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Dermer, Shari Joy, Ph.D.

City University of New York, 1992

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MULTIHORMONAL REGULATION OF PRO-OPIOMELANOCORTIN GENE EXPRESSION

Shari Joy Dermer

A dissertation submitted to the Graduate Faculty in Biomedical Sciences
in partial fulfillment of the requirements for the degree of Doctor of Philosophy,
The City University of New York

1992

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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

MULTIHORMONAL REGULATION OF PRO-OPIOMELANOCORTIN GENE EXPRESSION

by

Shari Joy Dermer

Advisor: Dr. James L. Roberts

Modulation of POMC gene expression occurs in response to a variety of stimuli. The POMC gene is subject to negative steroid feedback in two different neuroendocrine systems; by glucocorticoids in the hypothalamic-pituitary-adrenal axis and by estrogens in a subset of neurons in the hypothalamus. Both of these regulatory patterns are due to the genomic action of steroid receptors. Estrogen-mediated regulation of POMC gene expression was studied utilizing a model POMC-expressing cell culture system that expresses an exogenous estrogen receptor. ER-1 cells produced functional estrogen receptor as assessed by nuclear steroid binding assays. Time-, dose-, and receptor-dependence of estrogen-mediated POMC gene regulation was studied utilizing solution hybridization/nuclease protection assays. There was a dose-dependent decrease in long term POMC gene expression. The response to estrogen treatment was a biphasic, characterized by a significant transient increase in POMC biosynthesis with acute treatment, followed by a sustained long term decrease in gene expression to below control levels. These events were determined to be estrogen receptor-dependent. The mechanism of estrogen-mediated POMC gene inhibition was compared to the effects of glucocorticoids by examining the interaction of estrogens and a positive effector, corticotropin releasing factor (CRF). It was found that both estrogen and glucocorticoid treatments attenuated the stimulatory effects of CRF in ER-1 cells. The potential involvement of c-fos gene expression in acute estrogen-mediated stimulation of POMC expression was explored. While it was demonstrated that estrogen treatment in ER-1 cells resulted in induction of c-fos, a cause and effect relationship remains speculative. Delineation of a sequence which was able to

mediate CRF stimulation of a POMC reporter gene was performed using transient transfection studies. This sequence contains a nearly perfect palindromic element and may represent a binding site for a transcriptional regulatory protein. The complex array of regulation schemes which govern expression of the POMC gene require integration of signals from many different hormones, neurotransmitters, and second messengers. It is not surprising that this may involve interaction of multiple transcription factors that function within the context of the entire POMC promoter.

"that productive work is the process by which man's consciousness controls his existence, a constant process of acquiring knowlege and shaping matter to fit one's purpose, of translating an idea into physical form, of remaking the earth in the image of one's values -that *all* work is creative work if done by a thinking mind, and no work is creative work if done by a blank who repeats in uncritical stupor a routine he has learned from others-"

-Ayn Rand
Atlas Shrugged

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CHAPTER 1

INTRODUCTION

General overview

Control of transcription is the first step in gene expression, a cellular process which plays a significant role in coordination of the physiological status of an organism. Hormone-mediated gene regulation often involves the complex interactions of receptors, signal transduction systems, kinases, and transcription factors which propagate extracellular signals and result in concomitant alterations of transcription in the nuclei of affected cells. The various mechanisms by which transcription factors modulate gene transcription add further complexity to the scheme of gene regulation. It has been widely accepted that genes contain the information necessary for their own regulation and most of this information is present in the regions that are upstream from the coding portions.

The pro-opiomelanocortin (POMC) gene encodes the polypeptide precursor to several peptides that are of importance in regulation of neuroendocrine function. A single copy of the POMC gene is present in mammalian species per haploid genome (Lundblad and Roberts, 1988). The structure of the POMC gene is similar across species and it contains three exons and two introns. It is notable that the biologically active peptides produced by the POMC gene are all encoded by the third exon.

The POMC gene is expressed in numerous tissues including several regions of the brain, the gonads, the adrenal medulla, and the spleen. However, the most prevalent site of POMC gene expression is the pituitary where the precursor molecule is processed into adrenocorticotropic hormone (ACTH); ß-endorphin; and ß-lipotropin in the anterior lobe corticotrope; and α-melanocyte stimulating hormone; corticotropin-like intermediate lobe peptide; and acetyl-ß-endorphin in the intermediate lobe melanotrope (Eipper and Mains, 1980). Processing of POMC peptides occurs via a posttranslational mechanism.

Modulation of POMC gene expression occurs in response to a variety of stimuli. Hormones, releasing factors, and neurotransmitters influence the pattern of POMC transcription and thus reflect perturbations in the environment that elicit

physiological changes in this system. The most widely studied network involving the POMC gene is the response to stress. In a complex array of cellular and molecular mechanisms, the POMC gene product plays an integral role in coordinating this biological phenomenon. Hence a majority of the studies concerning regulation of POMC gene expression focus on the effects of stress-induced hormones such as glucocorticoids and corticotropin releasing factor.

