonucleotides covering different elements within POMC promoter region -240 to -105; the position of each of these elements in the rat POMC promoter is indicated.


ct
PCRH-RE MRE
-240/ -210
-210/ -170
-180/ -150
-160/ -130
-150/ -115
-125/ -105

Homology between different elements

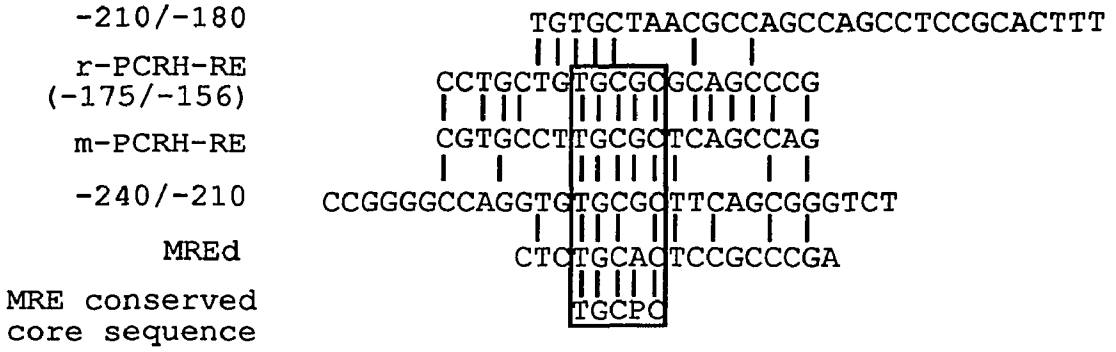


Figure 2-7: Sequence homology analysis between different elements. sequences of r-PCRH-RE (-175/-156), -210/-180, -240/-210, m-PCRH-RE and mouse metallothionein metal responsive element (MREd) are listed and compared. The sequences homologous to MRE conserved core sequence TGCPC (p,as purine) are boxed.

Figure 2-8

Divalent Cation Effects on Mouse PCRH-RE Gel-shift with Nuclear Proteins

100 mM of different divalent cations were used in gel shift reactions. In each reaction, 15 µg of nuclear extract from 10 nM CRH-treated AtT20 cells was used with ^{32}p-mouse PCRH-RE as in figure 2-3. Lane 1: control (ct), no divalent cations were added. Lane 2-6: $CaCl_2$, $MgCl_2$, $CuCl_2$, $CdCl_2$, and $ZnCl_2$ were added to each reaction as indicated.

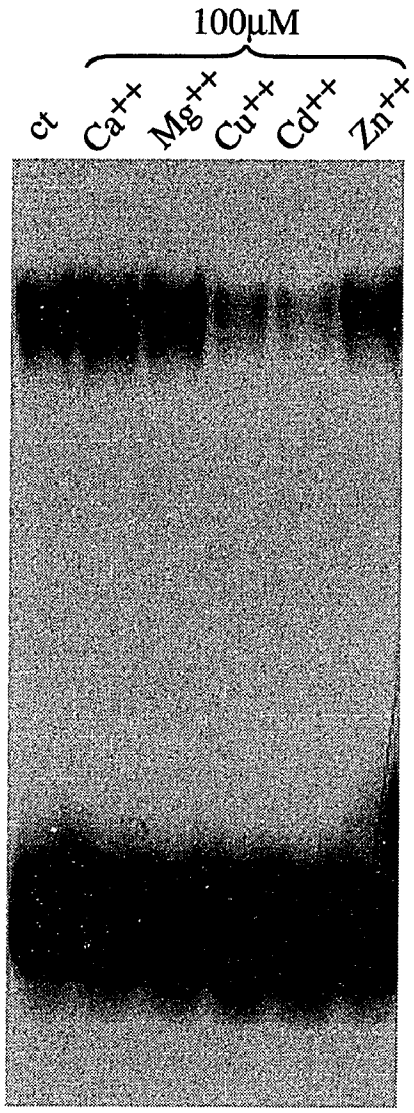

100μM
ct
Ca++
Mg++
Cu++
Cd++
Zn++

Figure 2-9

Comparison of the Bindings of the Nuclear Proteins to PCRH-RE with the Bindings to -180/-150 Element

Gel shift analyses with nuclear extracts from AtT20 cells using PCRH-RE (-175/-156) and element -180/-150 as probes. No competitor (0) or 50-fold cold different oligonucleotides were used in each lane, respectively, as competitor. The sequence of mutant PCRH-RE (ΔrP) and NSP1 were shown in legend of figure 2-5, the sequence of mutant -180/-150 oligonucleotide is: 5'-GCACATCTGCTGTAAACGCAGCCCCGACCGG-3'.

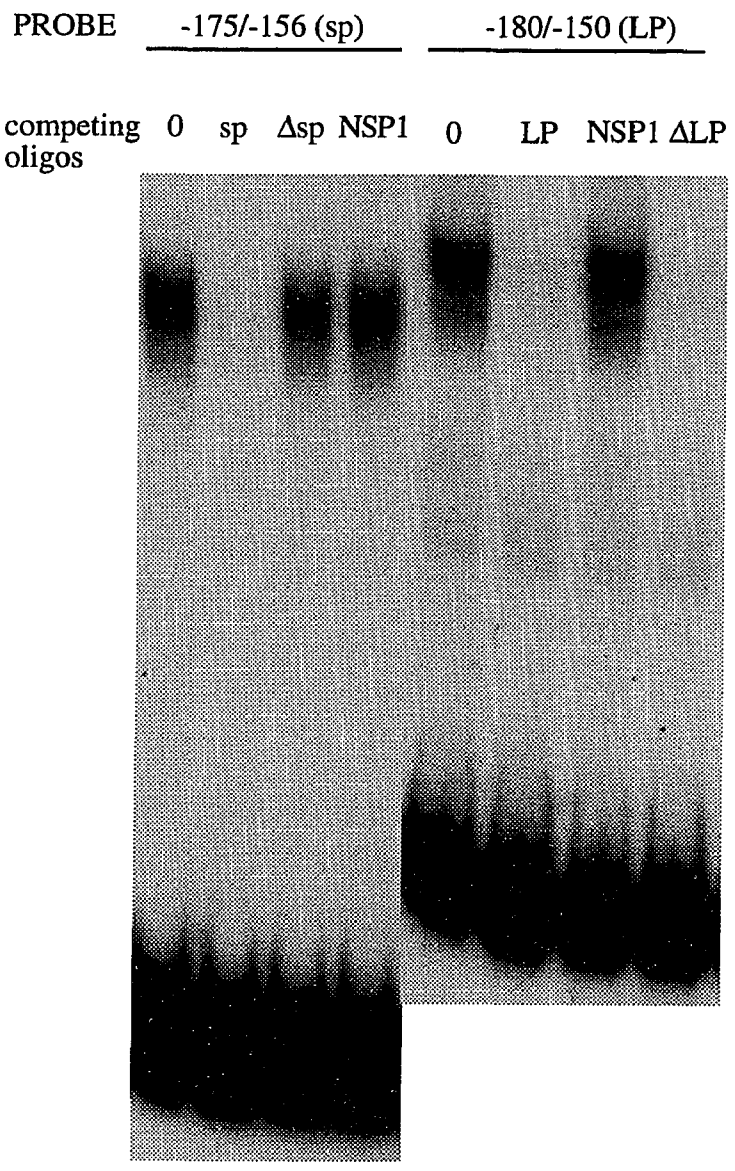
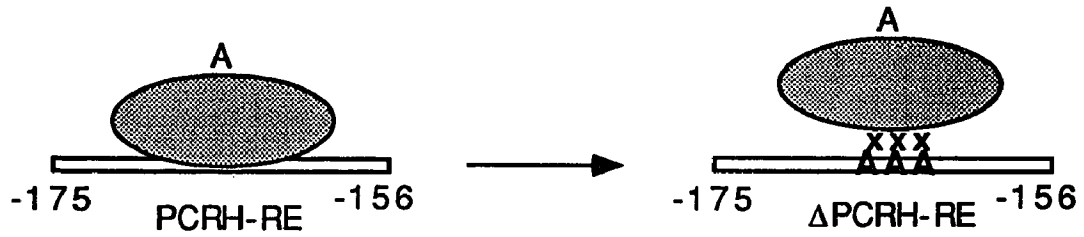

PROBE
-175/-156 (sp)
-180/-150 (LP)
competing oligos
0
sp
Δsp
NSP1
0
LP
NSP1
ΔLP

Figure 2-10

A Model of Protein Complex Binding to DNA Sequence Around -180/-150 Element

A model for interactions between the transcription factors binding to PCRH-RE and -180/-150 elements are diagrammed. Different transcription factors are shown in different shape of ellipses. Mutations within these elements are indicated, and the possible effects of these mutations on binding of transcription factors are stated as either "Binding" or "No Binding".

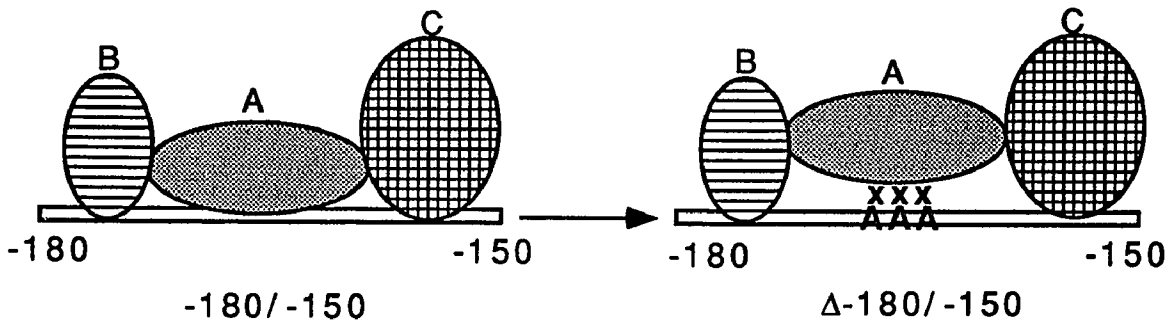
A.



Binding

No binding

B.



-180/-150

Δ-180/-150

Binding

Binding

Chapter III.
Molecular cloning and characterization of the PCRH-RE binding protein (PCRH-REB).

Introduction

Transcriptional regulation of gene transcription is critical for a cell to make adaptive changes to respond to different signals from a changing environment, and it is also the final step of a signal transduction pathway (Ginty et al. 1992). Regulation of gene transcription rate is mediated by both cis-acting elements and regulatory transcription factors. In order to understand the molecular mechanisms of a certain type of hormonal regulation of gene transcription, it is necessary to identify the cis-element sequences responsible for that kind of regulation as well as the transcription factors binding to that cis-element.

The stimulation of the gene expression of POMC, which encodes the precursor peptide of ACTH, by corticotropin releasing hormone (CRH), is part of the systemic response of the body to stress (reviewed in Antoni, 1986). The CRH stimulation of POMC gene expression has been proved to be happening at the transcriptional level (reviewed by Roberts et al. 1993). The CRH regulation of POMC gene transcription has been shown to be elicited through the cAMP as well as Ca^{2+} second messenger pathways (Lorang et al. 1994). While the molecular mechanisms of transcriptional regulation by cAMP/Ca^{2+} signals have been characterized (see chapter I), the nuclear mechanisms involved in CRH regulation of POMC gene transcriptions are still not clear.

The POMC promoter has been characterized by different groups (reviewed by Roberts et al. 1993). Multiple elements in the POMC promoter have been shown to bind nuclear proteins, and several of these elements have been found to be involved in either basal expression (Riegel et al. 1990) or tissue-specific expression (Therrin and Drouin, 1993). For CRH stimulation of

POMC gene transcription, studies from Loeffler and Roberts' labs have found c-fos to be involved in this regulation, and they identified a functional AP-1 element in the first exon which was shown to be able to mediate this regulation (Boutillier et al. 1994). But since the previous work from Roberts' lab has already shown that the major POMC CRH responsive region resided in the -236/-133 region (Roberts et al. 1987), there must be regulatory proteins other than c-Fos also involved in this regulation.

Actually, recent studies by our laboratory have identified a major CRH responsive element in the POMC -236/-133 region (see chapter II). It was found that the -180/-150 element had the ability to stimulate transcriptional activity of a TK heterologous promoter in response to CRH treatment. In gel shift assays, a smaller oligonucleotide within the -180/-150 (-175/-156, called as PCRH-RE) has been shown to be bound by AtT20 nuclear protein(s) and the binding was inducible by CRH treatment. Further analysis of the binding of this factor to various oligonucleotides showed it to be related in some fashion to the MRE conserved core sequence of the mouse metallothionein gene, but results from gel shift assays with divalent ions suggested that the factor binding to PCRH-RE is not one of the MRE binding proteins which have so far been defined (Furst et al. 1988; Imbert et al. 1989; Labbe et al. 1991; Foster et al. 1991; Saguin, 1991; Koizumi et al. 1992).

In order to identify what kind of factor is actually binding to the PCRH-RE and also to understand the molecular mechanisms of how this element is able to mediate the CRH responsiveness of the -236/-133 region, we decided to identify this factor. Here we report the cloning and characterization of a polypeptide that binds specifically to this element in the POMC promoter. Interestingly, while this work was in its final stages, several groups described the isolation of the same cDNA from mouse (Burbelo et al. 1993) and human (Lu et al. 1993; Luckow et al.

1994), suggesting that this cDNA encodes a protein also important in replication.

Results

Isolation of a cDNA coding for the PCRH-RE binding protein:

Previous studies by us (see chapter II) have identified a POMC CRH responsive element (PCRH-RE) in the -236/-133 promoter region, and the PCRH-RE binding factor appeared to be unique. To characterize it further, we screened a CRH-induced AtT20 cDNA expression library using Southwestern blotting with the specific PCRH-RE sequence. AtT20 cells were treated with 10 nM CRH for 30 min prior to extraction of RNA. A cDNA expression library was prepared from this RNA using a Bluescript expression vector (λ-Zap II) system (Stratagene). Approximately 10^6 plaques from the primary cDNA library were screened with a ^{32}P-labeled PCRH-RE concatemer. Filters were hybridized and washed as described in *Materials and Methods*. One clone that expressed a protein which bound to the PCRH-RE concatemer was isolated by this strategy, referred to as PCRH-RE Binding protein-1 (PCRH-REB-1). It was found to contain a 2.6 kb cDNA insert and to produce a fusion protein of approximately 105 kD. This protein was shown to bind the PCRH-RE oligonucleotide but not to an unrelated labeled oligonucleotide (N.Sp1, see figure 3-2 legend for sequence) in dot blot assays (data not shown). Polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the extracts showed that there was a low level of fusion protein expression, so the cDNA insert was excised and subcloned into a second expression vector pSEM (Knapp et al. 1990), containing 45 kD of ß galactosidase which has been observed to give a much higher level of expression. This construct was used in the studies described below.

PCRH-REB1 expression and analysis:

A Coomassie blue staining of proteins from unstimulated and IPTG induced bacterial lysates fractionated by SDS-PAGE (figure 3-1A) shows the IPTG induction of a major protein band at the expected molecular weight of 150 kD, PCRH-REB-1. This band as well as a control (ct) non-specific 40 kD band were excised, eluted, renatured and their binding activity to the PCRH-RE evaluated in gel-shift assay. Figure 3-1B and figure 3-2A show the efficient binding of the 150 kD fusion protein to the -180/-150 PCRH-RE containing oligonucleotides, while proteins eluted from a control 40 kD band did not bind (figure 3-1B), precluding any gel extraction artifact. Unrelated oligonucleotides (NSp1-3) bind poorly if at all to PCRH-REB-1 fusion protein (figure 3-2A). To test whether the bacterially expressed PCRH-REB-1 fusion protein displays characteristics similar to AtT20 nuclear extracts, its binding to the PCRH-RE was analyzed in the presence of various divalent cations. As shown for AtT20 nuclear extracts (figure 2-6), the binding of bacterially expressed PCRH-REB-1 is abolished by 100 μM Cu^{2+} and Cd^{2+}, while Zn^{2+}, Ca^{2+} and Mg^{2+} are without effect (figure 3-2B), suggesting that the protein we isolated is probably the same protein in the AtT20 nuclear extract which specifically binds to the PCRH-RE.

Sequence analysis of PCRH-REB:

In order to fully characterize the PCRH-REB encoding mRNA, a series of internal oligonucleotides were prepared as nucleotide sequence was obtained. Analysis of the sequence within the 2.6 kb insert revealed an open reading frame that extended throughout the entire sequence with a termination codon in the plasmid polylinker. Thus, the cDNA library was rescreened with a fragment derived from the 5' end of the cDNA clone, resulting in the identification of additional cDNA clones (figure 3-3). Through sequence analysis of these additional clones, an in-frame AUG start codon flanked by a consensus Kozak

sequence and an in frame UGA termination codon were identified and an approximately 127 kD protein was predicted to be encoded by the PCRH-REB mRNA open reading frame. Sequence comparison of PCRH-REB with Genebank revealed that the protein sequence of PCRH-REB was identical to the recently cloned cDNAs encoding MSW (Berbedu et al. 1993) and has one amino acid difference (Asn 945 Ser) from mRFC140 (Luckow et al. 1994), putatively the large 140 kD subunit of the replication factor C complex. It was also found to be 90% homologous to a human DNA binding protein PO-GA (Lu et al. 1993). In addition, a region of PCRH-REB (amino acids 400-490) was found to have significant homology to regions of E.coli and S. Pombe DNA ligases and human poly (ADP-ribose) polymerase.

The primary amino acid sequence was analyzed (GCG, program Motifs) to determine if there were any particular protein motifs or domains which may be indicative of our proposed function of PCRH-REB as a nuclear transcription factor. Analysis of the localization of cysteines and histidines in the protein showed no zinc fingers of the consensus structure. The protein is heavily charged with large numbers of basic and acidic residues with a predicted pKa of approximately 9.5. While in some regions the acidic and basic residues are evenly spaced, there are regions which are very basic or very acidic as outlined in figure 3-3. Several regions were predicted to form an alpha-helical structure, including two domains which were negatively charged. Further analysis of the alpha-helical regions did not, however, show any of them to contain appropriately spaced leucine or other hydrophobic residues to suggest that they may function as a "leucine zipper" as seen with other transcription factors (Lanschulz et al. 1988). Numerous consensus PKA phosphorylation sites, (R,K) 2X (S,T), were identified in the predicted PCRH-REB sequence, concentrated in the N-terminus, and an ATP/GTP binding site motif A (p-loop), (A, G) 4X G K (S, T), was found in

the central part of PCRH-REB. Finally, the deduced PCRH-REB protein is extremely rich in serine residues, comprising 10.4% of the total amino acid residues.

Expression of PCRH-REB mRNA in cells and tissues:

In order to define the size of the full length mRNA of PCRH-REB in AtT20 cells, primer extension and northern blot analyses were performed. Extension of a primer (nucleotide 202-219) revealed that there are 4 putative transcription initiation sites for the PCRH-REB mRNAs (figure 3-4A). In AtT20 cells the major initiation site is approximately 450 bases 5' of the primer, which suggests that the size of the full length mRNA should be approximately 4.9 kb. Using poly A+ RNA prepared from control as well as CRH-treated AtT20 cells, northern analysis revealed a single 4.9 kb mRNA (as estimated from single stranded DNA size markers) which hybridized to the PCRH-REB cDNA probe (figure 3-4B), in agreement with the primer extension observations. The quantity of this mRNA was not increased by treatment of AtT20 cells for 1 hour with CRH. This observation was verified by RT-PCR analysis as described below .

The expression of PCRH-REB mRNA in different tissues was investigated by reverse transcription of RNA and subsequent PCR analysis. 10 ug samples of total RNA from several different tissues were reverse transcribed using oligo dT as a primer and the resulting first strand cDNAs were subjected to PCR analysis using two oligonucleotide primers that corresponded to the 3' end of the PCRH-REB cDNA. As is shown in figure 3-5, PCRH-REB mRNA was found to be widely expressed in different tissues, although the expression in testis and heart was lower.

Functional studies of PCRH-REB by cotransfection:

In order to find out whether PCRH-REB can function as a transcriptional factor by itself, cotransfection experiments were performed. Full length PCRH-REB cDNA (which was made by ligating two partial PCRH-REB cDNA inserts) was cloned into a eukaryotic expression vector RSV-424 (Sadowski et al. 1989), which was driven by RSV promoter, fused in frame with Gal4 DNA binding domain (amino acids 1-147). DNA of this construct was cotransfected with the DNA of a CAT reporter construct which has 5 Gal4 binding elements in front of the CAT gene (figure 3-6). The Gal4 (1-147)-PCRH-REB fusion protein produced by the expression vector should be able to bind to the Gal4 binding elements adjacent to the CAT gene. So if the PCRH-REB has a transcriptional activation domain and can function as a CRH responsible transcription factor by itself, it should be able to stimulate the CAT reporter gene expression responding to CRH signal, just like the positive control protein (Gal4-CREB) does (figure 3-7). However, these studies showed that, while the Gal4-CREB dramatically stimulated CAT expression upon CRH treatment, Gal4-PCRH-REB failed to show any transcriptional activity by itself (figure 3-7).

Expression of the full-length PCRH-REB in bacteria and purification by His-tag resin column:

Full-length PCRH-REB cDNA was cloned into a bacterial expression vector, Pet 28 (Novagen), and the full-length PCRH-REB protein was expressed in the Dh21 bacteria by 1 mM IPTG induction. Protein extracts of the control and IPTG-induced bacteria were made and analyzed by SDS-PAGE. Figure 3-8A shows the IPTG induction of PCRH-REB expression at a size around 140 kD. Since the Pet 28 vector contains a His-tag sequence, which is a consecutive stretch of 6 histidine residues that can be expressed at the amino terminus of the target protein, bacterial expressed PCRH-REB was further purified using a His-

tag resine column (Novagen). The His-tag sequence can bind to divalent cations (Ni^{2+}) immobilized on the His-Bind resin. After unbound proteins were washed away, the target protein PCRH-REB was recovered by elution with imidazole. Figure 3-8B shows the PCRH-REB protein band after His-tag column purification.

Western blot:

Two polyclonal antibodies against PCRH-REB were raised in rabbit using SDS-PAGE gel purified PCRH-REB-1 as antigen. Briefly, PCRH-REB in a denatured form was obtained following preparative SDS slab gel electrophoresis and excision of the single band corresponding to the 150 kD b-gal-PCRH-REB-1 fusion protein. The protein was sent to Pocanol Farm for injection into rabbits. Initial injections of 200 μg of the protein were used for immunization, with three subsequent injections of 100 μg of this protein repeated at 2-week intervals. Blood was collected 1 week after the injections. Antiserum from each of the three bleeds were tested in western blots. These antibodies were used in western blot to characterize the expression of PCRH-REB in the AtT20 cells. Figure 3-9 showed that antibody 1 was able to cross-react with bacterial expressed 140 kD PCRH-REB in crude extract as well as in purified extract (right panel, lane IPTG and Purified P), and when this antibody was used in western blot with AtT20 nuclear proteins, it identified a putative PCRH-REB protein band at a size around 140 kD which was not cross-reacted by preimmune serum (middle panel, CT and CRF lanes). According to western blot results, PCRH-REB protein in AtT20 cells didn't appear to be induced by 1hr CRH (10nM) treatment.

Discussion

In this study we reported the isolation and characterization of a mouse cDNA encoding a PCRH-RE binding protein, PCRH-REB. PCRH-RE has been identified as the CRH responsive element in the POMC -236/-133 region, and the binding of protein (s) to this element has been shown to be sequence-specific, CRH inducible and metal ion sensitive (see chapter II). The cloning of a protein which binds to the PCRH-RE by Southwestern blot analysis initially revealed a protein which retained the binding specificity and metal sensitivity of nuclear extracts from the AtT20 cells, suggesting that, indeed, we had isolated the cDNA clone encoding the protein involved in the gel shift. That the gel shift of the PCRH-RE element could be achieved by the bacterially expressed, purified protein implied that no additional protein is necessary for eliciting the gel shift, although there may be other proteins involved in the normal *in vivo* binding. While the purified, cloned, PCRH-REB protein appeared to exhibit the proper binding characteristics, its structure did not readily suggest that it was a member of any of the well-known transcription factor gene families. No classical transcription factor motif, such as a zinc finger or leucine zipper, was identified in the protein. There are, however, several alpha-helical structures with basic charge predicted in the PCRH-REB protein which are also present in both zinc finger structures as well as in leucine zipper structures, and are involved in binding DNA through the major groove (Harrison, 1991). Possibly they serve that function in PCRH-REB protein. The presence of multiple PKA phosphorylation sites also suggests a mechanism by which CRH, which functions primarily through PKA activation, may activate the binding of PCRH-REB to the POMC promoter.

Sequence analysis of PCRH-REB by computer program also revealed that there was a putative ATP/GTP binding site in the middle domain of the protein

(figure 3-4). Preliminary dot blot studies suggested that this is an ATP binding site instead of a GTP binding site (data not shown). While this kind of ATP binding site is present in many proteins, such as replication factors and proteins in active transport (Linder et al. 1989; Higgins et al. 1990), few transcription factors have been shown to have this motif. However, there is one prokaryotic transcription factor, NTRC (nitrogen regulatory protein C, reviewed in Kustu et al. 1991), which has a similar motif structure like PCRH-REB. As we can see in figure 3-10, NTRC contains an N-terminal regulatory domain which has a protein phosphorylation site and a central transcriptional activation domain which contains a ATP binding site. It has been found that phosphorylation of the N-terminal domain is important for the transcriptional activity of the central domain, because the phosphorylation of the N-terminal domain is required for the ATPase activity of the central domain, and the ATPase activity allows the NTRC to make a DNA configuration change around the transcriptional start site which is important for transcription initiation. So by controlling the phosphorylation state of the N-terminal domain, the transcriptional activity of the NTRC can be regulated. Actually, studies have shown that responding to different nitrogen availability signals, the N-terminal domain can be either phosphorylated or dephosphorylated by phosphotransferase NTRB (Ninfa et al. 1986; Keener et al. 1988), so the transcriptional activity of this transcription factor was regulated. As a result, the expression of the downstream genes were also regulated, and in this way, the bacteria can adjust its cellular functions properly to adapt to different nitrogen availability environment. PCRH-REB also has an N-terminal domain which contains multiple consensus PKA phosphorylation sites and a central region which has a ATP binding site; whether they function in a mechanism similar to their counterpart in NTRC, that the phosphorylation of PCRH-REB N-terminal domain by PKA controls the transcriptional activity of this factor by

turning on the ATPase activity of the central region, remains to be elucidated.

Sequence comparison of the PCRH-REB with the Genebank revealed that protein sequence of PCRH-REB is 90% homologous with the human protein PO-GA (Lu et al. 1993) and 100% homologous with the recently identified mouse proteins MSW (Burbelo et al. 1993) or mRFC140 (Luckow et al. 1994). Thus PCRH-REB appears to be encoded by the same gene as mRFC140/MSW, which is probably the murine counterpart of the human PO-GA gene. All three plasmids were isolated by screening cDNA expression libraries with synthetic oligonucleotides, the same strategy as we used in this report. MSW cDNA was identified using a promoter element of the collagen IV gene, which has some homology with PCRH-RE, but not in the MRE region (3-11). Interestingly, the mRFC140 cDNA was isolated using a liver specific cAMP inducible enhancer element from the tyrosine aminotransferase gene, functionally similar to the PCRH-RE, but sharing only short homology to this element (figure 3-11). PO-GA was identified using a different DNA element in the POMC promoter (PO-B element, -15/-3) important in basal transcription. However, this element has no obvious sequence homology with PCRH-RE (figure 3-11). PO-GA and MSW were found to be DNA binding proteins whose binding to DNA showed preference for certain sequences, while mRFC140 was suggested to not have any sequence specificity for binding. We also found that the binding of PCRH-REB to DNA had preference for specific sequences (see figure 3-2), although some competition was seen with only mildly homologous sequences. Comparison of the sequences of the elements identified in each of these studies, however, failed to reveal a consensus DNA sequence to which this protein prefers to bind. So it appears that the protein PO-GA/MSW/mRFC140/PCRH-REB has some specificity for DNA binding, but the specificity is not as high as other transcription factors like CREB or c-Fos.

Burbelo et al. (1993) found that an antibody directed to MSW can cross-react with human A1-p145, a component of the five-protein complex called A1 (RF-C), which is required to assemble PCNA and polymerase δ on the DNA template (Lee et al. 1991; Tsurimoto et al. 1990). The more recent report of the mouse version of this clone by Luckow et al (1994) more clearly demonstrates that the encoded protein is indeed the large subunit (140-5 kD) of the replication factor C complex. Purified A1 proteins have been found to activate DNA replication in vitro, but the exact role A1-p145 plays in this kind of activity is still not clear. Sequence comparison analysis, as also reported by others (Lu et al. 1993; Burbelo et al. 1993; Luckow et al. 1994), showed PCRH-REB/mRFC140/MSW/PO-GA have regions of homology to E. coli and yeast DNA ligases and to proteins involved in DNA repair, which suggests that this group of proteins is homologous to other DNA binding proteins.

The fact that PCRH-REB, the protein we cloned as the putative factor binding to the PCRH-RE, is identical to mRFC140/MSW, a putative replication factor, was initially unexpected. But with the knowledge that there is a growing list of proteins that function in both transcription and replication, we have reason to think that PCRH-REB is probably another example. Two of the three cellular factors that are required for efficient adenovirus DNA replication, NFI (Rosenfeld et al. 1987; de Vries et al. 1987) and NFIII (O'Neill et al. 1988) later turned out to also be transcription factors (Gronstajski et al. 1988; Pruijin et al. 1987). ARS binding factor-1 can function either as a replication enhancer or in transcriptional activation and silencing (Newlon et al. 1993). Replication factor-A, a eukaryotic single-stranded DNA binding protein, has also been implicated as a transcriptional repressor (Luche et al. 1993). Recent reports that two of the components of the yeast replication origin recognition complex, ORC2 and ORC6, have also been identified as transcriptional regulators make it clearer that

the same protein can be involved in both replication and transcription (Foss et al. 1993; Bell et al. 1993; Li et al. 1993; Mickiem et al. 1993).

Bacterially expressed PCRH-REB appeared as a 140 kD protein when analyzed by SDS-PAGE (figure 3-8). Western blot with AtT20 nuclear extracts also revealed a putative PCRH-REB band at a size around 140 kD (figure 3-9). These results agreed with observations made by Burbelo et al. (1993) and by Luckow et al. (1994), suggesting that this factor is probably the same size in different cell types.

The mRNA expression of PCRH-REB was checked by RT-PCR, and it showed that PCRH-REB was widely expressed in different types of tissues. This result was not initially expected by us, because at that time we thought we were looking for a cAMP/Ca^{2+} responsive transcription factor which might be specific for POMC gene regulation. But after we saw the reports from those several groups which suggested that this factor is a putative replication factor, we felt it makes sense that, as a component of general replication factors, this factor should be expressed in different tissues.

Results from northern blot and RT-PCR failed to show the expression of PCRH-REB mRNA being stimulated by CRH treatment, and western blot with AtT20 nuclear extracts didn't show more PCRH-REB protein being present in CRH treated samples. Results from these studies suggested that the CRH stimulation of PCRH-RE gel shift is probably through the modification of PCRH-REB rather than enhancing the expression of this factor. Modification, such as phosphorylation or dephosphorylation, of PCRH-REB may be able to affect its DNA binding ability directly; or modification of PCRH-REB may affect its interaction with other factor(s), as a result, the total amount of protein complex binding to the PCRH-RE will be changed. In the western blot shown in figure 3-9, we could not detect any change of the mobility of PCRH-REB on SDS-gel after

CRH treatment, which might indicate modification of PCRH-REB. But this could be just because that the size of PCRH-REB protein is already very big, and modifications, such as phosphorylation, might cause a very small change of mobility on SDS gel, which is hard to detect.

Although the studies by Burbelo et al. (1993) and Luckow et al. (1994) have indicated that MSW/mRFC140 is the large subunit of the replication factor C complex (A1-p145), they didn't report any data showing the proteins they cloned can function in replication assays. For us, PCRH-REB was cloned as a putative transcription factor which can regulate POMC gene expression responding to CRH signal, so we wanted to see if this factor is able to function as a transcription factor. Unfortunately, preliminary results from the cotransfection experiments failed to show any transcriptional activities of PCRH-REB, suggesting that this factor can not activate gene transcription by itself. Although the possibility can not be ruled out that the cotransfection experiment didn't provide the right condition for PCRH-REB to function as a transcription factor, we tend to believe that PCRH-REB is not able to regulate POMC gene transcription responding to CRH signal by itself; probably PCRH-REB needs the interaction with other transcription factor(s) to achieve this activity.

As one component of the five subunits of replication factor C complex, A1-p145 is able to interact with the other protein subunits in the complex (Lee et al. 1991; Tsurimoto et al. 1990). It is believed that there should be a domain in its protein sequence which is mediating this protein-protein interaction. It is quite possible that, besides those proteins in the replication factor C complex, there are other proteins, such as transcription factors, which the A1-p145 can interact with through that protein interaction domain. So by interacting with different proteins, A1-p145 may function either in replication or transcription (figure 3-12).

Based on our results we hypothesize that this protein can participate in

regulating POMC gene transcription responding to CRH signal: PCRH-REB binds to the PCRH-RE and interacts with another factor(s) which has a transcriptional activation domain and is then able to activate POMC gene transcription. The interaction between PCRH-REB and these factors is not very tight, and this interaction can be enhanced by modification of PCRH-REB, such as phosphorylation of PCRH-REB by PKA or CaM kinase. So upon CRH treatment, PCRH-REB will be modified through cAMP/Ca2+ pathways, and more transcription factor(s) will be able to bind to PCRH-REB, which means that more transcriptional activities will be exerted from this element site to the general transcription machinery. As a result, POMC transcription will be stimulated. A further hypothesis can be made that the interaction of PCRH-REB and the transcription factor(s) may also be affected by the transacting factors which bind to the elements around the PCRH-REB, and this can explain why the CRH-responsiveness of the -236/-133 region can not be achieved fully by the -180/-150 element alone. As we can see in figure 2-3, while the PCRH-RE element and its binding protein appear to be a major POMC promoter component for eliciting the CRH transcriptional response in the -236/-133 region, this element alone can not regenerate all of the CRH inducing activity of this region. There are multiple other cis-acting elements surrounding the PCRH-RE that bind nuclear proteins, suggesting that the PCRH-RE and its binding protein(s) may interact with other nuclear factors to give the full CRH response. Analysis of multiple mutations in the -236/-133 region will be useful to address this issue. Thus, while the exact mechanism by which CRH activates transcription through this major responsive region (-236/-133) of the POMC promoter remains to be elucidated, at least we have identified the core CRH responsive element within it.

Material and Methods

Cell Culture: Mouse AtT-20 D16/16 tumor cells were cultured in Dulbecco's modified Eagle medium (DME, Gibco) supplemented with 10% fetal calf serum in a humidified 5% CO_2 incubator. Cells for transfection were cultured in 6-well dishes (Falcon) to 60% confluency and switched to serum-free medium (DMEM) for 24 hours before experiments. Cells for preparation of nuclear extracts were plated in 15 cm dishes.

Plasmid DNAs: The bacterial expression vector pSEM was used according to Knapp et al. (1990). An insert of 2.6 kb corresponding to the almost complete coding sequence of PCRH-REB was cut at Xbal and Kpn1 restriction sites and inserted into pSEM-3 to generate an in frame ß galactosidase-PCRH-REB fusion gene product. Full length PCRH-REB was cloned into the bacterial expression vector Pet-28 (Novagen) at restriction sites of Sal I and Not I. Eukaryotic expression construct was made by ligating full length PCRH-REB in frame into RSV-424 (Sadowsk et al. 1989) at sites of Xba I and EcoR I.

Expression screening for PCRH-REB: A I-ZAP II (Stratagene) expression library was constructed by using poly (A) RNA isolated from CRH treated (30 min, 10 nM) AtT-20 cells. The library was screened without amplification by the method of Vinson et al (49) with a slight modification. Briefly, bacteriophage (50,000 PFU/15-cm dish) were grown on lawn of E. coli (strain: Sure) for 3 to 4 hours at 42° C, overlaid with nitrocellulose filters (soaked in isopropyl β-D-thiogalactopyranoside), and incubated for an additional 6 hours at 37° C. After removal from culture plates, the nitrocellulose filters were allowed to air-dry for 30 min at room temperature and then subjected to denaturation-renaturation cycles

at 4° C as follows. Filters were first placed in a binding buffer (20 mM HEPES, pH 7.9, 40 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol) supplemented with 6 M guanidine hydrochloride and were gently shaken for 5 min. This step was repeated once, and the second wash was then diluted with an equal volume of binding buffer and shaken for 5 min. The filters were consecutively exposed to four additional two-fold dilutions of guanidine hydrochloride for 5 min per each wash. A final washing step was carried out with unsupplemented binding buffer. The filters were then transferred to a blocking solution containing 5% Carnation instant nonfat dry milk in binding buffer. After gentle shaking for 30 min, the filters were washed once more with binding buffer. Hybridization was carried out in binding buffer with ^{32}P-labeled DNA probe (10^6 cpm/ml) for about 4 hours at 4° C. Filters were then washed three times with binding buffer for 20 min per wash and finally dried and exposed to Kodak X-omat film overnight at -70° C with intensifying screen. The DNA probe used for hybridization was the double-stranded oligonucleotide spanning the PCRH-RE as described above. The oligonucleotide was phosphorylated with ($\gamma^{32}P$)-ATP by using polynucleotide kinase and concatenated with T4 ligase. The specific activity of the concatenated probe was around 5 x 10^8 cpm/μg. After PCRH-REB was isolated by expression screening, its DNA was used to rescreen the library and five more PCRH-REB encoding cDNA clones were isolated. All cDNA inserts were sequenced by the dideoxynucleotide chain terminator sequencing method.