Glucocorticoids and related steroid hormones exert effects upon developmental and homeostatic events. Steroid hormones impart their regulatory effects by binding to intracellular receptors that are able to modulate gene transcription. Steroid receptor-mediated gene regulation has been shown to occur through several mechanisms such as receptor-DNA interaction, synergism with other transcription factors, and inhibition of action of other transcription factors. The complex regulatory schemes that are governed by steroid hormones serve as an example of the many biological systems that are hormonally regulated.

My studies examined the interactions of two steroid hormones and other signal transduction systems in the regulation of POMC gene expression. Model cell culture systems were employed to facilitate the elucidation of molecular mechanisms involved in hormone-mediated POMC gene regulation. In particular, this endeavor focused on comparing the action of two steroid hormones known to effect POMC negative regulation *in vivo*.

Physiological regulation of POMC expression

The POMC gene is responsive to steroid feedback by glucocorticoids and estrogens in a receptor-mediated fashion in two different neuroendocrine systems.

Glucocorticoid regulation of POMC gene expression

Glucocorticoids have a significant role in regulation of the hypothalamic-pituitary-adrenal (HPA) axis in a classical negative feedback loop invoked during the stress response. In the anterior pituitary corticotrope, where the major product of the POMC gene is ACTH, glucocorticoids inhibit expression of the POMC gene. Both synthesis and secretion of ACTH are increased in response

to stressful stimuli due to the action of corticotropin releasing factor (CRF), which is released from the hypothalamus and reaches the anterior pituitary via the portal blood. ACTH secretion results in an increase in adrenal steroid production, which leads to glucocorticoid feedback inhibition of both synthesis and secretion of POMC gene products in the anterior pituitary (Sayers and Portanova, 1974; Dallman, 1972), as well as a reduction in hypothalamic CRF content (Jones, 1976; Buckingham, 1979).

Glucocorticoid inhibition of POMC biosynthesis has been studied in a number of biological systems including the rat, primary rat pituitary cultures, and the AtT20 mouse anterior pituitary-derived cell line. Studies performed *in vivo* have shown that glucocorticoid-mediated decreases in POMC synthesis are due to reductions in cytoplasmic mRNA levels (Nakamura, 1978; Roberts, 1979; Herbert, 1981; Schachter, 1982). Glucocorticoid inhibition of POMC mRNA requires a minimum of 6 hours for effects to be reflected in changes of mRNA content (Roberts, 1979), and these effects are independent of new protein synthesis (Roberts, 1982). The observed glucocorticoid-induced reductions in POMC mRNA levels have been demonstrated to be a result of transcriptional repression as measured by nuclear transcription run-on studies which examined the effects of steroid treatment in anterior pituitary cells of the rat. Eberwine and Roberts showed that treatment with dexamethasone (DEX), a synthetic glucocorticoid, in the intact rat, resulted in rapid (15 min) inhibition of POMC gene transcription that was 6- to 7-fold below control levels. Transcription remained inhibited at least 2- to 3-fold for 6 hours following DEX administration (Eberwine and Roberts, 1984).

Pioneer studies using the paradigm of adrenalectomy, a process which is known to reduce levels of glucocorticoids and thus increase ACTH release, resulted in a 3-fold increase in POMC mRNA as measured by cell-free translation and immunoprecipitation (Nakanshi, 1977). Adrenalectomy followed by glucocorticoid treatment for several days caused a reduction in pituitary POMC mRNA to levels that were comparable to those found in intact animals. Other groups examined the effects of adrenalectomy and glucocorticoid treatment using either hybridization with cDNA probes or *in situ* hybridization. These groups found that glucocorticoid-sensitive POMC gene expression is limited to the anterior lobe pituitary (Schachter, 1982; Jingami, 1985; Birnberg, 1983; Hollt and Haarmann 1984; Bruhn, 1984; Gee and Roberts 1983; Fremeau, 1986). Furthermore, *in situ* hybridization studies demonstrate that areas of silver grains,

which correspond to regions that have POMC mRNA that has hybridized to a POMC-specific probe, are altered by glucocorticoid replacement in adrenalectomized rats. These areas are limited to the anterior lobe corticotropes and not to the intermediate lobe pituitary (Gee and Roberts, 1983; Fremeau, 1986). Two separate *in situ* hybridization studies show that 10 day or 2 week adrenalectomy results in an increase in silver grain density in anterior lobe corticotropes, and that acute treatment (30 min to 2 hour) with dexamethasone did not significantly reduce the level of grain intensity (Fremeau, 1986). It can be concluded that levels of POMC mRNA change more slowly in response to glucocorticoid treatment than do levels of transcription.