Purification of bacterial expressed protein: Bacterial expressed fusion protein β-Gal-PCRH-REB-1 was isolated by a modification of the procedure of England et al. (1990) and Knapp et al. (1990). PCRH-REB1-pSEM expression vector was transformed into its host bacteria, w301. Bacteria were grown in LB medium at 37° C until OD_{600} reached 0.4, then IPTG (Sigma) was added to a final

concentration of 1 mM, and the bacteria were allowed to grow for another 6 hours before harvest. Bacterial pellets were resuspended in TEN buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl), lysozyme (Sigma) was added at a concentration of 1 mg/ml and incubated at room temperature for 30 min with gentle agitation. Then bacteria were pelleted again and resuspended in TEN buffer supplemented with 0.1% deoxycholate, 2 mM $MgCl_2$ and DNase (Worthington) 1 mg/ml, gently shaken at room temperature for 30 min. After spinning at 12,000 g for 10 min, the pellet was suspended in TEN supplemented with 0.5% Triton X-100, and sonicated on ice. The bacterial extracts were pelleted by centrifugation at 12,000 g for 10 min, and finally suspended in PBS supplemented with 2% SDS and 5% β-mercaptoethanol. Bacterial proteins was fractionated on 8% SDS-PAGE gels. The part of gel which contains the β-galactosidase-PCRH-REB fusion protein was cut out and crushed with a Teflon pestle in an Eppendorf tube containing 0.25 ml elution buffer (0.1% SDS, 50 mM Tris-HCl pH 7.6, 0.1 mM EDTA, 5 mM DTT, 0.1 mg/ml BSA, 0.15 M NaCl) and incubated for one hour at room temperature. After the supernant was recovered, the gel was washed with 0.1 ml elution buffer. Protein from the 0.3 ml pooled supernant was precipitated with 1.2 ml acetone. To renature the protein, the pellet was resuspended in 10 µl 6 M guanidine-HCl made in dilution buffer (25 mM HEPES pH 7.9, 0.1 M NaCl, 0.5 mM DTT, 10% glycerol, 0.1 mg/ml BSA) for 30 minutes at room temperature. 0.5 ml dilution buffer was added and incubated for another 50 min at room temperature. The renatured protein was concentrated in a centricon-30 filter from 0.5 ml to 50 µl. Finally, 50 ul renatured protein was brought to 100 ul by adding 50 µl dilution buffer, 4 µl was used in each reaction of gel shift assay.

The bacterial expressed full length PCRH-REB protein from Pet-28 vector was purified by His-Tag column (Novagen). Briefly, bacterial expressed PCRH-

REB was induced by 0.4 mM IPTG at OD_{600} of 0.4 followed by continued for incubation for 2-3 hr before harvest. Bacterial pellets were resuspended in cold binding buffer (5 mM imidazole, 500 mM Nacl, 20 mM Tris-HCl, pH 7.9) and were subjected to sonication. After centrifugation, the pellet containing the PCRH-REB inclusion bodies were resuspended in binding buffer plus 6 M guanidine HCl, incubated on ice for 1hr to dissolve the protein, and the supernatants after centrifugation were used to load the His-Tag columns. The columns were prepared by loading His-Tag resin, washed with 3 volumes of dH_2O, 5 volumns of charge buffer (50 mM $NiSO_4$) and 3 volumes of binding buffer. After the protein extracts were loaded, the columns were washed with 25 ml of binding buffer and 15 ml of 20mM imidazole buffer, and then the proteins were eluted with elution buffer (1 M imidazole, 400 mM Nacl, 20 mM Tris-HCl pH 7.9). All these buffers contained 6 M guanidine HCl.

Gel shift assays: All oligonucleotides were synthesized by the MSSM DNA synthesis facility. Rat PCRH-RE (5'-TCGACCTGCTGTGCGCGCAGCCCG-3'), mouse PCRH-RE (5'-TCGACGTGCCTTGCGCTCAGCCAG-3'), MRE (5'-CTCTGCACTCCGCCCGA-3') Five p moles of double stranded oligonucleotide were end-labeled with (γ-^{32}P) ATP (3000 Ci/m mole, NEN) using polynucleotide kinase, resolved on a 10% acrylamide gel and eluted from the gel at 4° C in TE buffer (10 mM TRIS-HCl, pH 8, 1 mM EDTA) or dH_2O (when used for testing effects of divalent cations on gel shift). Approximately 0.2 ng of labeled DNA (15,000 to 20,000 cpm) were added to the pre-incubated bacterially expressed PCRH-REB.

For the expressed PCRH-REB1, 4 µl (containing about 200 ng PCRH-REB1 protein) of purified protein solution were pre -ncubated for 10 min on ice in 15 µl of binding buffer (22 mM HEPES, pH 7.9, 6 mM KCl, 10 mM DTT, 5 mM

spermidine, 8% glycerol and 2% Ficoll). 0.2 ng of labeled probe was added and the binding reaction was incubated for an additional 15 min on ice. The protein-DNA complexes were resolved on a 4% polyacrylamide gel in 0.5X TBE. The gels were dried and autoradiographed with intensifying screens at -70° C with Kodak X-OMAT AR films.

Transfection and Chloramphenicol Acetyl-transferase Measurement: Transfection experiments were performed using a lipopolyamine-based method (TRANSFECTAM ™, Promega). Briefly, cells were transfected with 2 ug (1.5 ug of each construct DNA in cotransfection experiments) of reporter gene DNA for 6 hours. Cells were then grown for 12 hours in fresh serum-free medium for 6 to 8 hours with or without 10 nM CRH. CAT activity was determined by the method of Gorman (1982). After autoradiography, the different forms of chloramphenicol were located, cut out of the silicate gel and counted.

Primer extension assays: Primer extension analysis was performed as described previously (Ausubel et al. 1989). About 20 ng of labeled primer from the 5' end of PCRH-REB-2 (nucleotide 202-219, 5'-GTTTGGAGGCGTCCTCT-3') was hybridized with 40 ug of total cytoplasmic RNA isolated from AtT20 cells and allowed to incubate at 37° C for 24 hours. Reverse transcription was performed in a mixture of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 0.5 mM dGTP, dATP, dTTP, and dCTP, 40 U of RNAsin (Promega), and 200 U of Moloney murine leukemia virus reverse transcriptase (BRL); incubation was at 37° C for 60 min. The samples were analyzed on a DNA sequencing gel.

Reverse transcription of mRNA and PCR amplification: 10 μg of total RNA was reverse transcribed by MMLV reverse transcriptase (Gibco, BRL) with oligo

dT as primer, and one-tenth of the first-strand cDNA was used subsequently for PCR. For detecting the expression of PCRH-REB, two oligonucleotides in its 3' terminal region were used as PCR primer. The PCR conditions used were 94° C 1min, 45° C 1min, 72° C 1min, 30 cycles, and the PCR products were analyzed by 1% agarose gel.

Northern Blot: Total RNA was extracted by the LiCl method, according to Auffray and Rougeon (1980). RNA was quantitated by UV absorption, and 5 μg polyA$^+$ RNA was used for Northern analysis, as previously described (Loeffler et al. 1985).

Preparation of polyclonal antibody: Protein in denatured form was obtained following preparative SDS slab gel electrophoresis and excision of the single band corresponding to the protein. Initial injections of 200 ug of protein were used for immunization, with three subsequent injections of 100 ug of the same protein repeated at 2-week intervals. The protein was mixed with Freund's complete adjuvant and injected intradermally into New Zealand White rabbits. Blood was collected in heparinized tubes from the terminal ear vein 1 week after the injections. Antiserum was stored in 200-ul aliquots at -20° C·

Western Blot: Proteins were electrophoresed on 8% SDS-PAGE mini gel at 100 volts for 1 hr at room temperature, and were transblotted onto nitrocellulose membrane overnight at 40 V in cold transfer buffer containing 25 mM Tris glycine (pH 8.3) and 20% methanol. After transfer, the membrane was blocked with Blotto containing 5% Carnation instant nonfat dry milk in a solution of 150 mM Nacl, 50 mM Tris pH 8.0 for 2 hr, and then the membrane was incubated with primary antibody with 3% Blotto overnight at 4° C. The membrane was then

washed in washing buffer (150 mM Nacl, 50 mM Tris, pH 7.5, 0.05% Tween-20) and incubated with secondary antibody (^{125}I-Goat anti-Rabbit IgG) in 3% Blotto for 2 hr. Finally, the membrane was washed in washing buffer, air dried and autoradiographed withKodak X-OMAT films.

Figure 3-1

Bacterial-expressed Fusion Protein of PCRH-REB-1 and Its DNA Binding Ability

A. Bacterial-expressed fusion protein of PCRH-REB

Bacterial extracts were analyzed by 8% SDS-PAGE gel. Lane I: Prestained molecular weight markers. Lane 2: Bacterial extract from untreated cells. Lane 3: Bacterial extract from IPTG-Induced cells. The full-length of PCRH-REB-1 fusion protein (150 kD) or the non-specific 40 kD protein (ct) which were excised for subsequent analysis are indicated.

B. DNA binding ability of PCRH-REB-1 fusion protein

Gel shift assays with proteins cut and eluted from the SDS-PAGE gel bands shown in panel A. One band is coresponding to the place of the full lengh of fusion protein (PCRH-REB-1). The other is the low molecular weight band which presented eaqually in control and IPTG-induced lane (CT), representing the indulgent bacterial protein (its position on the SDS-PAGE gel is indicated, which is around 43 kD). Lane 1: fusion protein PCRH-REB-1,. Lane 2: Control protein was used.

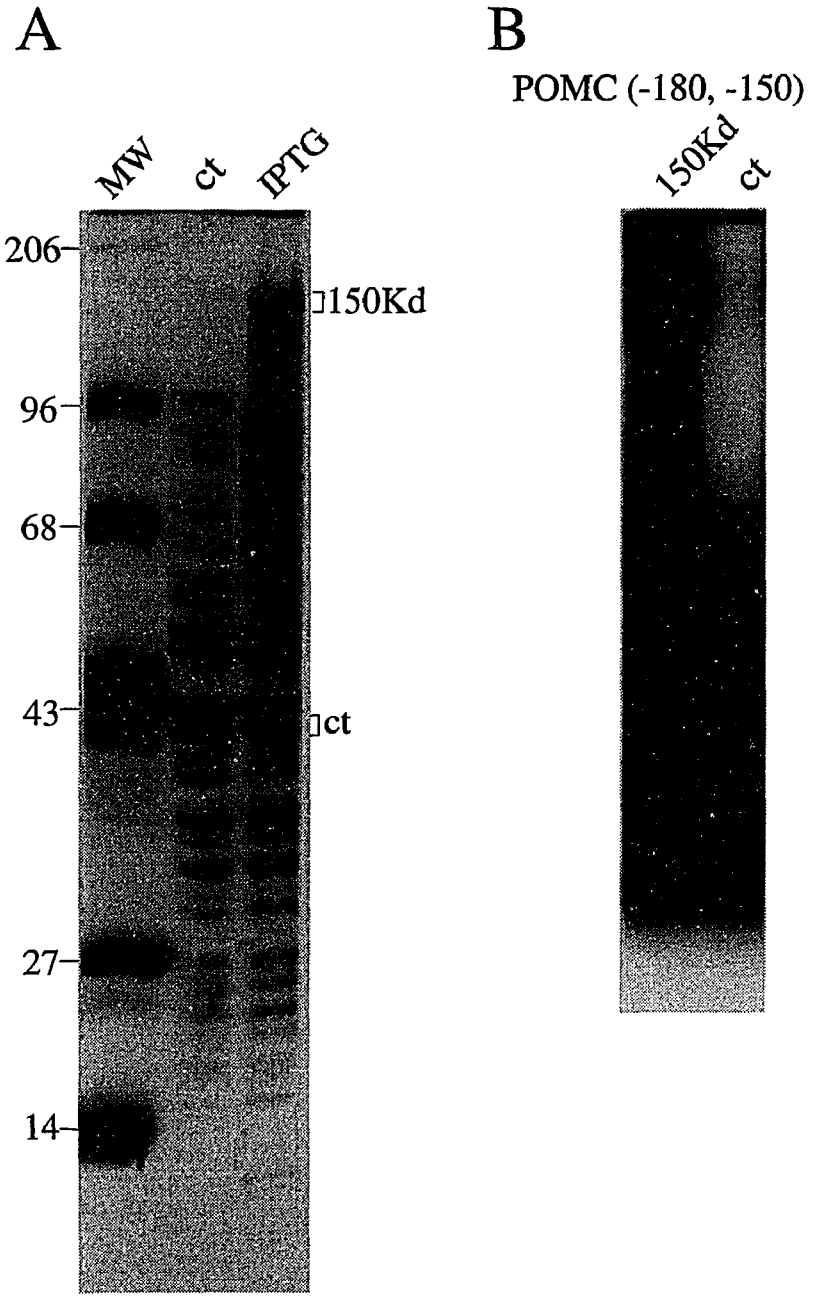
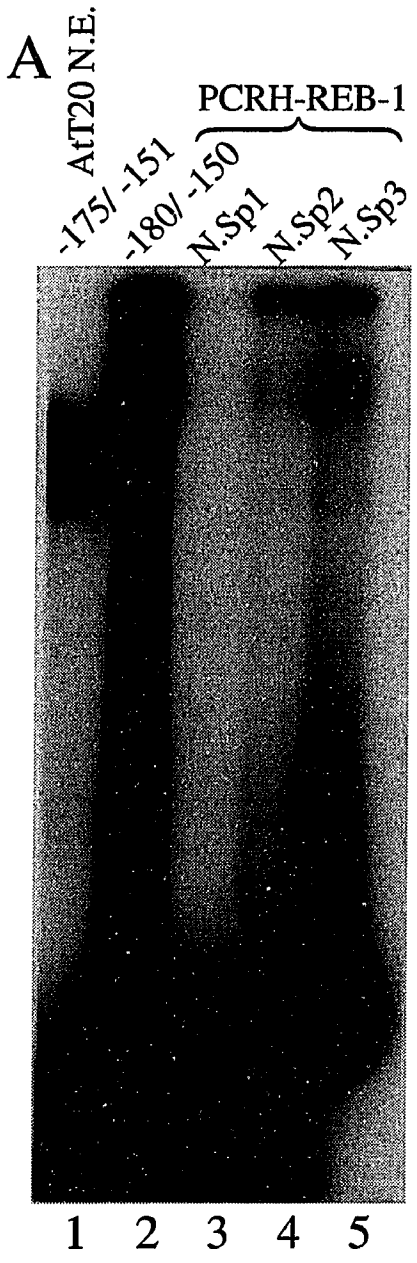

A
MW
ct
IPTG
206
96
68
43
27
14
150Kd
ct
B
POMC (-180, -150)
150Kd
ct

Figure 3-2

Characterization of Bacterial Expressed PCRH-REB-1 Fusion Protein

Left panel (A): The DNA binding specificity of PCRH-REB-1 fusion protein. The gel purified PCRH-REB-1 fusion protein was used in gel shift assays with several different oligonucleotides as probes. Lane 1: 15 μg AtT20 nuclear extract with mouse PCRH-RE as probe. Lane 2: oligonucleotide (-180/-150) which contains the PCRH-RE element used as probe. lane 3-5: three different oligonucleotides unrelated to PCRH-RE were used as probe. The sequences of these oligonucleotides are: NSp1: 5'-AGCTTCGGCAGGACAATTATTCGA-3'; NSp2: 5'-AGCTTAGCCTTATCCTGATGTA-3'; NSp3: 5'-GTCGACGCAGCACCGTCTCAAGGTCGCCGAGTAGGAGAA-3'.

Right panel (B): Divalent cation effects on mouse PCRH-RE gel-shift with bacterial expressed PCRH-REB-1 fusion protein. 100 μM of different divalent cations were used in the gel shift reaction with PCRH-REB-1 fusion protein with the mouse PCRH-RE as probe. Lane 1: control. Lane 2: $CaCl_2$. Lane 3: $MgCl_2$. Lane 4: $CuCl_2$. Lane 5: $CdCl_2$. Lane 6: $ZnCl_2$.


A
AtT20 N.E.
-175/ -151
-180/ -150
PCRH-REB-1
N.Sp1
N.Sp2
N.Sp3
1 2 3 4 5

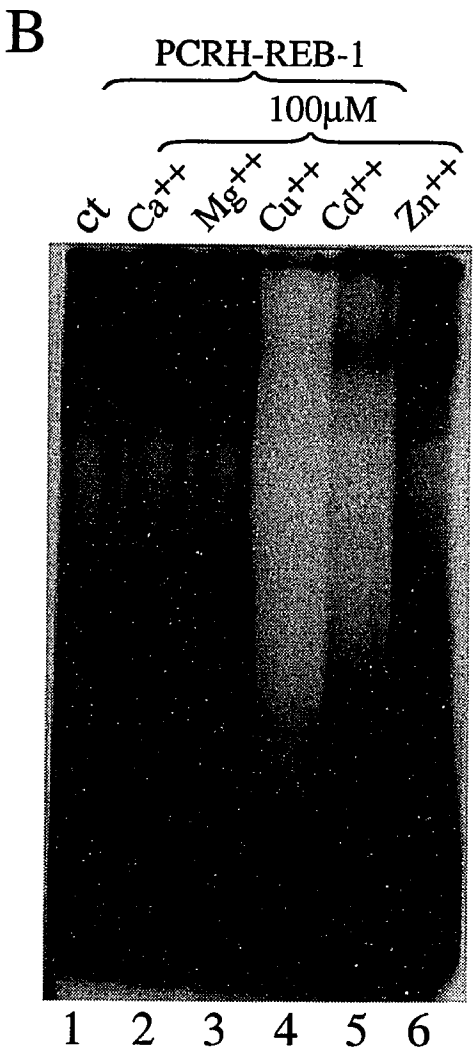

B
PCRH-REB-1
100µM
ct
Ca++
Mg++
Cu++
Cd++
Zn++
1 2 3 4 5 6

Figure 3-3

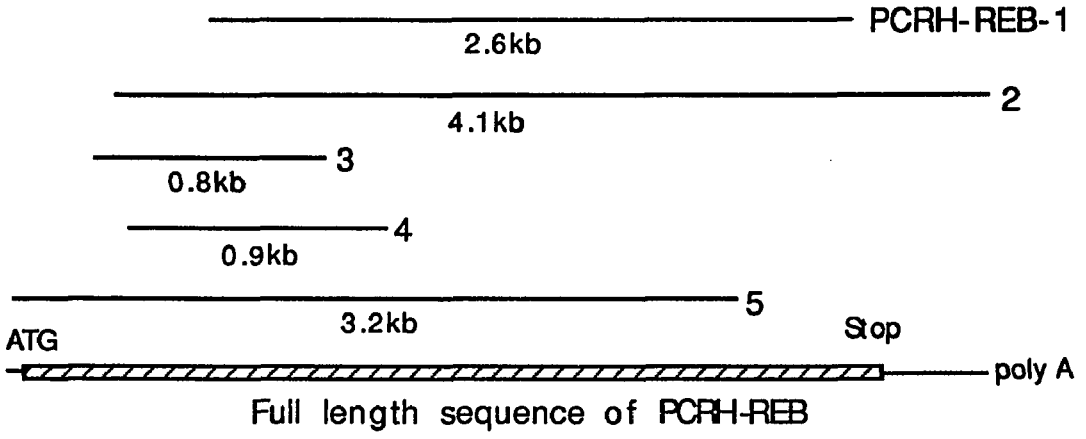
PCRH-REB Protein-Cloning Strategy and Sequence Analysis

A) Sequencing strategy. The predicted 4.6 kb PCRH-REB cDNA derived from the different cDNA clones is diagrammed and the putative translation start and stop sites are indicated.