Estrogen regulation of POMC gene expression

The initial observation that stress affects reproductive function was indicative of a role for hormones that are involved in the HPA axis in modulation of the hypothalamic-pituitary-gonadal (HPG) axis (Selye, 1939). It has been shown that prolonged stress, which activates the HPA axis, inhibits luteinizing hormone (LH) release and blocks ovulation (reviewed in Rivier and Rivest, 1991). A relationship between POMC-derived peptides and reproductive function exists. In the hypothalamus, POMC-containing neurons are involved in modulating the reproductive axis by directly innervating gonadotropin releasing hormone (GnRH) neurons, modulating the inhibitory tone. POMC gene expression is found in a subset of neurons in the arcuate nucleus of the hypothalamus (Mezey, 1985), a region that is known to project to the preoptic area where GnRH neurons are located (Leranth, 1988; Wilcox, 1986). These GnRH neurons receive synaptic input from ß-endorphin containing neurons (Chen, 1989). This activation of μ opiate receptors which has been demonstrated to be involved in physiological inhibition of gonadotropin secretion in several species (Cicero, 1979; Petraglia, 1986).

POMC gene expression in the hypothalamus is altered by fluctuations in estrogen levels such as those that occur during the estrous cycle (Weimann, 1989), resulting in an inhibition of estrogen-modulated POMC gene expression (Wilcox and Roberts, 1985; Chowen-Breed, 1989). Receptors for the sex steroids estrogen, progesterone, and testosterone, have been co-localized with POMC-derived peptides in the hypothalamus (Morrell, 1985). Furthermore,

estrogen receptors have not been detected in GnRH neurons (Shivers, 1983), implying that the effects of estrogen on GnRH release are mediated by some estrogen-sensitive system such as the POMC-derived opiate, ß-endorphin.

Measurements of POMC-derived peptides, mRNA, and transcription rates agree with the inhibitory effect of estrogen on POMC gene expression. Researchers found that levels of ß-endorphin in estrogen replaced ovariectomized rats were decreased, as were levels of POMC mRNA; as quantified by dot blot hybridization (Wilcox and Roberts, 1985). Reductions in arcuate nucleus POMC mRNA were determined to be 40% below those in control animals and they required 24 hours for maximal effects to be detected. Using the transcription run-on assay, a direct measurement of the rate of transcription, it was demonstrated that estrogen inhibited POMC transcription, in the periarcuate region of the hypothalamus, within 60 min to a level 60% of control (Roberts, 1985). Following solution hybridization and nuclease protection assay techniques, it was shown that in female rats ovariectomy with estrogen replacement, for 2 or 3 weeks, reduced POMC mRNA levels as compared to untreated animals (Treiser and Wardlaw, 1992). Interestingly, it was found that medial basal hypothalamic α-MSH and ß-endorphin content of the 3 week estrogen-replaced, but not the 2 week estrogen-replaced rats was significantly reduced (Treiser and Wardlaw, 1992). Similar results were found in male rats that were castrated and given testosterone for either 2 or 4 weeks. The two week treatment group had significant changes in POMC mRNA levels, but not in ß-endorphin content (Blum, 1989b). It can therefore be concluded that changes in POMC gene expression precede alterations in POMC peptide content in the hypothalamus. Moreover, it appears that there are both acute and long term inhibitory effects of estrogen on POMC gene expression *in vivo*. It has also been shown that there are positive effects of estrogen on POMC gene regulation which relate to the role of POMC gene expression in reproductive function. Estrogen-modulated fluctuations in POMC gene expression as measured by *in situ* hybridization were found to occur in the arcuate nucleus. Ovariectomized estrogen-replaced rats exhibit a diurnal rhythm in POMC mRNA levels at proestrous, which is similar in timing to the LH surge (Wise, 1990). Hence, while the major influence of estrogen on POMC gene expression in this system is an inhibitory one, positive regulation of the POMC gene by estrogen does occur.

Gene regulation by steroid hormones

Steroid hormones elicit their effects by binding to and activating nuclear receptors that are members of a family of transcription factors that have been studied extensively (Yamamoto, 1985; Evans, 1988; Beato, 1989; O'Malley, 1990). As modulators of gene expression, steroid receptors serve as signal transducers that acquire the ability to bind to specific cis-acting sequences of DNA within genes that are steroid-regulated. Members of the family of nuclear receptors are related by conservation of structure, as well as by the mechanistic similarity of transcriptional regulatory function. Furthermore, current theory based upon sequence analysis and comparison of evolutionary history argues that the nuclear receptors originated from a multi-domain precursor that was originally involved in signal transduction (Amero, 1992).

Consensus sequences

Binding sites for steroid receptors exist within the chromatin structure of hormonally responsive genes. These hormone responsive elements (HREs) are characterized as being cis-acting enhancer-like elements of DNA, typically located in the 5' flanking regions of genes that are steroid-responsive. HREs were localized using gene transfer techniques and DNA footprinting methodologies (reviewed in Yamamoto, 1985). Consensus nucleotide sequences to which steroid receptors bind, are usually 15 base pairs (bp) in length and they are fairly well conserved for each receptor type (Berg, 1989; Evans, 1988; Beato, 1989). HREs are divided into three separate classes dependent upon receptor type. One class includes binding sites for the glucocorticoid receptor (GR), progesterone receptor (PR), mineralocorticoid receptor (MR), and androgen receptor (AR). (von der Ahe, 1985; Cato, 1986; Dabre, 1986; Arriza, 1987; Cato, 1987; Strahle, 1987; Cato and Weinmann, 1988; Ham, 1988; Chalepakis, 1988; Slater, 1988). The estrogen receptor (ER), retinoic acid receptor (RAR), vitamin D receptor, and thyroid hormone receptor (TR) bind a similar consensus sequence that differs only in its spacing for these various nuclear receptors (Strahle, 1987; Klock, 1987). Lastly, an HRE exists for the v-erbA, chicken ovalbumin upstream promoter-transcription factor (COUP-

TF) and the *Drosophila melanogaster* gap gene "knirps"-related DNA binding proteins, all of which are orphan receptors, by virtue of their currently unidentified ligands. Although consensus binding sequences exist, several functional HREs deviate from their specified sequences and this has been shown to affect the efficiency by which they direct hormonal regulation (Klock, 1987; Martinez, 1987; Ham, 1988; Klein-Hitpass, 1988).

Steroid receptor-DNA interactions

The portion of steroid receptors that contact DNA assume a zinc finger structural motif that was first described for the *Xenopus laevis* 5S transcription factor, TFIIIA (Brown, 1985; Miller, 1985). A salient feature of nuclear receptor zinc fingers is the tetrahedral arrangement of four cysteine residues surrounding a zinc ion. This structure was confirmed by the use of extended X-ray fine absorption spectra (EXFAS) (Freedman, 1988; Severne, 1988). The DNA binding domain in steroid receptors possess two non-identical zinc fingers each of which has a different functional role. The proximal finger contacts the major groove of one half of the HRE and the distal finger lies on the the sugar phosphate backbone of the DNA (Chalepakis, 1988). Recently it was found using two dimensional NMR that the finger structures are two amphipathic α-helices that cross at right angles near their centers (reviewed in Schwabe and Rhodes, 1991). It has been proposed that the proximal most finger forms a DNA recognition helix that permits differentiation of an estrogen response element (ERE) from a glucocorticoid response element (GRE) (Schwabe and Rhodes, 1991).

The specificity of DNA binding sites used by the different steroid receptors is determined by various residues in the finger region. Initially, it was shown that the entire first finger was responsible for target HRE binding as exemplified by studies utilizing swapping of the fingers of the ER and GR and reporter test genes containing either an ERE or GRE (Green, 1988). Several laboratories later elucidated three residues, in particular: gly ^{25}, ser ^{26}, and val ^{29}, dictate the preferential binding to a GRE type motif, whereas glu, gly, and gly in these positions specify thyroid hormone response element (TRE) binding; and glu, gly, and ala indicate an ERE (Umesono and Evans, 1989; Danielson, 1989; Mader, 1989). More specifically, in DNA recognition sequences, there is some contact of

the GR to the thymidine in position 12 of the GRE; whereas the ERE contains an adenosine in the 12 position (Beato, 1989). It is possible that this difference in consensus sequences affects the specificity of target gene binding since it has been demonstrated that the human GR fails to activate an ERE reporter gene; and conversely, the human ER cannot induce a GRE reporter (Green and Chambon, 1987; Green, 1988; Umesono and Evans, 1989); however changing one or two bp in each half site of an ERE can convert its hormonal responsiveness to that of a GRE (Klock, 1987; Martinez, 1987).

Steroid receptors bind to DNA as dimers as demonstrated by DNase I and methylation protection footprinting experiments (Scheidereit and Beato, 1984; Scheidereit, 1986). Receptor dimers contact four turns of the DNA helix, with the conserved hexanucleotide motifs located centrally within the regions of the HRE. The palindromic dyad axis of symmetry of HREs permits binding of a single receptor to each half site. Contact is made between the receptor protein and specific bases in the HRE. Receptor dimerization has been demonstrated *in vivo* (Tsai, 1989) and *in vitro* (Tsai, 1988; Kumar and Chambon, 1988).

A role for HRE half sites as functional elements has recently been recognized. The ovalbumin gene contains several upstream half palindromic elements that confer estrogen inducibility upon either the homologous or heterologous promoter (Kato, 1992). These binding sites are widely spaced and thus they are thought to function synergistically within the context of the entire promoter. A requirement for a proximal binding site working in conjunction with the distal elements could be replaced by binding sites for other transcription factors. Evidence has been found that the proximal ERE half site is involved in phorbol ester induction of the ovalbumin gene (Gaub, 1990). This supports the notion that there are interactions between different transcription factors and DNA sequences, which mediate gene expression of complex promoters. Recent evidence shows that the TR and RAR can bind to half sites depending upon the orientation of the half site and space between them (Forman, 1992). This is not surprising in light of the fact that natural TREs and RAREs occur as two or more imperfect direct or inverted repeats.

Structure-function

The structure of steroid receptors is modular. The various domains correspond to regions of different functional significance. The carboxy terminal ligand binding domain differs in length (220-250 amino acids) and amino acid sequence for each of the receptor types. However, there is considerable conservation of this region despite the differential preference for ligands. The assignment of ligand binding to this particular region of the GR and ER proteins was resolved using mutational analysis of cloned receptor molecules expressed in cell lines which were free of endogenous receptors (Rusconi and Yamamoto, 1987; Giguere, 1986; Kumar, 1986). Hormone-dependent receptor dimerization function has been assigned to the ligand binding region of the ER (Kumar and Chambon, 1988).