B) Sequence analysis of PCRH-REB. The protein sequence of PCRH-REB was searched by computer program (GCG) for consensus motifs. A represents a consensus PKA phosphorylation site which is (R,K)-x-x-(S,T). The positions of these sites are amino acid 88, 185, 241, 337, 360, 371, 513, 519 and 1,104. P-loop refers to an ATP/GTP binding site motif A, which is (A,G) X_4 GK (S,T). The position of this site starts at amino acid 635. Acidic and basic regions are indicated by different boxes. The region which has homology to DNA ligases and poly (ADP-ribose) polymerase was underlined.

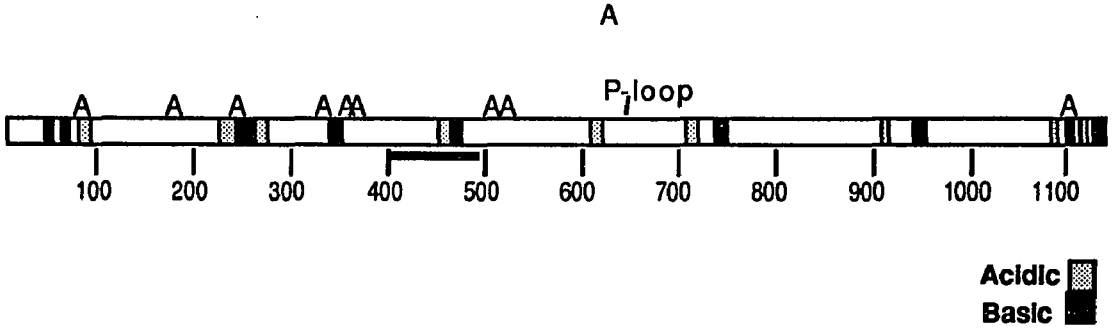
PCRH-REB Protein-
-Sequencing Strategy and Sequence Analysis

A. Sequencing strategy of PCRH-REB cDNA



Full length sequence of PCRH-REB

B. Sequence analysis of PCRH-REB



A-PKA phosphorylation site

P-loop ATP/GTP binding site

Figure 3-4

Size of PCRH-REB mRNA

A) Primer extension. 40ug of total RNA from AtT20 cells (A) as well as from mouse liver tissue (L) were used for primer extension analysis using reverse transcriptase. Four putative transcription initiation sites are marked on the left, the major one is 450 bp from the primer. The sizes were determined by a known DNA sequence ladder (M).

B) Northern blot. 30 μg of total RNA was electrophoresised and hybridized with 32p-labeled probe. Lane 1: Total RNA from control AtT20 cells (CT). Lane 2: RNA from 10nM CRH treated AtT20 cells (CRF). The size was determined according to known molecular weight markers (indicated on the left).

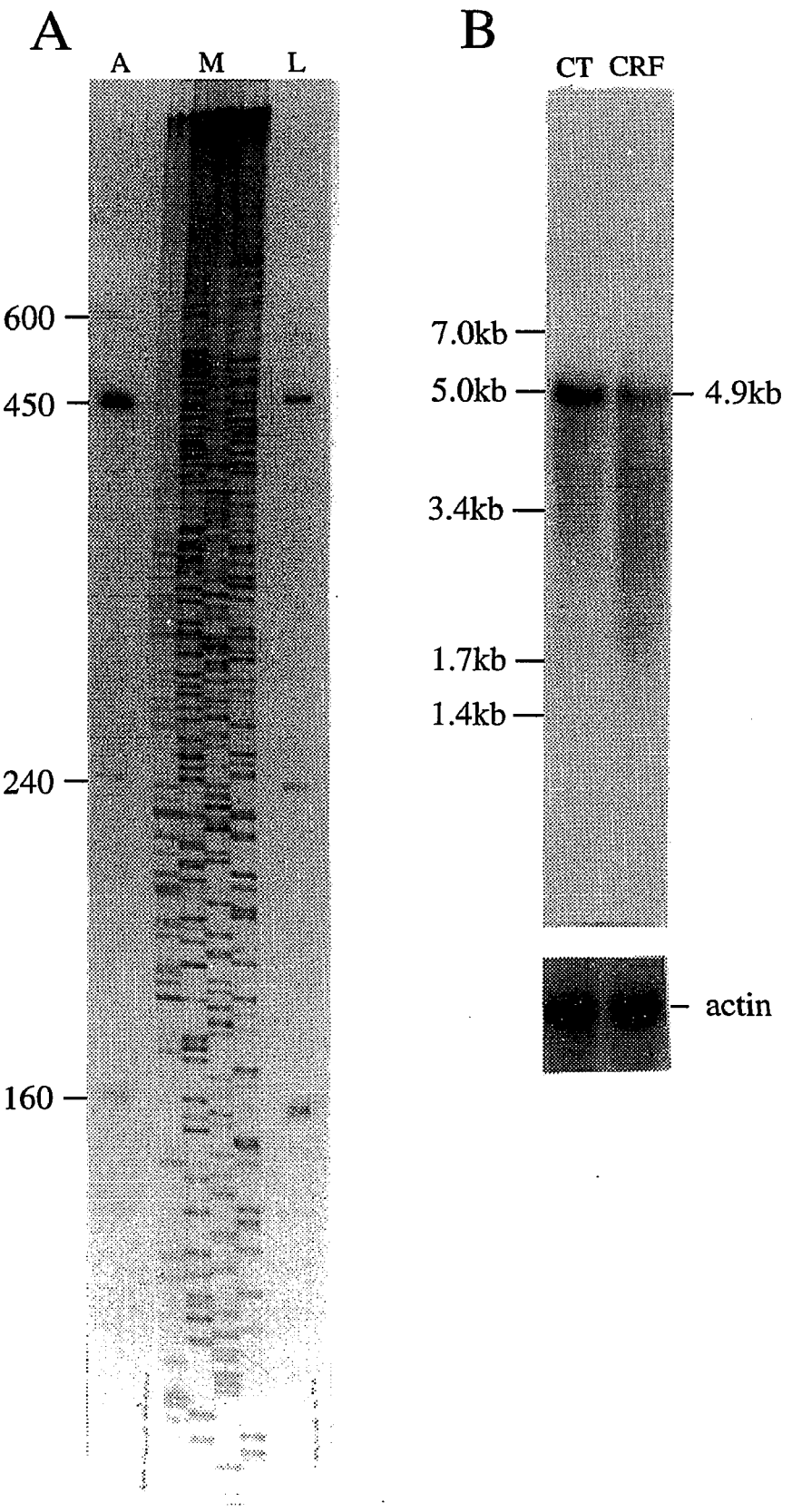
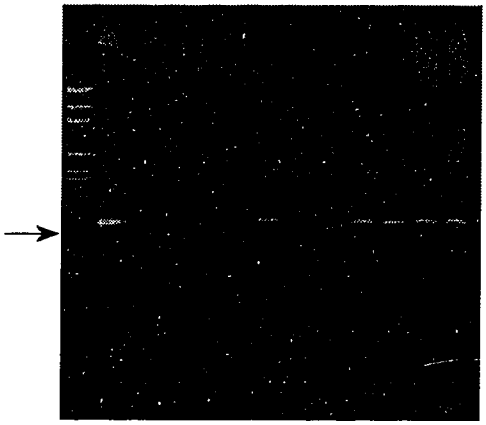

A
A
M
L
600
450
240
160
B
CT
CRF
7.0kb
5.0kb
3.4kb
1.7kb
1.4kb
4.9kb
actin

Figure 3-5

mRNA Expression of PCRH-REB in Different Tissues

10 μg of total RNA from different mouse tissues and cell lines were reverse transcribed and the resulting first strand cDNA was PCR amplified for PCRH-REB with two oligos in the 3' region of PCRH-REB. The sequences of these two oligos are: 5'-AAAGACAGAGCTTGGCC-3' and 5'-CTGTGATGGAGTCTATA-3'. The PCR products were analyzed by 1.2% agarose gel electrophoresis. Lane 1, the molecular weight markers. Lane 2, positive control, 10 ng of PCRH-REB DNA was used for PCR. Lane 3, negative control, no template was added. Lanes 4-13, RNA from tissues or cell lines as marked.

C



1 2 3 4 5 6 7 8 9 10 11 12 13

1. marker
2. +
3. -
4. brain
5. liver
6. testis
7. kidney
8. heart
9. spleen
10. GT_1
11. αH3
12. control } AtT20
13. CRF

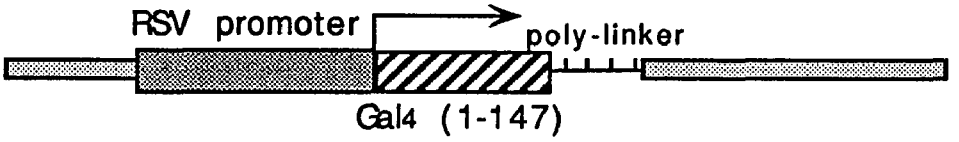
Figure 3-6

Diagram of the Mechanism of Co-transfection Experiment

A. Diagram of the structures of the vectors. The structures of the vectors used in co-transfection experiment are shown. The regions for promoter, domain, element, or reporter gene are indicated by different boxes.

B. Diagram of the presumed effects of co-transfection. The possible effects of Gal4-PCRH-REB fusion protein on the transcription efficiency of the Gal4-CAT reporter vector are diagrammed. Transcription factors are shown in different ellipses.

A.Diagram of the structures of the vectors



RSV expression vector

Gal4-CAT reporter vector

B.Diagram of the presumed effect of co-transfection

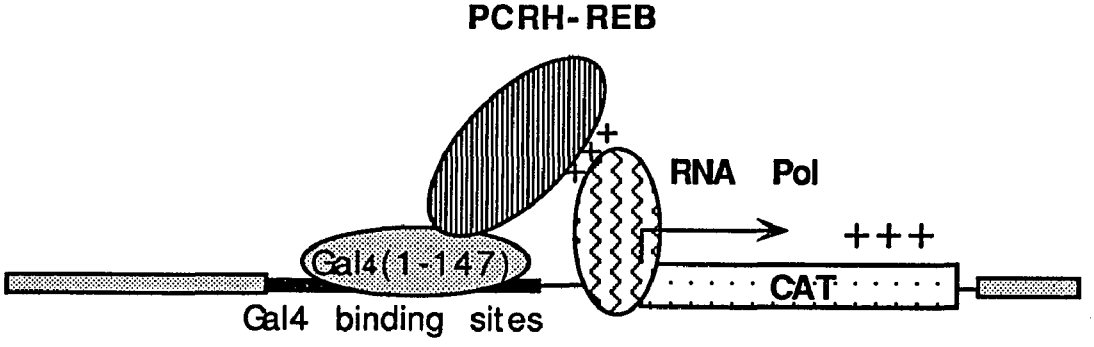
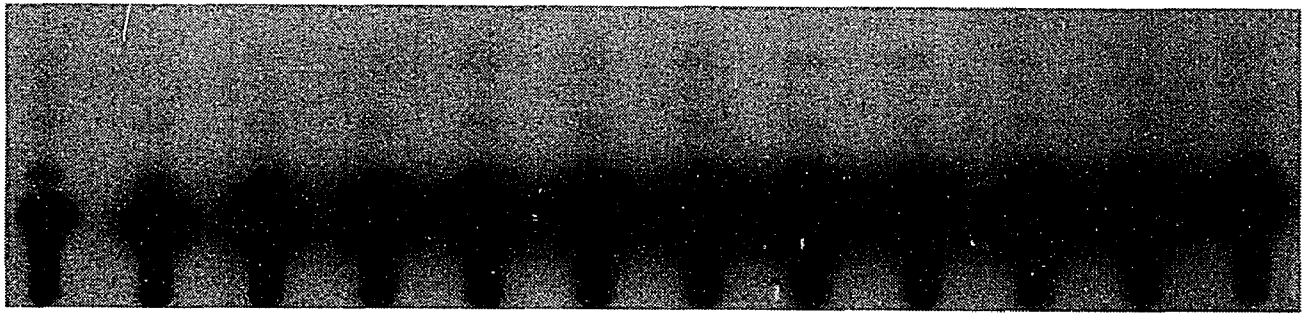


Figure 3-7

Results of Co-transfection Experiment

The CAT assay results of the cotransfection experiments are shown. In each cotransfection, DNA of Gal4-CAT reporter vector was used in combination with each of the four constructs shown in the figure. For each condition, cotransfection was carried out in triplicate. AtT20 cells transfected with these constructs were then either untreated or treated with 10 nM CRH for 9 hrs and harvested for CAT assay by the TLC/^{14}C-chloramphenicol method.


Ct
CRF
Ct
CRF
Bluescript
RSV-Gal4(1-147)

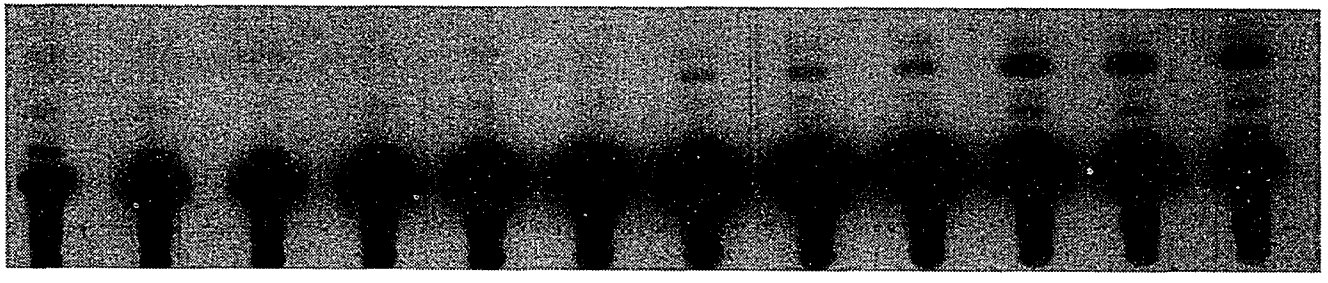

Ct
CRF
Ct
CRF
RSV-Gal4-PCRH-REB
RSV-Gal4-CREB

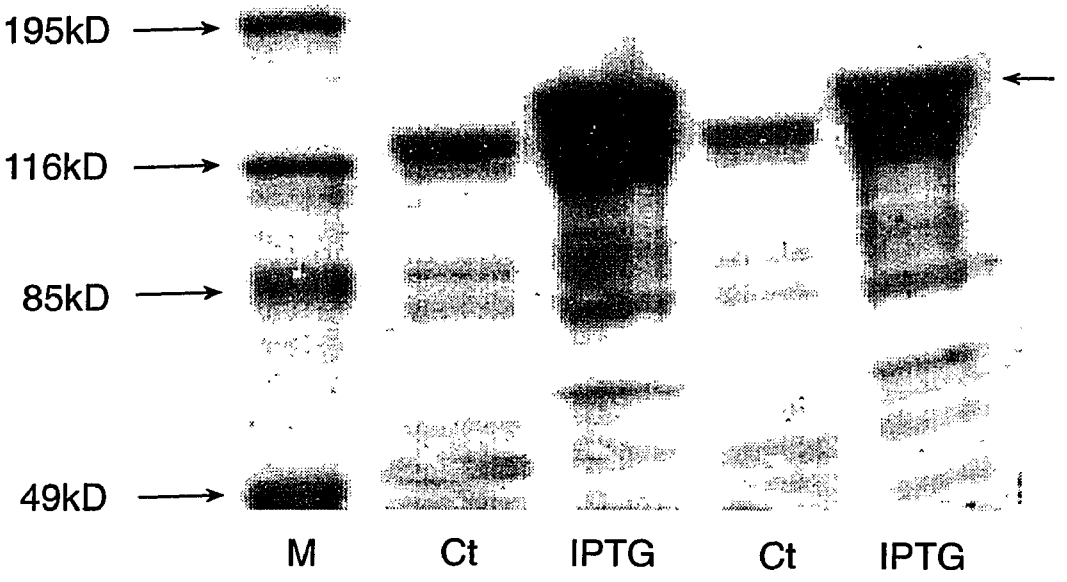
Figure 3-8

Bacterial Expressed and Purified Full-length PCRH-REB Protein

Upper panel: Bacterial extracts were analyzed on 8% SDS-PAGE gel. The band around 140 kD induced by IPTG, indicating the full length PCRH-REB, is marked by arrow.

Lower panel: Crude bacterial extracts as well as His-Tag column purified PCRH-REB protein were analyzed on 8% SDS-PAGE gel. The band indicating the full length PCRH-REB is shown by arrow.

A.


195kD
116kD
85kD
49kD
M
Ct
IPTG
Ct
IPTG

B.