It has been suggested that the ligand binding domain may impart an inhibitory action on receptor-mediated transcriptional activation. When the C-terminal region was removed from the GR, the receptor displayed constitutive gene activation (Hollenberg, 1987; Godowski, 1987). This phenomenon may be explained in two ways, firstly as a conformational alteration in the receptor protein such that the DNA binding domain is exposed; secondly as a release of the receptor molecule from proteins such as heat shock protein (hsp) 90 that may confer an inactive receptor.

Regions within the steroid hormone receptors that are necessary for transcriptional activation have been localized by several groups. The human GR possesses two trans-activation (tau) domains, one in the C-terminal portion of the receptor that forms an amphipathic α-helix and another in the N-terminal region (Hollenberg and Evans, 1988). The rat GR contains an activation region in the N-terminal portion of the receptor termed enh2 which is able to impart trans-activation on an heterologous LexA DNA binding domain (Godowski, 1988). A hormone-dependent trans-activation function (TAF-2) exists for the human ER and GR in the ligand binding domains (Webster, 1988). Additionally, a hormone-independent trans-activation function (TAF-1) was localized to the N-terminal portion of the human ER (Tora, 1989b). While assignment of these activation functions to particular regions of the nuclear receptors have been made, the mechanism of transcriptional activation is still not fully understood.

Negative regulation

Steroid receptor-dependent transcriptional activation has been the subject of numerous studies. Cell culture systems have been employed to measure the effects of steroids on expression of either transfected reporter test genes or endogenous hormonally regulated genes. It is known that the same receptors are able to mediate both positive and negative gene expression; however, it is not universally accepted that inhibition of gene expression occurs entirely by virtue of DNA binding.

Several models exist that offer explanations of negative regulation. One example is the steric interference of binding of a positive effector protein. This model is used to explain the glucocorticoid inhibition of the gene encoding the α-subunit of the glycoprotein hormones, luteinizing hormone and follicle stimulating hormone, which is positively regulated by the cAMP regulatory element binding (CREB) protein. DNA binding sites for the two different regulatory proteins overlap in a 52 bp region of the gene. This region does not function as a direct inactivator of transcriptional activity, there is the requirement for the induction of a CRE and a cAMP-responsive cell type to effect negative regulation (Akerblom, 1988). The mechanism of inhibition is thought to be interference of CREB protein binding imposed by GR binding. Regions of the GR that are thought to be involved in mediating this regulatory scheme are the DNA binding domain and the ligand binding domain, not the N-terminal portion of the GR. However, replacement of the C-terminal region of the receptor by a corresponding part of the mineralocorticoid receptor or the ß-galactosidase protein results in negative regulation of the glycoprotein hormone α-subunit gene (Oro, 1988). This observation may be due to the removal of the inhibitory ligand binding region which has been suggested to impart a transcription repression function (Hollenberg, 1987; Godowski, 1987).

The bovine prolactin gene is negatively regulated by glucocorticoids. This gene contains several negative GREs (nGREs) identified by footprint analysis. The nGREs function in concert with one another to inhibit transcription within the context of the whole promoter (Sakai, 1988). Interestingly, the nGREs are able to mediate positive regulation by glucocorticoids when placed in front of an heterologous promoter. The possibility exists that these binding sites may be used by other transcription factors which are not yet identified.

The mouse proliferin gene offers another example of a gene which is repressed by glucocorticoids and induced by another transcription factor, activator protein-1 (AP-1). There is a 31 bp region that has been shown to contain overlapping binding sites for the GR and AP-1. This juxtaposition of DNA elements was initially thought to imply a competition for the same cis-elements by the two transcription factors (Mordaq and Linzer, 1989). However, regulation of proliferin gene expression appears to be more complex than originally thought, because of the discovery of interaction between GR and AP-1 proteins (discussed later).

Another method of gene repression employed by steroid receptors requires occupation of a DNA binding site by an unactivated nuclear receptor. This occurs with the non-ligand bound TR and genes that are induced by thyroid hormone and retinoic acid. In this instance, the ligand-free receptor occupies DNA sequences normally bound by the hormonally induced receptor (Damm, 1989). The interference of activated receptor binding by an unactivated receptor may be a means of acutely regulating the hormonal responsiveness of the cell.

Interaction of steroid receptors with other transcription factors

Members of the steroid-thyroid family of receptors have been found to associate with other proteins that function to modulate transcription. Gene transfer experiments demonstrated interaction between the GRE/PRE of the mouse mammary tumor virus (MMTV) and binding sites for Sp1, NF1, CaCCC factor, and OTF transcription factors (Schule, 1988a; Schule, 1988b). The result of these interactions varies depending upon the position and strength of the individual binding sites. However, it is thought that interaction of multiple factors aids in establishing and stabilizing a productive transcription complex (Maniatis, 1987; Lewin, 1990; Lillie, 1988; Mitchell and Tjian, 1989).