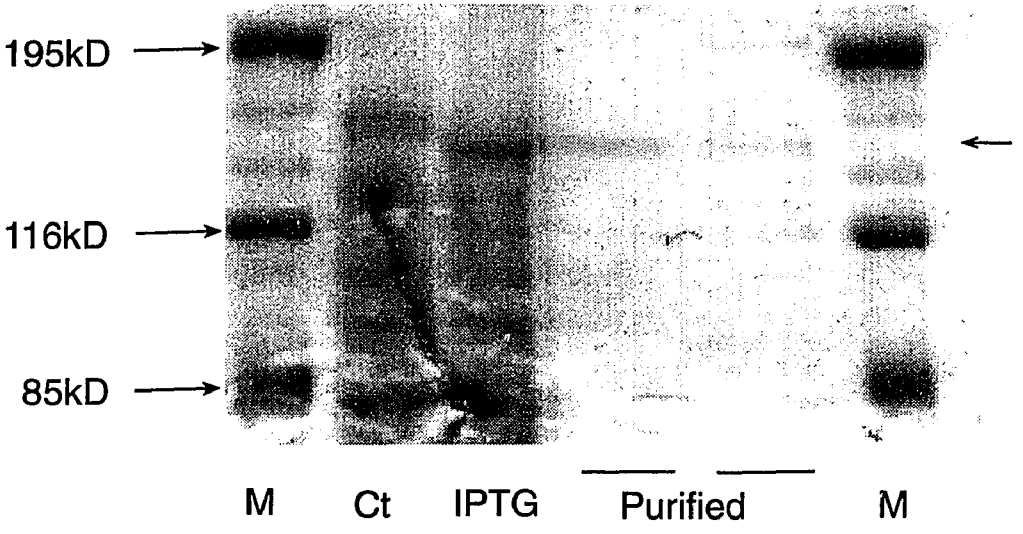
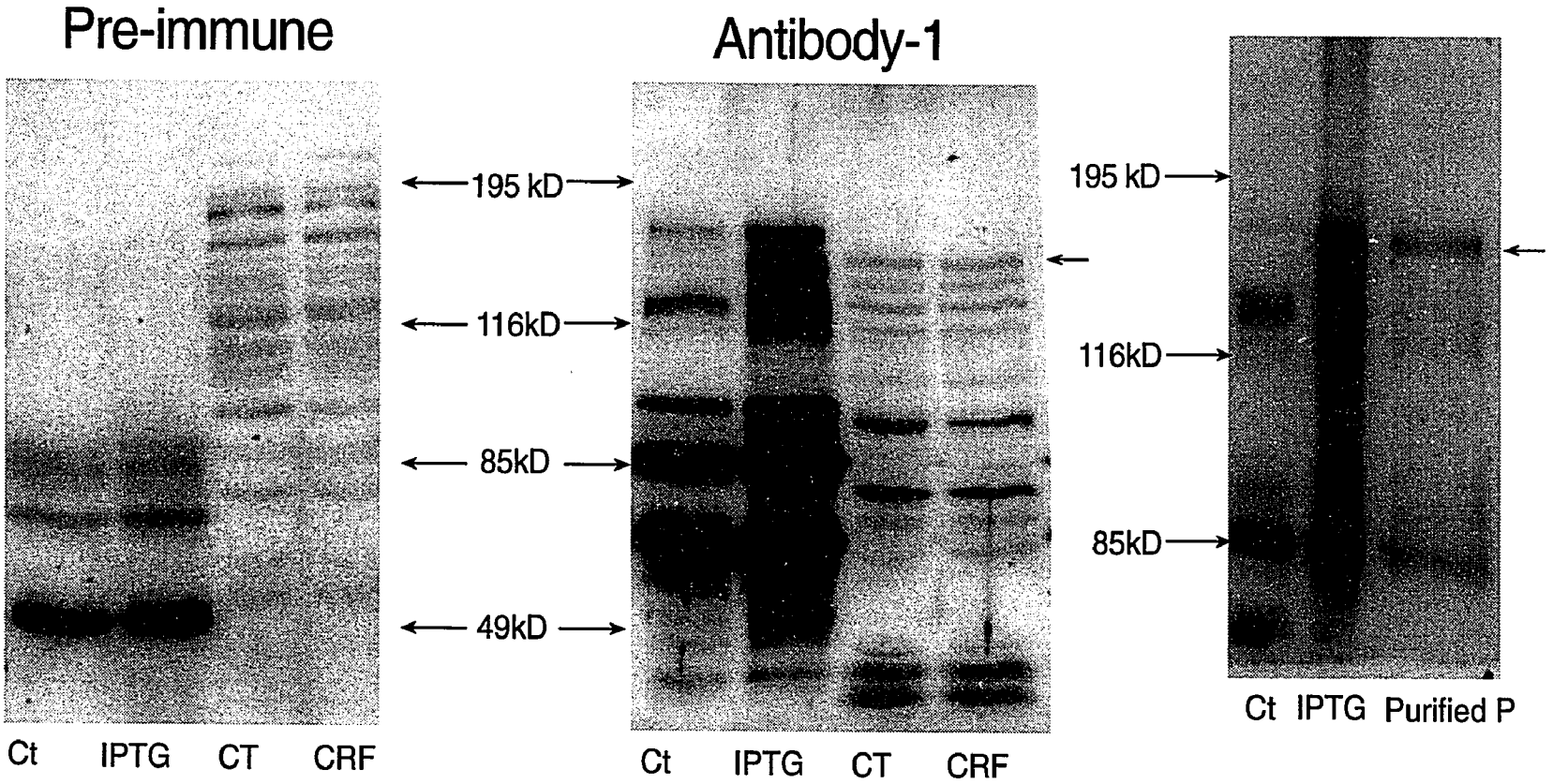

195kD
116kD
85kD
M
Ct
IPTG
Purified
M

Figure 3-9

Western Blot of PCRH-REB

Bacterial extracts and AtT20 nuclear extracts were electrophoresised on SDS-PAGE gel and transfered onto nitrocellulose membrane for western blot using either pre-immune or anti-PCRH-REB serum. The molecular weight marker is indicated and the band representing the PCRH-REB is pointed out by arrow.


Pre-immune
Antibody-1
195 kD
116kD
85kD
49kD
Ct
IPTG
CT
CRF
Ct
IPTG
CT
CRF
195 kD
116kD
85kD
Ct
IPTG
Purified P

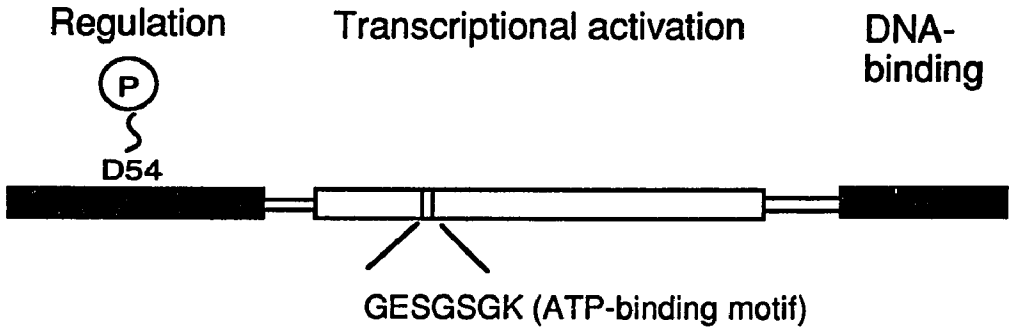


Figure 3-10. Diagram of the structure of NTRC. The three domains of NTRC are shown. The phosphorylation site (D54) and the ATP-binding motif are indicated (This figure is copied from Kustu et al. 1991).

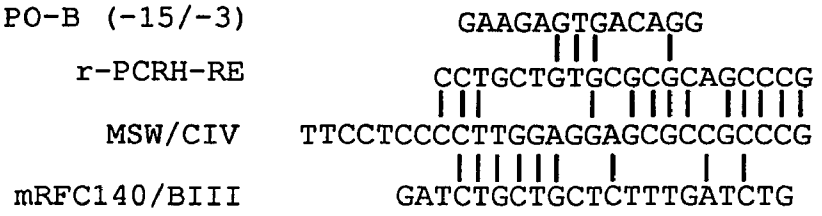
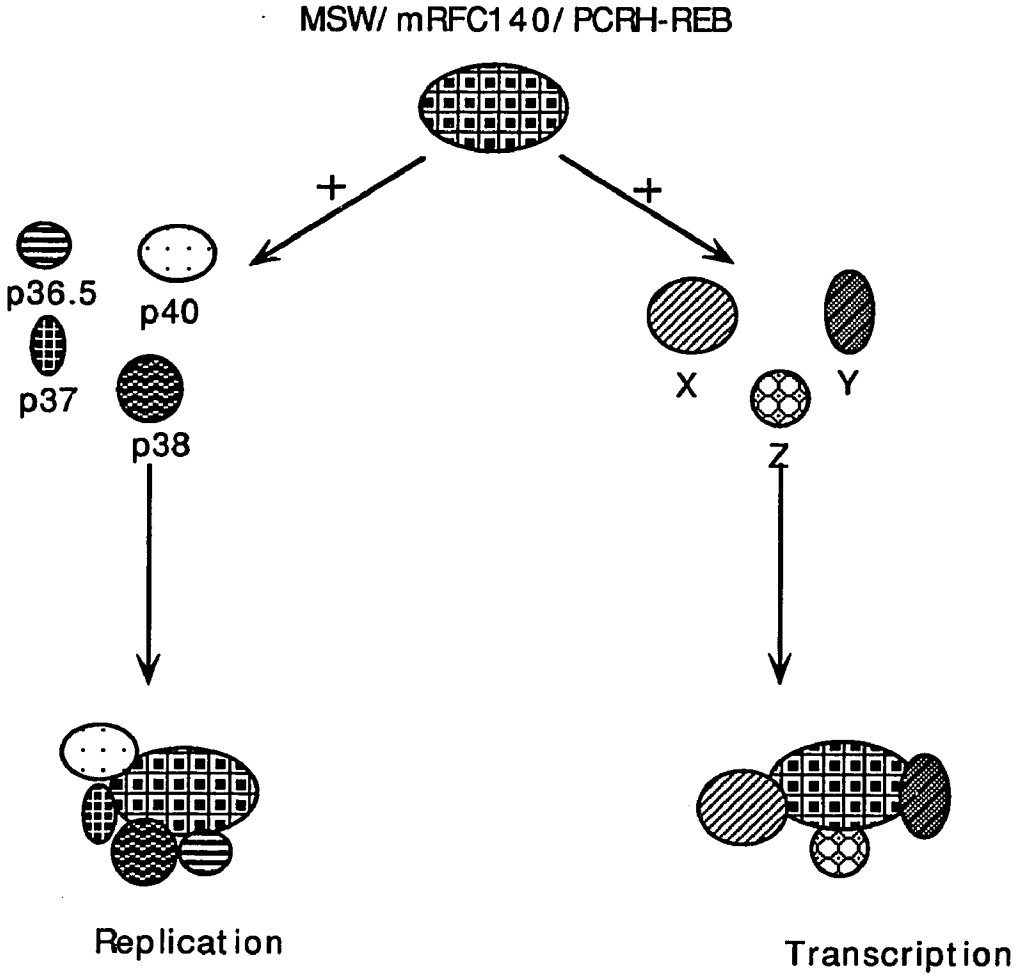


Figure 3-11: Sequence homology analysis between different elements. The sequences of element PO-B, r-PCRH-RE, MSW/CIV and mRFC140/BIII are listed and compared (mRFC140/BIII is compared to r-PCRH-RE).

Figure 3-12

Diagram of the Putative Mechanism of PCRH-REB to be Involved in Both Transcription and Replication

A model for PCRH-REB to be involved in both transcription and replication is diagrammed. Different factors involved either in transcription or replication are shown as different ellipses.


MSW/ mRFC140/ PCRH-REB
+
+
p36.5
p40
p37
p38
X
Y
Z
Replication
Transcription

Chapter IV.
Studies on other cis-elements which may interact with PCRH-RE.

Introduction

CRH is able to stimulate POMC gene transcription through cAMP/Ca2+ pathways (Reviewed by Roberts et al. 1993). Previous studies from our lab have mapped a major CRH responsive region in the flanking sequence -236/-133 of the POMC promoter (Lundblad et al. 1988). DNAse-footprint analysis revealed that multiple elements in this region were bound by nuclear proteins from the POMC expressing AtT20 cell (see figure 2-1), and when these individual DNA elements were separately tested in heterologous reporter constructs for CRH induction, one element (-180/-150, which contains the PCRH-RE, see chapter II) was found to be most inducible. However, the induction of this smaller element is still less than (about half) observed with the -236/-133 region (figure 2-3), suggesting that in order to achieve the full CRH response, the PCRH-RE and its binding protein may need to interact with other nuclear factors.

The putative PCRH-RE binding factor (PCRH-REB) has been cloned (see chapter III). It is a 140 kD protein, and its sequence is identical to the large subunit (A1-p145) of replication factor C complex. Functional studies of PCRH-REB suggested that this factor might need other transcription factors to work together to give the CRH responsiveness (see discussion of chapter III).

Sequence analysis of the -236/-133 region showed multiple DNA elements that are homologous to several consensus transcription factor binding sites, such as NFκB site (-152/-140) and AP2 site (-144/-133, see figure 2-2). The NFκB element was first identified as an enhancer sequence in the immunoglobin κB gene (Sen and Baltimore, 1986). It is bound by a NFκB protein complex which contains a 50 kD peptide and 65 kD peptide, both of which are widely expressed

in different tissues. NFκB activity is inducible by cellular-activating stimuli such as antigens for lymphocytes, cytokine stimulation, or viruses infection (Baeuele et al. 1991; Griffin et al. 1989; Osborn et al. 1989). After stimulation, NFκB proteins can translocate from cytoplasm into nucleus and bind to its specific site in the promoter to activate gene transcription (Ghosh et al. 1990). But so far, no report has shown NFκB to be able to respond to a cAMP signal directly.

Activating protein 2 (AP-2) binding sequence has been found to be responsible for induction by either phorbol esters which activate protein kinase C, or by forskolin which raises the concentration of cAMP (Imagawa et al. 1987). The AP-2 element binds a 52 kD protein which has a proline and glutamine clustered transcriptional activation domain (Williams et al. 1988). This AP-2 protein was found to be able to interact with other proteins such as T-antigen and was suggested to also be able to mediate the control of developmentally regulated gene transcription (Mitchell et al. 1991). But so far, the mechanism by which AP-2 protein mediates this transcriptional regulation is still not clear.

In the POMC promoter, we don't know whether the NFκB and AP-2 consensus elements in the -236/-133 region are actually bound by NFκB protein and AP-2 protein, respectively; and whether it is one of these two elements or even other elements in the -236/-133 region that is interacting with the PCRH-RE to achieve the full CRH responsiveness of this big region. To address these issues, preliminary studies were performed with the POMC NF-kB and AP-2 elements.

Results

Characterization of nuclear proteins bind to POMC kB element:

Sequence analysis of POMC promoter region -236/-133 by computer program has shown that there is a consensus NF-κB element present at -152/-144 (figure 2-2). In order to find out whether it is actually the NF-κB protein that binds to the POMC NF-κB, gel shift experiments were carried out to study the nuclear proteins binding to this site. Nuclear extracts from control and CRH treated (10 nM, 60 min) AtT20 cells were prepared as described (Dignam et al. 1983) and shown to bind efficiently to the POMC NF-κB element (figure 4-1, left panel). The binding was not enhanced by CRH treatment. The interaction between proteins and POMC NFκB appeared specific, since the binding was competed efficiently by 100-fold excess cold oligonucleotides of probe itself, as well as by the immunoglobin κB element which represents the consensus sequence that NFκB proteins bind, but not competed at all by unrelated oligonucleotides like TRE. A similar gel shift complex was also formed between AtT20 nuclear extracts and immunoglobin kB element (figure 4-1, right), and POMC κB element and immunoglobin κB element was shown to be able to compete with each other in the cross-competition experiments, implying that the proteins which bind to POMC promoter region -152 to -144 may be NFκB proteins.

In order to confirm this, super-shift experiments were performed with antibodies against the mouse NFκB protein p50 and p65, respectively. The p50 antibody is able to form a supershifted band by interacting with the p50 component in the NFκB-DNA gel shift complex, while p65 antibody works by interacting directly with the p65 DNA binding domain to abolish the band formed by NFκB protein and its binding oligonucleotides. As shown in figure 4-2, when in vitro transcribed/translated p50 and p65 protein were used in positive control

lanes, both of p50 and p65 antibodies worked well in the expected ways. But when AtT20 nuclear extracts were used in the gel shift reactions, there were no clear effects exerted by these antibodies on the shifted band, which suggested that, in AtT20 cells, the factors binding to the POMC NFκB element are not exactly the NFκB proteins, although they can be related.

Characterization of nuclear proteins binding to POMC AP-2 element:

In POMC promoter region -236/-133, there is also a consensus AP-2 element. In order to characterize the protein binding to this element and to see whether it is the AP-2 protein, gel-shift assays and southwestern blots were performed. A gel shift experiment with the POMC AP-2 oligonucleotide as probe revealed a specific band formed by AP-2 element and AtT20 nuclear extracts (figure 4-3, left panel). The mobility of this band is quite different from the one formed by the NFκB element. The AP-2 shifted band migrated faster, which suggested that the protein binding to this element probably has a smaller size than the one binding to NFκB.

In order to find out what size protein is specifically binding to the POMC AP-2 element, southwestern experiments were performed. AtT20 nuclear extracts were electrophoresed by SDS-PAGE before transfer onto nitrocellulose membranes. After denaturation and renaturation the membrane was hybridized with radiolabeled AP-2 oligonucleotides. Figure 4-3 (right panel) shows that a protein at a size around 50 kD appeared to be able to bind to the AP-2 element specifically: the binding was competed by cold AP-2 oligonucleotides, while the POMC NFκB oligonucleotide was not able to compete. This result suggested that the protein binding to the POMC AP-2 element is probably the AP-2 protein, because the cloned AP-2 protein has a size of 52 kD (Williams et al. 1988).

Construction of multiple mutations in the -236/-133 region:

Our previous studies (chapter II and III) have suggested that within the POMC promoter region -236/-133, PCRH-RE may need to interact with other elements in this region, such as NFκB and AP-2, to achieve the full CRH responsiveness of the larger region. In order to find out what role each of these elements in the -236/-133 region is playing for the CRH responsiveness and whether any of them is interacting with PCRH-RE to achieve this CRH responsiveness, six sites in the -236/-133 region which locate to the footprinted elements were designed to be mutated (figure 4-4, upper panel). Each mutation made each of these elements lose their ability to bind trans-acting factors (checked by gel-shift, data not shown). A PCR method was used to create these mutations either singly or in combination with a second mutation. Five double mutations were created, which are the mutation in PCRH-RE element combined with the mutation in each of other five elements. The procedure of how these mutations were made by PCR reactions is shown in lower panel of figure 4-4. All of these mutant products were then subcloned into the TK-CAT heterologous reporter gene (see material and methods) and were used in the studies described below.

Effects of the mutations in the -236/-133 region on CRH responsiveness:

DNA from the TK-CAT reporter constructs containing different single or double mutations in the POMC promoter region -236/-133 was transiently transfected into AtT20 cells, and CAT activities in extracts of untreated and 10 nM CRH-treated (1 hr) cells were measured. Figure 4-5 is a summary of the results from studies on constructs containing single mutations. As we can see, none of these single mutations was able to affect the CRH responsiveness of the -236/-133 region, even the mutation in the PCRH-RE element had no effect,

which suggests that the CRH responsiveness of -236/-133 region is not solely dependent upon a single enhancer element.

Figure 4-6 shows the transfection and CAT assay results from the studies on double mutant constructs. While the combination of M4 (in PCRH-RE) with either M1, M2 or M3 had no significant effect on the CRH responsiveness of the -236/-133 region, combination of M4 with M5 (in NFκB element) was able to reduce the CRH induction fold by half. Results from these studies suggested that CRH responsiveness of -236/-133 region requires interaction of PCRH-RE with the NFκB element; however, other sequences in this region can also provide CRH responsiveness, since the double mutation of these two elements was not able to totally abolish the CRH induction.

Discussion

Results from our previous studies (see chapter II and III) have already suggested that the activity of the POMC promoter region -236/-133 for CRH induction is probably mediated by a complex and multi-element involved mechanism. We extended our earlier studies on the PCRH-RE element to other elements in the -236/-133 region, and tested the possible interactions between these elements.

Characterization of the POMC NFκB element revealed that there are proteins related to NFκB proteins which are binding to this element. In gel shift experiments, the POMC NFκB element and a NFκB consensus element, immunoglobin κB element, showed similar characteristics for AtT20 nuclear protein binding, suggesting that the protein(s) binding to this element is very similar to the NFκB proteins. But further analysis by super-shift assay showed them to be different, because the AtT20 nuclear protein(s) binding to the NFκB element were not able to be recognized by antibodies to NFκB proteins (p50 and

p65). Supershift is caused by the interaction of antibody with the protein which binds the DNA probe, and since NFκB proteins (p50 and P65) belong to an expanding transcription factor family called the rel-family (Inoue et al. 1992). The protein binding to the POMC κB element may be one of this family members, in which the antibody interacting region is not identical to the ones in NFκB proteins, thus not being able to be recognized by those antibodies in the supershift assay.