Recently several laboratories have described interactions between the glucocorticoid receptor and the AP-1 (Fos/Jun) protein. AP-1 is a nuclear transcription factor that was originally identified as mediating gene induction by phorbol esters, growth factors, and inflammatory agents (Angel, 1987a; Angel, 1987b; Brenner, 1989; Kerr, 1988). The AP-1 protein complex is comprised of the oncoproteins c-Fos and c-Jun which exist as either heterodimers or Jun-Jun homodimers. This complex belongs to a family of transcriptional regulatory

proteins containing the leucine zipper structural motif (Bohmann, 1987; Landschultz, 1988; Angel, 1988; Franza, 1988).

The mouse proliferin gene contains a 25 bp region to which the GR binds. This region confers either positive or negative gene regulation depending upon the cellular milieu of transcription factors. The proliferin GRE is inactive in the absence of c-Jun, is positively regulated in its presence. When c-Jun and a high level of c-Fos are present there is negative glucocorticoid regulation of reporter gene expression (Diamond, 1990). The relative amount of the regulatory proteins determines the transcriptional effect. This mode of gene regulation may represent a squelching phenomenon which has been previously described for several other systems (Gill and Ptashne, 1988).

The collagenase gene is also positively regulated by phorbol esters and negatively regulated by glucocorticoids which repress phorbol ester-mediated induction (Yang-Yen, 1990). In this system it has been shown that there is mutual repression of each of these activities by the GR and AP-1. Furthermore, direct interaction between these two protein complexes has been demonstrated by protein cross-linking and immunoprecipitation. There is additional evidence that the antagonism between the GR and AP-1 does not require DNA binding (Schule, 1990). Analysis using mutant proteins demonstrated that the ligand binding domain and the DNA binding domain of the GR as well as the leucine zipper region of the AP-1 protein are essential for trans-repression. Another group found that the GR was able to inhibit repression of the serum response element by the Fos protein and that this activity was also dependent upon the presence of the DNA binding domain of the GR (Lucibello, 1990). Specificity of the oncoprotein component is exemplified by the discovery that repression of GRE reporter gene activity requires a region of the c-Fos protein that is not found in the FosB or Fos related antigen (Fra-1) oncoproteins.

The ER and AP-1 proteins have similarly been shown to interact to influence gene regulation. The ovalbumin gene contains an ERE that overlaps with a binding site for AP-1. As demonstrated in gene transfer experiments, Fos and Jun (AP-1), and the ER promote increased gene activity. However, there is not a requirement for the DNA binding domain of the ER for these results (Gaub, 1990). Another group investigated the effects of c-Fos and/or c-Jun on GR, PR, or AR-mediated reporter gene activation. They found that there was cell type, receptor type, and promoter type specificity of regulation (Shemshedini, 1991). Furthermore, it was shown that inhibition of steroid receptor trans-activation was

influenced by the presence of the oncoproteins, while the converse was not observed. Interaction between AP-1 and the ER *in vitro*, has been described as interference at the level of DNA binding (Tzukerman, 1991). Interestingly, while TPA or AP-1 proteins were able to inhibit ER activity in several cell lines, the converse was not observed.

cAMP regulation of gene expression

Gene regulation that results from increased levels of cAMP has been shown to be mediated by transcription factors that are members of the leucine zipper containing dimeric protein complexes (reviewed in Ziff, 1990; Struhl, 1989; Landschultz, 1988). Members of this gene family have been characterized by various laboratories and the group includes activator transcription factor (ATF) proteins, the Fos and Jun oncoproteins, C/EBP, and CREB proteins. Some members of the family are as much as 80% homologous. A consensus DNA sequence, the cAMP regulatory element (CRE), was identified as the DNA motif that was necessary for transcriptional regulation by cAMP (Lin and Green, 1988). This element is the one to which the cAMP regulatory element binding (CREB) protein binds (Montminy and Bilezikjian, 1987). It is notable that a consensus CRE differs from a consensus TRE by only a single base addition in the CRE.

Phosphorylation of the CREB protein has been shown to regulate transcription activation function of CREB. The negatively charged domain of CREB contains potential phosphorylation sites for PKA, PKC, casein kinase II, and glycogen synthase kinase III and is termed a P box (Hoeffler and Habener, 1990). Calcium-mediated transcriptional regulation has been demonstrated to occur through calcium calmodulin (CaM) kinase-mediated phosphorylation of the CREB protein in PC12 pheochromocytoma cells (Sheng, 1991) and *in vitro* (Dash, 1991). Furthermore, phosphorylation of CREB induces dimer formation and engenders a complex that has a 10-fold greater affinity for the somatostatin CRE (Goodman, 1990).

Regulation of POMC gene expression in the AtT20 model system

The study of the regulation of POMC gene expression has been aided by the use of the AtT20 mouse anterior pituitary-derived cell line. This POMC-expressing cell line represents most closely the corticotrope cells of the anterior pituitary because it possesses receptors for both glucocorticoids and CRF, however as an established cell line it offers a homogeneous population of cells in which transcriptional fluxes can be readily measured. Several laboratories have utilized AtT20 cells to study both regulation of the endogenous POMC gene, as well as regulation of exogenous reporter genes. Although most hormones that transcriptionally regulate POMC gene expression also affect secretion of POMC-derived peptides, this review will be limited to hormonally modulated effects on gene transcription.