Southwestern studies on the POMC AP-2 element identified a protein with a size around 50 kD that is able to bind to this element specifically. Since the AP-2 protein was cloned as a 52 kD protein (Williams, et al. 1988), the AtT20 nuclear protein binding to the POMC AP-2 element may actually be this 52 kD AP-2 protein or a related protein. Supershift assay with antibody to this 52 kD AP-2 protein will be useful to confirm this.

Functional studies on the mutant -236/-133 promoter region revealed that none of six single mutations were able to knock out the CRH inducibility of this region, suggesting that the CRH responsiveness of the -236/-133 region is not just dependent on a single element within this region. The disruption of one element can be "rescued" by the rest of the promoter sequence of this region. One would expect that the mutation of the PCRH-RE should have some effect on the CRH responsiveness, because this element had been shown to be CRH inducible by itself (see chapter II). But if we analyze the results of studies on this element carefully, we will find that it is not surprising that mutation in this element didn't affect its function. As we can see in chapter II, although the three base mutation in the PCRH-RE (-175/-156) element abolished its ability to bind nuclear protein (figure 2-4), when the mutant -180/-150 oligonucleotide was used in gel-shift assay, the same three base mutation didn't affect the nuclear protein binding at all (figure 2-9). As we discussed in chapter II, this is caused probably because

the proteins which bind to the sequence adjacent to these three bases are interacting with the factor binding to the PCRH-RE, possibly causing the binding of this factor to the DNA to be stabilized, even if the three bases which are important for this binding are mutated (figure 2-10). So when the mutant -236/-133 promoter region containing these three mutant bases was tested in a functional promoter assay, the binding of the PCRH-RE binding protein(s) to this promoter region was not affected; and as a result, the activity of this region is not affected either.

Functional studies on the double mutations in -236/-133 region revealed that only the combination of mutations in PCRH-RE and the NFκB element affected the CRH inducibility (figure 4-6), suggesting that these two elements are probably interacting with each other in some way. Probably, the proteins binding to the NFκB element interact with the proteins binding to the PCRH-RE, and this interaction makes the whole binding complex stable; mutation of either of these two elements is not sufficient to disrupt this complex, only when these two sites are mutated together, will the binding complex be dissociated. Recently, there are reports showing that NFκB protein is able to interact with other transcription factors such as Sp1 and ATF family members (Perkins et al. 1994; Kaszubska et al. 1993). In human HIV-1 long terminal repeat (LTR), the two NFκB binding sites are in close proximity to three SP1 binding sites. It has been shown that a protein-protein interaction must occur between NFκB and SP1 in order to induce the HIV gene expression, and this protein-protein interaction is dependent on the orientation and position of their binding sites (Perkins et al. 1994). In another study, Kaszubska et al. (1993) reported that in the promoter of the E-selection gene, NFκB protein needs to interact directly with a transcription factor of the ATF family to mediate the regulation of the cytokine-induced expression of this gene.

Our studies also showed that double mutation of PCRH-RE and NFκB elements was still not able to totally abolish the CRH responsiveness of the -236/-133 region, suggesting that besides these two elements, other sequences within this region are probably also required for the full activity of this region. Combination of triple mutations containing these two mutated sites will be useful to identify these sequences.

From the results of this study, it appears that like basal promoter expression and glucocorticoid negative regulation, there are complex and probably redundant elements within POMC promoter -236/-133 region that are responsible for its CRH response. Possibly, it resulted from a need of POMC regulation to respond to different hormonal and environmental signals in various tissues.

Material and Methods

Cell Culture: Mouse AtT-20 D16/16 tumor cells were cultured in Dulbecco's modified Eagle medium (DME, Gibco) supplemented with 10% fetal calf serum in a humidified 5% CO_2 incubator. Cells for transfection were cultured in 6-well dishes (Falcon) to 60% confluency and switched to serum-free medium (DMEM) for 24 hours before experiments. Cells for preparation of nuclear extracts were plated in 15 cm dishes.

Plasmid DNAs: Constructs containing multiple mutations in the -236/-133 region were made by ligating restriction enzyme digested and gel purified PCR product into Hind III and Xba I digested TK-CAT vector. Each mutation throughout the -236/-133 region was verified by DNA sequencing.

Nuclear extracts: AtT 20 cells were grown until 70-80% confluency and serum deprived 24 hours prior to a 1 hour treatment with or without 10 nM CRH (pennesula labs). Cells were then harvested in cold phosphate-buffered saline (PBS). Nuclear extracts were prepared as described (Dignam et al. 1983). Final concentrations were typically 2 to 3 µg/µl as determined by the Biorad protein assay (FRG, Germany).

Gel shift assays: All oligonucleotides were synthesized by the MSSM DNA synthesis facility. Immunoglobin κB - 5'-TCGACAGAGGGGACTTTCCGATTG - 3', rat POMC κB - 5' GCCCCGACCGGGAAGCCC-3', POMC AP-2, 5'-CCCCCCTCCCGCGGCC-3'. Five pmoles of double stranded oligonucleotide were end-labeled with (γ-^{32}P) ATP (3000 Ci/m mole, NEN) using polynucleotide

kinase, resolved on a 10% acrylamide gel and eluted from the gel at 4° C in TE buffer (10 mM TRIS-HCl, pH 8, 1 mM EDTA) or dH_2O (when used for testing effects of divalent cations on gel shift). Approximately 0.2 ng of labeled DNA (15,000 to 20,000 cpm) were added to the preincubated bacterially expressed PCRH-REB.

5-15 µg of proteins were preincubated 10 min at room temperature in 12 µl of binding buffer with 1 µg of poly (dI-dC) and 1 mg of nonspecific single stranded DNA. 0.2 ng of ^{32}P-labeled probe was added and the binding reaction was left at room temperature for 15 min. In competition experiments, 50-fold molar excess of unlabelled competitor oligonucleotides were added in the preincubation reaction. In supershift experiments, antibodies were preincubated with nuclear extracts for 1 hour on ice before gel shift reaction was performed (anti mouse p50 and p65 antibodies are from Dr. David Baltimore's laboratory). The protein-DNA complexes were resolved on a 4% polyacrylamide gels in 0.5X TBE. The gels were dried and autoradiographed with intensifying screens at -70° C with Kodak X-OMAT AR films.

Site-specific mutagenesis of POMC -236/-133 region by PCR: Site-specific mutagenesis was made by overlap extension using PCR technology (Steffan et al. 1989). The principle is shown in figure 4-4. The polymerase chain reactions were carried out according to the standard protocol (Innis et al. 1990). In PCR reaction 1 (Rx1), oligo a and b were used as primers; in reaction 2 (Rx2), oligo d and c were used as primers. M indicates the introduced mutant sites. The products from Rx1 and Rx2 were gel-purified and used as templates in PCR reaction 3 (Rx3) with oligo a and d as primers. Rx3 is an overlap extension step generating the full size DNA template carrying the mutations as designed in oligo c and b. The DNA template used in Rx1 and Rx2 was plasmid DNA JL-169

(Lundblad et al. 1987). The primers a and d indicated in figure 4-4 correspond to the sequence of the pUC 18 plasmid and CAT gene, respectively. The sequence of these two oligonucleotides are: Primer a, 5'-GGGGCTGGCTTAACTATGC-3', and primer d, 5'-CTTTACGATGCCATTGGGA-3'. Six pairs of mutant oligonucleotides were synthesized; the mutant sites are shown in the upper panel of figure 4-4. Each pair of mutant oligonucleotides was used as primers in PCR reaction as primer c and b shown in lower panel figure 4-5. The products from PCR reaction 3 were cut with Xba I and Hind III, and subcloned into the TK-CAT vector, which served as the template for the next round of site-specific mutagenesis. In this way, multiple mutations in -236/-133 promoter region were generated. Each mutant DNA construct was confirmed by DNA sequencing.

Transfection and Chloramphenicol Acetyl-transferase Measurement: Transfection experiments were performed using a lipopolyamine-based method (TRANSFECTAM ™, Promega). Briefly, cells were transfected with 2 μg (1.5 μg of each construct DNA in cotransfection experiments) of reporter gene DNA for 6 hours. Cells were then grown for 12 hours in fresh serum-free medium for 6 to 8 hours with or without 10 nM CRH. CAT activity was determined by the method of Gorman (1982). After autoradiography, the different forms of chloramphenicol were located, cut out of the silicate gel and counted; or after phosphoimage exposure, the different forms of chloramphenicol were located and quantified by computer program (Molecular Dynamics).

Southwestern blot assay: Nuclear proteins prepared from AtT20 cell lines (20-60 μg) were separated on SDS-8% polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.). Protein samples were boiled for 3 min before being loaded. After being air dried at room

temperature for 30 min, the filters were washed stepwise to renature proteins in 6, 3, 1.5, 0.75, and 0.37 M guanidine hydrochloride solutions containing 25 mM HEPES (pH7.9), 3 mM MgCl2, 40 mM KCl, and 1 mM DTT. Each wash lasted 5 min at 4°C. Prehybridization followed in a solution containing 10 mM $NaPO_4$ (pH7.4), 150 mM NaCl, 5% powdered milk, 1% BSA, 2.5% polyvinylpyrrolidone-40, and 0.1% Triton X-100 at 4°C for 1 hour. Hybridization was performed with 10^6 cpm of probe per ml in 10 mM Tris-HCl (pH7.5) containing 0.5% powdered milk, 0.5% BSA, 50 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, and 5% glycerol (hybridization buffer) at 4°C for 6 hours. Probes were labeled with T4 DNA kinase and separated from the free γ-^{32}P-ATP by elution through a sephadex G-50 column. The filters were washed twice for 30 min each time in hybridization buffer at 4°C and then autoradiographed. A prestained molecular size marker was used to determine approximate molecular weights. For competition assays, cold oligonucleotides were added after prehybridization (Liu et al. 1992).

Figure 4-1

Binding of AtT20 Nuclear Proteins to POMC κB and Immunoglobin κB Elements

Gel shift analyses with nuclear extracts (15 μg of protein) from untreated (CT, lane 1, lane 8) or 10 nM CRF-treated AtT20 cells. Lane 1-7, Immunoglobin κB (IGKB) element was used as probe. Lane 8-14, POMC κB (PKB) element was used as probe. lane 3, 100-fold cold IGKB was added as competitor. lane 4-6, 10-fold, 50-fold, and 100-fold, respectively, PKB cold oligos were used as competitor. Lane 7, 100-fold cold TRE oligos was used as nonspecific competitor. Lane 10, 100-fold PKB. Lane 11-13, 10-fold, 50-fold, and 100-fold IGKB, respectively. Lane 14, 100-fold TRE.

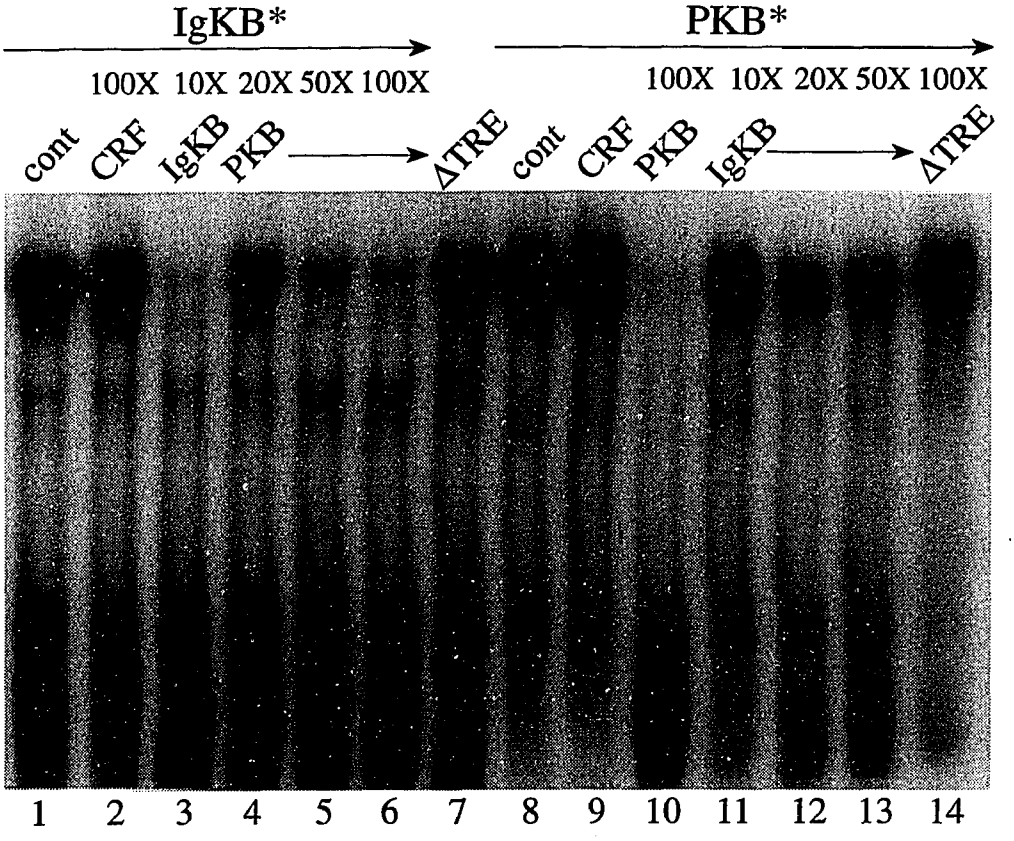

IgKB*
PKB*
100X 10X 20X 50X 100X
100X 10X 20X 50X 100X
cont
CRF
IgKB
PKB
ΔTRE
cont
CRF
PKB
IgKB
ΔTRE
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 4-2

Supershift Experiments with NFκB Antibodies

15 μg of nuclear proteins or 100 ng of in vitro translated p50 proteins were incubated with either P50 antibody, p65 antibody, or a preimmune serum for 30 min on ice before probes were added in the gel shift reactions. Lane 1-8, nuclear extracts. Lane 9-12, in vitro translated p50 proteins. Lane 1-4, PκB as probe. Lane 5-12, IGKB as probe. In lane 1, 5 and 9, no antibody was added. Lane 2, 6, 10, P50 antibody was added. Lane 3, 7, 11, p65 antibody was added. Lane 4, 8, 12, preimmune serum was added.

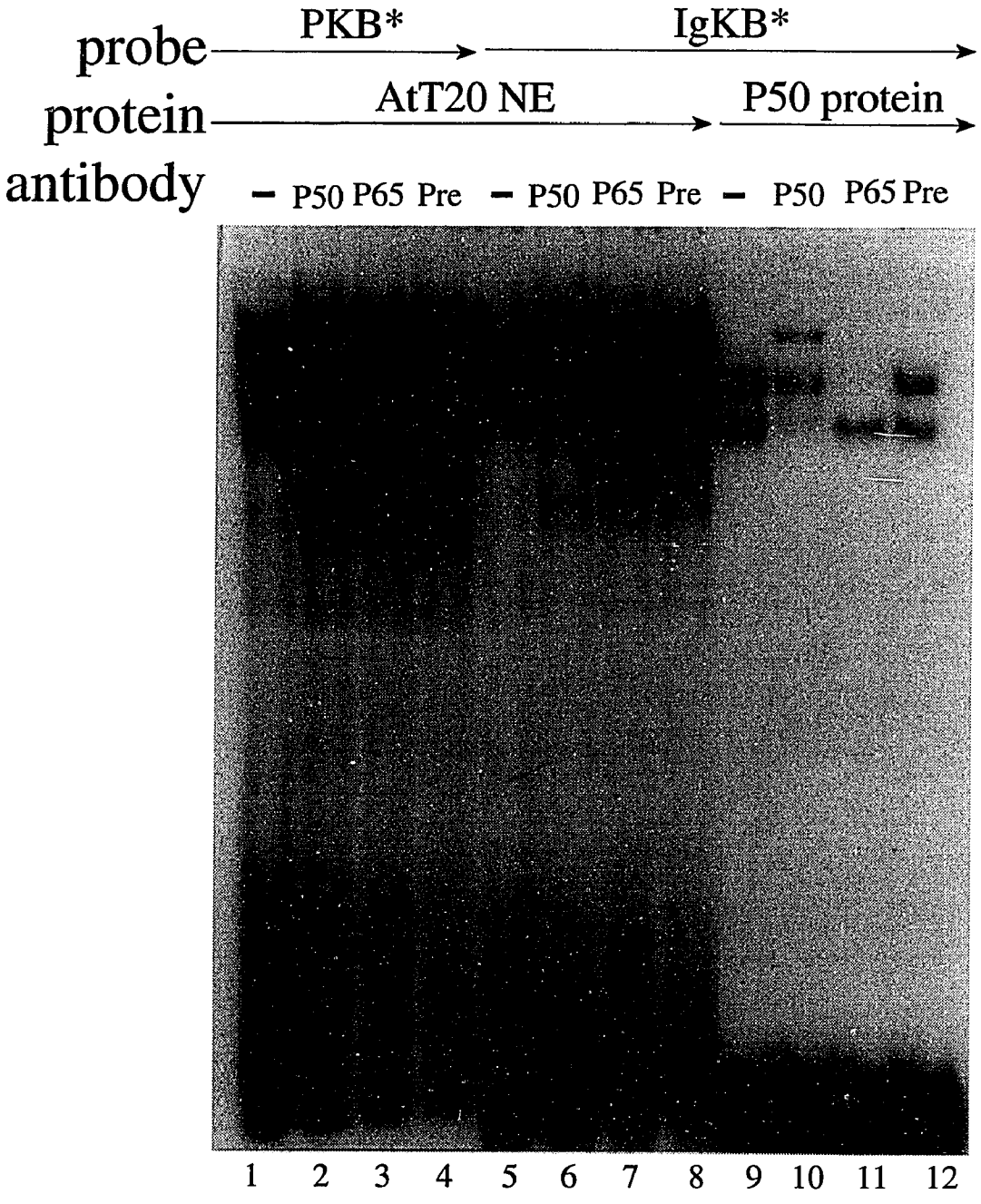
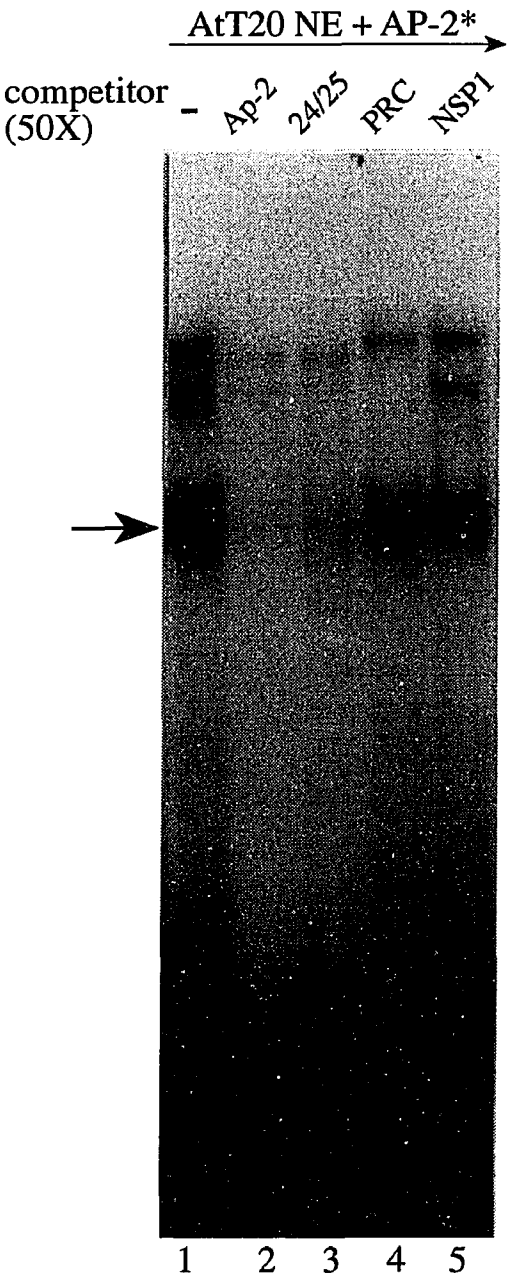

probe
PKB*
IgKB*
protein
AtT20 NE
P50 protein
antibody
– P50 P65 Pre – P50 P65 Pre – P50 P65 Pre
1 2 3 4 5 6 7 8 9 10 11 12

Figure 4-3

Binding of AtT20 Nuclear Proteins to POMC AP-2 Element

A. Gel shift assay: 15 μg nuclear extracts from 10 nM treated AtT20 cells were used in each gel shift reaction with the ^{32}-P labeled POMC AP-2 element as probe. Lane 1, no cold competitor was added. lane 2, 100-fold cold POMC AP-2 oligos was added. Lane 3, -140/-105. lane 4, PCRH-RE. Lane 5, NSP1 as nonspecific oligo1. The position of the specific band formed by nuclear protein and AP-2 probe is indicated by arrow.

B. Southwestern blot with POMC AP-2 element: 30 μg of nuclear extracts from 10 nM CRF treated AtT20 cells was analyzed in each lane of the 8% SDS-PAGE gel. The proteins on the gel were transferred to a nitrocellulose membrane and hybridized with the ^{32}P-labeled POMC AP-2 element. Lane 1, molecular weight markers. Lane 2, no cold competitor DNA was added. Lane 3, 500-fold cold POMC AP-2 oligos was added as specific competitor. Lane, 500-fold cold IgκB oligos were added as nonspecific competitor.


AtT20 NE + AP-2*
competitor (50X)
-
Ap-2
24/25
PRC
NSP1
1
2
3
4
5

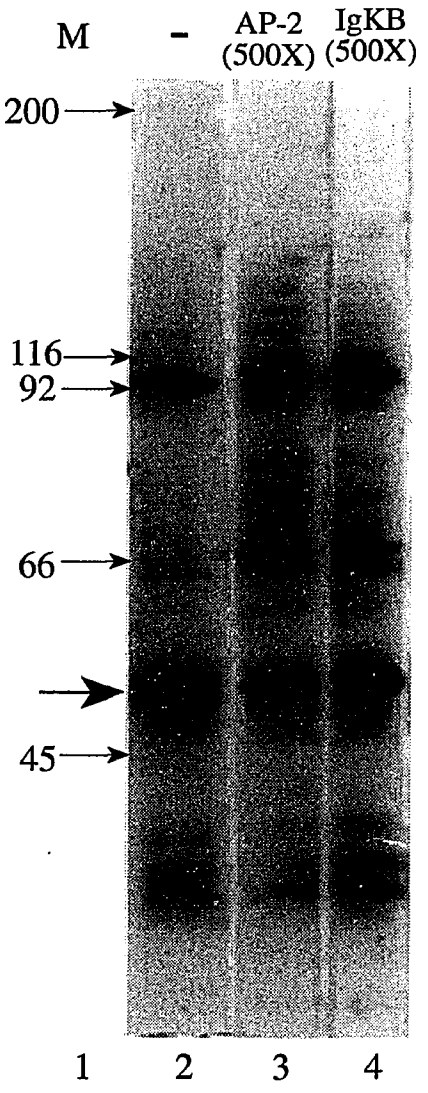
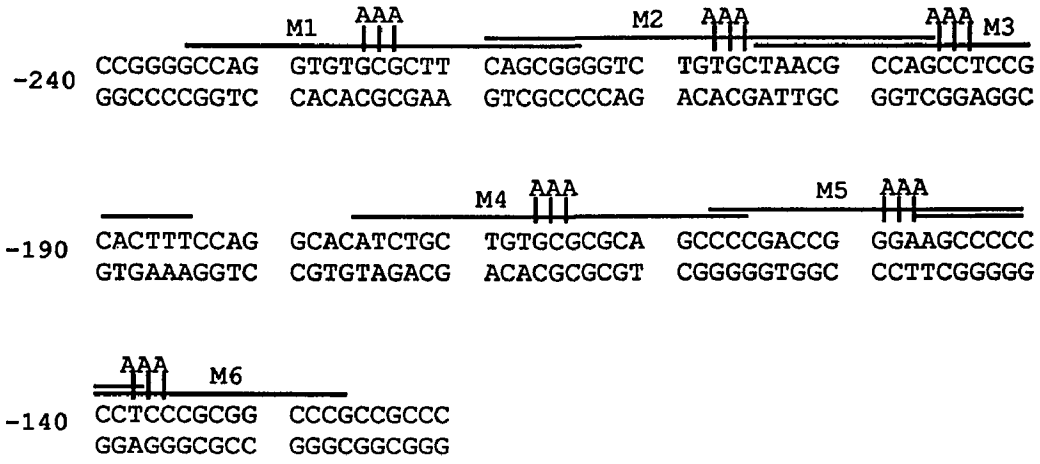

M
-
AP-2 (500X)
IgKB (500X)
200
116
92
66
45
1
2
3
4

Figure 4-4

Site-directed Mutagenesis of POMC Promoter -236/-133 Region by PCR

Upper panel: Sequence of the POMC -236/-133 region is shown, and six selected mutation sites are indicated. Lower panel: Diagram of mechanism of Site-specific mutagenesis by PCR. In PCR reaction 1 (RX1), oligo a and b, in reaction 2 (RX2) , oligo d and c are used as primers. M indicates the introduced mutant sites. The products from Rx1 and Rx2 will be gel purified and used in Rx3 with oligo a and d as primers. Rx3 is an overlap extension step generating the full size DNA template carrying the mutations as designed in oligo c and b.


M1
AAA
M2
AAA
AAA
M3
-240
CCGGGGCCAG GTGTGCGCTT CAGCGGGGTC TGTGCTAACG CCAGCCTCCG
GGCCCCGGTC CACACGCGAA GTCGCCCCAG ACACGATTGC GGTCGGAGGC
M4
AAA
M5
AAA
-190
CACTTTCCAG GCACATCTGC TGTGCGCGCA GCCCCGACCG GGAAGCCCCC
GTGAAAGGTC CGTGTAGACG ACACGCGCGT CGGGGGTGGC CCTTCGGGGG
AAA
M6
-140
CCTCCCGCGG CCCGCCGCCC
GGAGGGCGCC GGGCGGCGGG

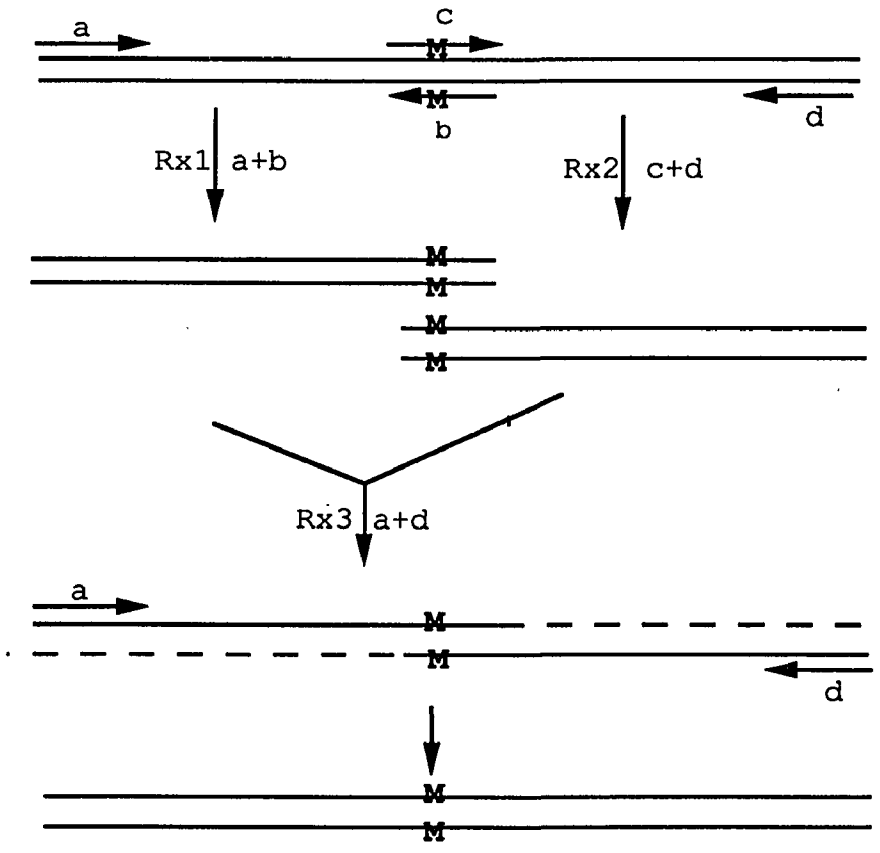
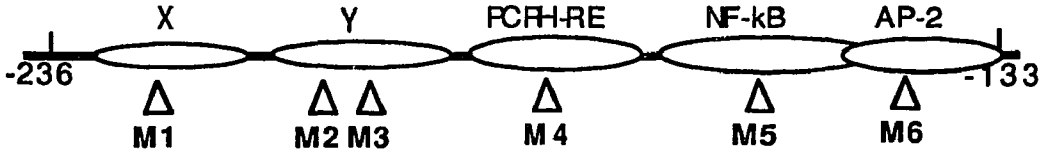

a
c
M
M
b
d
Rx1 a+b
Rx2 c+d
M
M
M
M
Rx3 a+d
a
M
M
d
M
M

Figure 4-5

Effects of Single Mutations within -236/-133 Region

The results of the CAT assays are summarized. DNA of constructs containing POMC promoter fragment -236/-133 and those with different single mutations within this fragment were analyzed in the presence and absence of 10 nM CRH after transfection into triplicate AtT20 cell cultures. The fold induction of CAT activities by CRH are shown by different size of bars.

Functional studies on single mutants



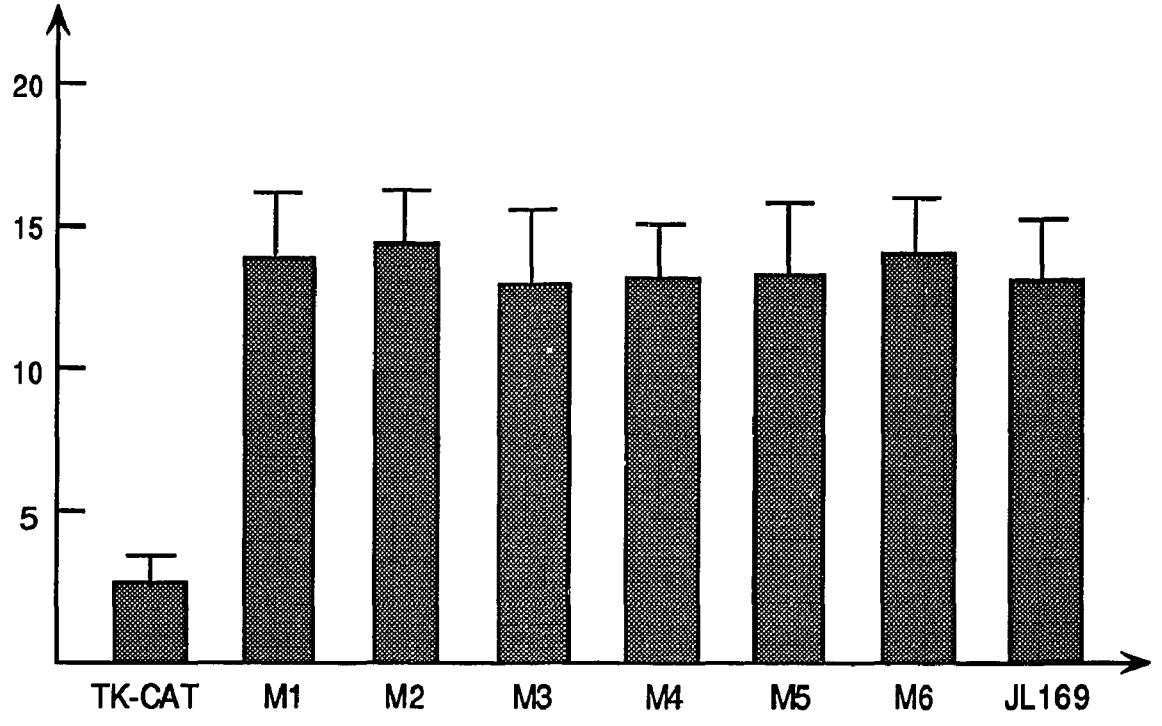
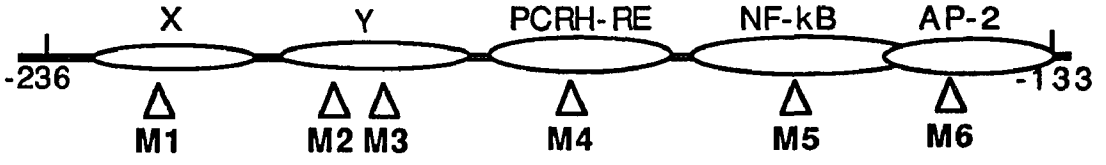


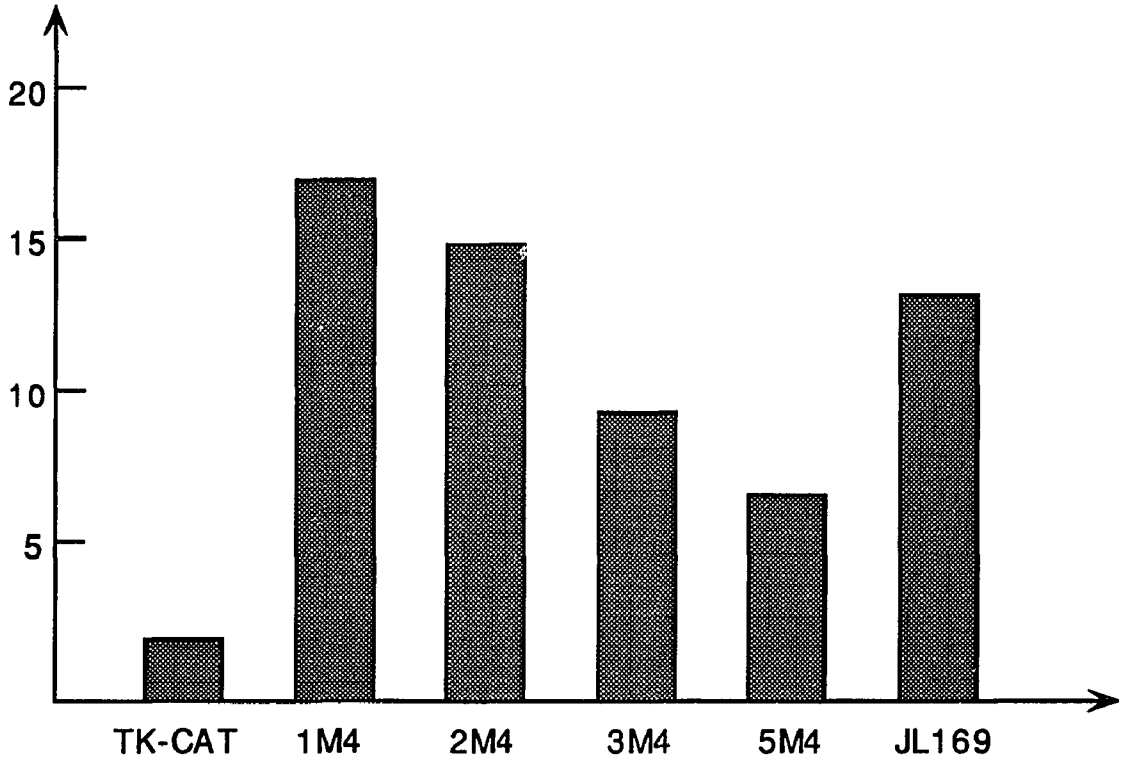
Figure 4-6

Effects of Double Mutations within -236/-133 Region

The results of the CAT assays are summarized. DNA of constructs containing POMC promoter fragment -236/-133 and those with different double mutations within this fragment were analyzed in the presence and absence of 10 nM CRH after transfection into triplicate AtT20 cell cultures. The fold induction of CAT activities by CRH are shown by different size of bars.

Functional studies on double mutants





Chapter V. General Discussion

Transcriptional regulation of POMC gene expression by CRH, like its basal expression and negative regulation by glucocorticoids, is mediated by a complex, multi-element involved mechanism (see review of Roberts et al. 1993; chapter II and chapter III). In this thesis, we focused our studies on a 100 base area within the POMC promoter, the -236/-133 region, which has previously been shown to be the major CRH responsive region in the POMC promoter (Lundblad et al. 1988). Within this region, we identified a major CRH responsive element (PCRH-RE), and characterized its binding factor (PCRH-REB) by biochemical and recombinant DNA techniques. Further functional studies of multiple mutations within this 100 base region revealed that the CRH responsiveness is mediated by multiple elements, probably through the interaction of PCRH-RE and its surrounding elements like NFκB and AP-2.