CRF regulation

Similar to the effects observed *in vivo*, CRF is a positive regulator of POMC gene expression in the AtT20 cells. The effects of CRF on POMC gene expression can be detected in several parameters, such as mRNA, heteronuclear RNA, and primary transcript levels, and transcription rate. The accumulation of POMC mRNA has been reported in response to CRF treatment in the AtT20 cells (Reisine, 1985; Affolter and Reisine, 1985). CRF maximally stimulated levels of POMC mRNA 2- to 3-fold after 8 hours of persistent treatment and levels remained elevated for a total of 24 hours (Affolter and Reisine, 1985). Increased levels of POMC mRNA reflect changes induced by an increased transcription rate. It has been shown that CRF causes a rapid stimulation in transcription of the POMC gene in AtT20 cells as measured by nuclear run-on assay (Roberts, 1987; Lorang, 1992b). Maximal transcriptional stimulation was observed to occur subsequent to 60 min CRF treatment.

CRF treatment also results in increases in levels of POMC hnRNA and primary transcript. The POMC primary transcript contains two introns that are rapidly spliced to result in a mature mRNA species. The order of intron splicing was determined using intron-exon junctional probes and solution hybridization techniques in the AtT20 cells and in primary cultures of anterior pituitary cells. It was found that intron B is spliced out first, followed by the loss of intron A (Levin,

1989; Lorang, 1992b). Because of the rapid processing of POMC primary transcript and heteronuclear RNA into mRNA, the rate of gene transcription is thought to be accurately reflected by levels of primary transcript and heteronuclear RNA. Studies have shown that this is indeed true in the AtT20 cell line for various hormonal and pharmacological treatments (Lorang, 1992b) and in primary cultures with CRF treatment (Levin, 1989). Furthermore, studies in rats, using adrenalectomy and glucocorticoid replacement paradigms prove that this phenomenon exists *in vivo* and thus represents the physiological mode of POMC gene processing (Autelitano, 1989).

Glucocorticoid regulation

Glucocorticoids elicit an inhibitory effect upon POMC gene expression that is reflected both by transcription rate and mRNA levels in AtT20 cells. The effects of glucocorticoid treatment are mediated by the type II glucocorticoid receptor in AtT20 cells because it has been shown that the cell line contains this type of receptor and not the type I GR (Gannon, 1990). Direct transcriptional repression by glucocorticoids has been demonstrated using the synthetic glucocorticoid, dexamethasone (DEX), which exerts maximal 2- to 4-fold inhibition within 15 to 20 min (Roberts, 1987; Lorang, 1992b). Moreover, direct transcriptional inhibition of POMC has been shown to occur in primary cultures of anterior pituitary (Eberwine, 1987; Gagner and Drouin, 1987) as well as *in vivo* (Eberwine and Roberts, 1984). It has been shown that in the AtT20 cell line acute DEX treatment resulted in significant time-dependent decreased levels of heteronuclear RNA and primary transcript which occurred at 45 min and were maximal at 60 min (Lorang, 1992b).

Long term effects of glucocorticoid treatment in AtT20 cells are reflected by decreased POMC mRNA levels that are first detected subsequent to 10 hour treatment and are maximally reduced to 30-40% of control culture levels following 48 hours of treatment (Roberts, 1979; Nakamura, 1978). Similar suppression of POMC mRNA levels is found in primary cultures (Eberwine, 1987), however the magnitude of *in vitro* inhibition is considerably less than that observed *in vivo* .

Interaction between CRF and glucococorticoids

Another means of glucocorticoid-mediated inhibition of POMC gene expression is attenuation of CRF induction by glucocorticoids. Studies using primary cultures showed that acute DEX pretreatment followed by cotreatment with CRF and DEX resulted in levels of POMC transcription that were reduced as compared to those of CRF treated cells alone (Eberwine, 1987; Gagner and Drouin, 1985; Gagner and Drouin, 1987). Utilizing AtT20 cells, it was demonstrated that POMC heteronuclear RNA levels were reduced as compared to CRF treated cells when pretreated for 15 min with DEX and subsequently cotreated with both CRF and DEX (Lorang, 1992b). Interestingly, there appears to be a temporal hierarchy of hormonal regulation with these two hormones. When primary cultures were treated with CRF for a time period that was maximally stimulatory, subsequent cotreatment with CRF and DEX did not result in attenuation of CRF-induced stimulation (Eberwine, 1987). Furthermore, the same regulatory pattern is observed in AtT20 cells (Lorang, 1992b). Although the mechanism of this regulation pattern is not understood, it is possible that there is interaction between transcription factors such as c-Fos, which is elevated by CRF (Boutillier, 1991), and the GR that are responsible for mediating these hormonal and second messenger effects. The complex nature of the POMC promoter is likely to be a confounding factor in this regulatory scheme.