The sequence of PCRH-RE is not identical to any known consensus sequence for transcription factor binding, except it contains a MRE core sequence. But characterization of this element has shown that it doesn't function like the classical MRE: although the binding of nuclear proteins to PCRH-REB was sensitive to divalent cations such as Cd^{2+} and Cu^{2+}, the effects of these divalent ions to the binding are not the same as the MREs which were characterized by other groups (Furst et al. 1988; Imbert et al. 1989; Labbe et al. 1991; Foster et al. 1991; Saguin, 1991; Koizumi et al. 1992; and see chapter II). So PCRH-RE appeares to be novel element which can respond to cAMP/Ca^{2+} signals. Not only did PCRH-RE appeares to be novel, but its putative binding protein, PCRH-REB, also showed novel characteristics. Sequence analysis of PCRH-REB showed that there is no classical transcription factor structure motifs such as leucine zipper or zinc finger in its protein sequence. Instead, sequence

comparison with genebank showed that its sequence is identical to a newly identified DNA replication factor (Lu et al. 1993; Burbelo et al. 1993; Luckow et al. 1994). But since none of these groups that cloned this putative replication factor has ever tested its function in transcription, the studies we did to characterize the PCRH-REB as a putative transcription factor which could be involved in transcriptional regulation of POMC gene expression responding to cAMP/Ca^{2+} signal, would contribute to a better understanding of the mechanism of how a factor can function in both of transcription and replication. Transcriptional regulation of gene expression is mediated by transcription factors binding to cis-acting DNA elements. Although most of the transcription factors were only found to have transcriptional regulation activities, there is a increasing number of transcription factors which have been found to be also involved in DNA replication (Foss et al. 1993; Bell et al. 1993; Li et al. 1993; Mickiem et al. 1993). But so far, the mechanism of how these factors to be involved in both of transcription and replication process is not well understood. One of these factors which have dual functions in transcription and replication, T antigen, has been extensively studied for years for its functional mechanisms. T antigen was first identified as an SV 40 produced protein which is important for DNA replication (Tjian, 1978). It can bind specifically to the minimal core origin of replication, and in the presence of ATP, it forms an oligomeric structure which is capable of binding to an extended region of the origin and locally unwinding the early palindrome region which will lead replication to start (Dean et al. 1987; Dodson et al. 1987). Besides its role in replication, T antigen was also found to be able to control transcription. Through binding to sequences in the origin of DNA replication, T antigen represses transcription from the SV40 early promoter (Rio et al. 1980). During the lytic infection cycle, T antigen regulates transcription from the SV40 late promoter in a process that does not require T antigen binding

to viral DNA (Keller et al. 1985). Besides the viral promoter, large T antigen also activates many cellular promoters to varying degrees, when the promoters are presented in a reporter plasmid (Alwine, 1985; Gilinger and Alwine, 1993). Gilinger et al. (1993) showed that the promoter structure necessary for T antigen-mediated transcriptional activation was very simple: a TATA or initiator element was required, in addition to an upstream factor-binding site, which can be quite variable, such as SP1-, ATF-, AP1-, or TEF-I-binding site. Since T antigen activates promoters containing many different upstream activating sequence (UAS) elements, it seems unlikely that T antigen transactivation is mediated primarily by direct interaction between T antigen and proteins that bind to UAS elements, since this would require that T antigen be able to interact with multiple proteins containing different types of activation domains. More likely, transactivation by T antigen involves interactions between T antigen and components of the basic transcription machinery. It has been found that T antigen is able to bind TBP (TATA binding protein), so it was suggested that T antigen mediates transcriptional activation by interacting both with components of the basal transcription machinery and with factors that recognize upstream activating element, and in so doing, may stabilize the transcription complex (Gruda et al. 1993; Rice et al. 1993). But so far, no direct evidence has been provided to prove this hypothesis.

T antigen has been found to bind ATP and can also be posttranslationally modified by phosphorylation; both processes which can affect its activities in replication (Bramhill and Kornberg, 1988; Klausing et al. 1988). Besides these modifications, the activity of T antigen can also be modulated by interaction with other DNA binding proteins such as P53, retinoblastoma (RB) protein, transcription factor AP2 and c-Jun (Mitchell et al. 1987; deCaprio et al. 1988; Martin et al. 1993; Bharucha et al. 1994). So it seems that complex mechanisms

are involved in the regulation of T antigen activities in both replication and transcription.

PCRH-REB has been suggested to be a replication factor based on sequence homology and immuno-recognition (Lu et al. 1993; Burbelo et al. 1993; Luckow et al. 1994), although no functional study has been carried out to prove this activity. On the other hand, as a putative transcription factor binding to POMC PCRH-RE, our functional studies with cotransfection experiments (chapter III) failed to show any transcriptional activity of PCRH-REB by itself. So unlike T antigen, which has transcriptional activities by itself, PCRH-REB appears to need other transcription factors to serve as a mediator to regulate gene transcription (see discussion of chapter III). Recently, a mediator transcription factor for CREB has been identified (Kwok et al. 1994), this protein is called CBP (CRE binding protein). It was found that CBP can bind specifically to the PKA-phosphorylated form of CREB, and at the same time, the c-terminal region of CBP binds specifically to the basal transcription factor TF IIB. Through this interaction, CBP serves as a coactivator for the phosphorylated form of CREB by enhancing the activity of basal transcriptional machinery. For PCRH-REB, it is possible that a mediator transcription factor is limittedlly expressed. So in cotransfection experiments, although PCRH-REB full length protein was overexpressed, no mediator protein was available for these extra PCRH-REB to bind; as a result, no enhancement of transcriptional activity was able to be seen.

The interaction of CREB and CBP is found to be mediated through a protein interaction domain within CREB, which is amino acids 101-160 within the CREB activator domain (Chrivia et al. 1993). In PCRH-REB, we believe that there must be a protein-protein interaction domain within its protein sequence, which mediates its interaction with other protein subunits within the replication factor C complex. It is possible that this same domain mediates the interaction with other

transcription factors. Sequence analysis of PCRH-REB revealed that several regions within this protein were predicted to form alpha-helical structures. Whether it is one of these regions that serves as the protein-protein interaction domain remains to be tested. A recent report (Kroll et al. 1993) showing that DNA topoisomerase II was able to form direct protein-protein interactions with CREB as well as ATF-2 and c-jun, concluded that the interactions between these proteins were not mediated by the alpha-helical leucine zipper domain within the DNA topoisomerase II protein, which suggested that some uncharacterized structure can be involved in these kinds of protein-protein interactions.

There was some discrepancy about the DNA binding specificity of PCRH-REB/PO-GA/MSW/mRF140 reported from different groups (Lu et al. 1993; Burbelo et al. 1993; Luckow et al. 1994; chapter III). In our hands, bacterial expressed and gel purified PCRH-REB-1 bound to POMC -180/-150 much stronger than several other oligonucleotides we tested, but still, we feel that the binding specificity of this protein is not very high, since when we put excess poly(dI-dC) as non-specific competitor in the gel-shift reaction, the binding of PCRH-REB was abolished. Even in the gel shift assays when AtT20 nuclear extracts were used, the binding to PCRH-RE was able to be competed to some degree by several different oligonucleotides (figure 2-8), and the sequence of some of these oligonucleotides are just mildly homologous to PCRH-RE (figure 2-7). Results from these studies suggested that the PCRH-REB/PO-GA/MSW/mRF140 is not a highly sequence specific DNA binding protein, and it should be able to bind to multiple sequences in genomic DNA. In the sense of this protein as a putative replication factor, it is not surprising that this protein can bind to different DNA sequences. But as a putative transcription factor, how can this protein achieve its specificity to exert its activity as a transcription factor to a specific gene? Here we hypothese that, for PCRH-REB, when it functions as a

transcription factor, it is through interactions with other protein factors, and it is through this protein-protein interaction, that the DNA binding specificity of this protein is able to be increased. A recent report from Sogarrd-Anderson et al. (1993) may help us to understand this hypothesis. In that report, they showed that E. coli transcription factor Cyt R bound with a low affinity to a specific binding site, but in the presence of cAMP-CRP, which itself can bind strongly to a site close to the Cyt R binding site, the binding of Cyt R was enhanced more than 1000-fold. They found that this happened because of the protein-protein interaction between the Cyt R and cAMP-CRP. Mutations in the cAMP-CRP binding site or changing of the helical phasing between these two sites, which would interfere with the protein-protein interaction between them, prevented the Cyt R DNA binding ability to be increased. They further showed that the protein-protein interaction between these proteins was more important for Cyt R exerting transcriptional activity than the binding of Cyt R itself to its binding site. Mutation of the Cyt R binding site or deletion of the DNA binding domain within Cyt R protein had little effect on its transcriptional activity, as long as the protein-protein interaction happened between Cyt R and cAMP-CRP. Results from these studies remind us of the mutation studies reported in chapter II and chapter IV. In chapter II, we found that a three base mutation within the -180/-150 oligonucleotide had no effect on protein binding in gel shift assays. In chapter IV, we found that the same three base mutation within the -236/-133 promoter region had no effect on the CRH responsiveness of this region. As we discussed in these two chapters, PCRH-REB is probably like Cyt R protein: its DNA binding ability can be increased by the protein-protein interaction with factors binding to the nearby sequences. The PCRH-RE, the site of action of PCRH-REB, can be specified by protein-protein interactions to factors binding to nearby sites, whereas PCRH-REB-DNA interactions may primarily serve to stabilize the

nucleoprotein complex. This type of specificity mechanism may represent a general concept in the recruitment of DNA-binding proteins in combinatorial regulatory systems.

PCRH-REB binding to its DNA element has been shown to be sensitive to divalent cations (see chapter II); the binding was inhibited by Cu^{2+} and Cd^{2+}. While the molecular basis for this kind of metal sensitivity of PCRH-REB binding is still not clear, sequence analysis of PCRH-REB revealed that there is a putative metal ion binding motif present in its protein sequence, which is Cys(734) -X- Cys-X_{12} - His - Cys that has some similarity to the metal ion binding motif which may facilitate DNA binding (Vos et al. 1988). Some kind of metal ion is probably able to bind to this domain, and facilitate the binding of PCRH-REB to DNA, either by directly interacting with the DNA or by promoting protein-protein cooperatively in DNA binding. In the gel shift assay, Cu^{2+} and Cd^{2+} were probably competing for binding to this metal ion binding domain, so inhibited the binding ability of PCRH-REB . So far, we have no information as to what kind of metal ion this domain normally binds in vivo. We have tested different concentrations of Ca^{2+}, Mg^{2+} and Zn^{2+} in PCRH-RE gel shift with either AtT20 nuclear extracts or gel-purified PCRH-REB (data not shown). Result from these studies failed to show any of these metal ions to be able to enhance the PCRH-RE binding. Probably, that is because the ion concentration in the extract buffer was already high enough and all the metal binding sites in the PCRH-REB were already saturated. Experiments with careful designation to exclude these metal ions in the reaction system will be helpful to identify the exact metal ion that PCRH-REB binds.

Results from previous studies have shown that POMC gene transcription was able to be stimulated by Ca^{2+} signal (Eberwine et al. 1987; Lorang et al. 1994). One interesting finding by Lorang et al. (1994) is that Cd^{2+} (50-500 uM)

was able to block the CRH induced POMC hnRNA, but only when it was allowed to enter the cell, which indicates that the blockade was due to some intracellular event and not to blocking Ca^{2+} influx through Ca^{2+} channels. Since in the same study, it has been shown that this kind of Ca^{2+} mediation of POMC transcription happened in a calmodulin and protein kinase C (PKC) independent manner, we suspect that the Cd^{2+} blockade effect is due to a direct inhibition of transcription factor activity. PCRH-REB has been shown to be sensitive to Cd^{2+}, and PCRH-REB is a putative transcription factor which is responsible for CRH regulation of POMC transcription. So this factor can be the potential target of Cd^{2+} action. If this is true, then a possible mechanism of Ca^{2+} stimulating POMC transcription will be to activate the transcriptional activity of PCRH-REB, probably by direct binding of this ion to the protein. For future studies, it will be important and interesting to check whether PCRH-REB is actually a Ca^{2+} binding transcription factor, and whether its activity can be regulated by Ca^{2+} binding.

PCRH-RE has been shown as the major CRH responsive element in the POMC -236/-133 region, but since its CRH responsiveness is still less than the -236/-133 region (chapter II), there must be other elements and transcription factors also involved in this regulation. Results of functional studies on constructs containing double mutations within -236/-133 region suggested that NF-kB element is possibly interacting with PCRH-RE. However other sequences in the -236/-133 region are also required for the full CRH responsiveness, since a double mutation of the PCRH-RE and NFκB elements still can not knock out the full CRH induction (chapter IV). It appeared that CRH regulation of POMC gene transcription through the -236/-133 promoter region is mediated by many elements and factors in a complex mechanism. In figure 5-1, we present a model of how this mechanism might work to regulate POMC gene transcription. Briefly, this model shows that PCRH-REB and probably another factor (X) which has a

transcription activation domain, bind to the PCRH-RE, and this protein complex, at the same time, is interacting with factors binding to nearby elements such as NFκB and an unknown sequence (Y). CRH signal will cause the modification of PCRH-REB either by phosphorylation or Ca^{2+} binding, and these modifications will enhance the interaction between PCRH-REB and X , as well as with factors binding to NFκB and Y elements. This causes more transcriptional activation to be achieved by the -236/-133 region, which will finally exert on the basal transcription machinery and increase the initiation rate of POMC gene transcription.

Protein-protein interactions between members of diverse families of transcription factors have been found to be involved in the transcriptional regulation of many gene systems. Glucocorticoid receptor has been found to be able to interact with the AP1 protein, Jun-Fos complex (Diamond et al. 1990). Through this interaction, two different signal transduction pathways, mediated by hormone binding and protein kinase C activation, respectively, can directly intersect. Some viral transcriptional regulators, while unable to bind DNA themselves, have profound effects on transcription as the result of forming protein-protein complexes with cellular transcription factors. For example, aTIF/VP16 from herpes simplex virus can interact specifically with the homeodomain of the POU domain protein Oct-1 (Stern et al. 1989) and alters the transcriptional activity of Oct-1 by virtue of tethering its highly acidic transcriptional activation domain. For PCRH-REB, it may be interactions with different sets of proteins that determines whether its transcriptional or replicational activity will be exerted on a DNA sequence.

In this thesis, we report the identification of a major CRH responsive element PCRH-RE in the POMC promoter region -236/-133, and characterization of its binding protein PCRH-REB. We also studied the possible interactions

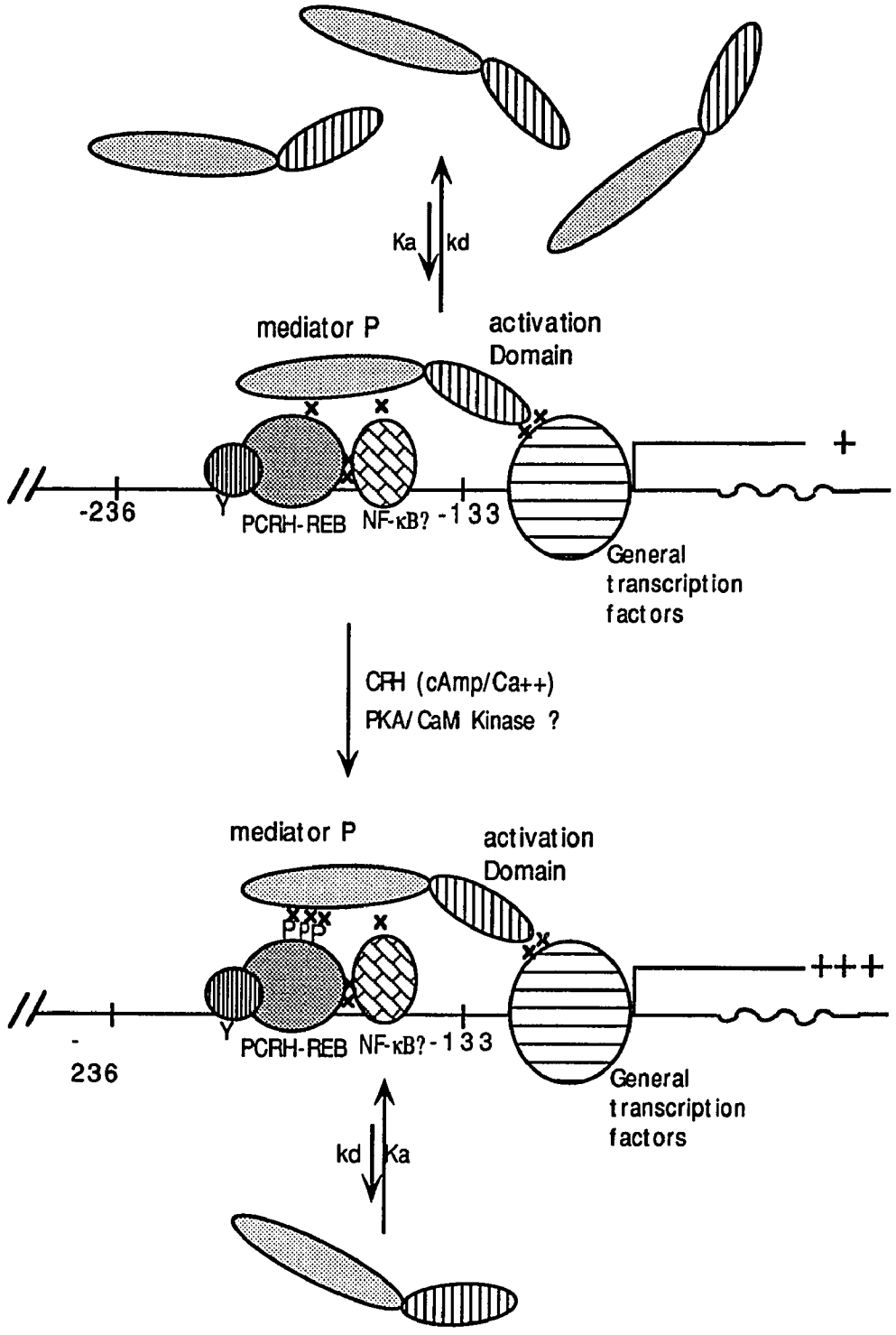
between the PCRH-RE and other elements within the -236/-133 region, which can be part of the mechanism of how this region can be the major CRH responsive region in the POMC promoter. Characterization of PCRH-REB has revealed some interesting characteristics of this factor, and has led us to propose a model of how this factor can possibly be involved in both replication and transcription, the mechanism of which may also apply to other factors that have dual functions of replication and transcription. To prove this model and have a clearer understanding of the mechanism of how this factor can be responsible for CRH regulation of POMC gene transcription, it will be important to further study the transcriptional activity of PCRH-REB, trying to understand how a CRH signal can activate this factor to enhance its transcriptional activity. Since we have finally been able to prepare bacterial expressed, purified PCRH-REB protein, we can use it in different experiments for further characterization of this factor. For example, we will be able to determine whether protein kinase A can directly phosphorylate it, and what effect the phosphorylation will have on PCRH-REB characteristics: whether it increases its DNA binding ability or its interaction with other proteins. We can use the purified PCRH-REB to check whether it is a Ca^{2+} binding protein and study the kinetics of this binding. We can use the protein to purify antibodies to this protein, and then use the purified antibody to characterize the activity of the endogenous PCRH-REB in AtT20 cells. Also, we can use the purified PCRH-REB and its antibody to identify the factors which it interacts with and to check whether CRH signal can affect the interaction. Hopefully, after we complete these studies we will be able to understand the mechanisms of how PCRH-REB can function both in replication and in transcriptional regulation of POMC gene expression. This may give us a clue to the mechanisms used by other factors which have dual activities in replication and transcription. So by elucidating the mechanism of how the POMC -236/-133 region mediates the

transcriptional regulation of POMC gene expression responding to CRH signal, and what kind of role PCRH-REB is playing in this process, we will have a better understanding of how a cell regulates its gene expression in response to outside signals.

Figure 5-1

Diagram of the Model for PCRH-REB Regulating POMC Gene Transcription in Response to CRH Signal

The model for PCRH-REB regulating POMC gene transcription responding to CRH signal is diagrammed. Different transcription factors are shown as different shapes of ellipses. The activation domain in the mediator protein (mediator P) is marked in different colors. Phosphorylation of PCRH-REB is shown as P. Transcriptional activation of the promoter is marked as + in front of the arrows standing for transcription. Ka and Kd stand for association and dissociation rate constants of the mediator P between the promoter transcription factor complex and its surrounding nuclear environmental solution.


Ka
kd
mediator P
activation
Domain
+
-236
PCRH-REB
NF-κB?
-133
General
transcription
factors
CRH (cAmp/Ca++)
PKA/ CaM Kinase ?
mediator P
activation
Domain
+++
-
236
PCRH-REB
NF-κB?
-133
General
transcription
factors
kd
Ka

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