Estrogen regulation

Regulation of POMC gene expression by estrogen (E2) has been examined in preliminary studies using a derivative of the AtT20 cell line, ER-1, that has been stably transfected with an expression vector for a human ER and in the CV-1 monkey kidney-derived cell line (Lundblad, 1992). When ER-1 cells were treated with E2 for 18 hours there was a reduction in POMC mRNA levels as compared to untreated cells. Similarly, a reduction in POMC-ß-globin reporter gene activity was observed in CV-1 cells that were transiently transfected with a POMC reporter gene and treated with E2 for 16 hours. However the studies in the CV-1 cells employed a reporter gene that contained a downstream SV40 enhancer in order to achieve adequate basal POMC expression. These studies demonstrate that the effects of E2 treatment are indeed transcriptional and that

they require only a region of the POMC promoter which spans from -704 to +63 of the rat gene.

cAMP regulation

In addition to the effects of CRF and glucocorticoids, other agents are able to elicit transcriptional regulation of POMC expression. It is now understood that the effects of CRF are mediated in part by cAMP. It has been shown that CRF activates adenylate cyclase via the CRF receptor and presumably its coupling to a Gs G-protein effector, leading to a rise in levels of intracellular cAMP (Mizayaki, 1984). This implies involvement of protein kinase A (PKA) in a signal transduction pathway which regulates POMC gene expression. In AtT20 cells treatment with 8-bromo-cAMP, forskolin, and isoproterenol, agents that result in elevated cAMP levels, produce an increase in POMC mRNA levels that is comparable to increases caused by CRF treatment (Affolter and Reisine, 1985). When a PKA inhibitor was administered to AtT20 cells, the action of cAMP activating agents was blocked (Reisine, 1985) thus implying the involvement of PKA in the stimulatory action of cAMP. Furthermore, 8-bromo-cAMP and forskolin similarly induced POMC primary transcript and heteronuclear RNA levels, respectively in AtT20 cells (Lorang, 1992b).

Calcium regulation

Intracellular calcium has been implicated in the modulation of POMC gene expression. It has been demonstrated that the calcium ionophore A23187 can increase POMC mRNA levels (Loeffler, 1986; Dave, 1987). The effects of raising intracellular calcium levels is also reflected by increased POMC transcription both in anterior pituitary cells in culture (Eberwine, 1987) and in AtT20 cells (Lorang, 1992a). CRF-induced increases in POMC mRNA levels are repressed by pretreatment with voltage dependent calcium channel blockers, thus implying a role for entry of extracellular calcium in CRF stimulation (von Dreden, 1988). The mechanism by which calcium participates in CRF-stimulated POMC gene expression is not yet clear. However, it has recently been demonstrated that CRF induces expression of the c-fos gene in a calcium-dependent manner

involving activation of CaM kinase, and overexpression of the c-Fos protein results in elevation of POMC gene expression (Boutillier, 1991).

POMC promoter elements

The regulatory elements of the POMC gene have been the subject of several investigations. Studies have focused on elements responsible for basal, CRF-stimulated, and glucocorticoid-repressed gene expression and identification of the transcription factors that bind to these particular elements.

Tissue specific and basal elements

Early gene transfer studies in the AtT20 cell line using neomycin to select cells expressing a rat POMC promoter construct fused to the gene for neomycin resistance showed that promoter elements which spanned 4.8 kilobases (kb), 706bp, or 480bp of the 5' flanking region all yielded the same amount of promoter function (Drouin, 1987). Similar results were found when -704 to -38 of the rat POMC promoter was fused to the herpes simplex virus (HSV) thymidine kinase (tk) minimal promoter and this construct was transiently expressed in AtT20 cells (Roberts, 1987). Furthermore, the sequences from -706 to +63 were deemed necessary to direct tissue specific expression of the POMC gene in pituitaries of transgenic mice (Trembly, 1988) and gonadotropin regulated expression of the transgene in granulosa cells in primary culture (Young, 1989). More recently, three functional regions of the rat POMC promoter were delineated. These elements correspond to sequences -480 to -323 (distal), -323 to -166 (central), and -166 to -34 (proximal) of the rat gene and they appear to require the presence of all regions for full promoter activity (Therrien and Drouin, 1991). It was notable that both the central and proximal elements could maintain basal promoter expression, but the distal element could not. In contrast to these results, another group found that deletion of the region between -480 and -320 resulted in a significant loss in basal promoter activity (Reigel, 1991). Additionally, they showed that further 5' deletion of the promoter reduced reporter gene activity to greater extent. It must be noted that although within these

elements there are several identified binding sites for transcription factors, they may or may not play a role in mediating POMC gene regulation. For example, although a binding site for the COUP-TF protein exists within the aforementioned proximal element, destruction of this binding site by mutagenesis did not reduce basal promoter activity (Therrien and Drouin, 1991).

CRF-responsive region

Mapping the elements of the POMC gene that are responsible for mediating CRF-inducibility has been a fairly new endeavor. A region of the POMC promoter that participates in CRF induction was identified by transient transfection of POMC-chloramphenicol acetyltransferase (CAT) reporter genes into AtT20 cells (Roberts, 1987). Recent studies have delineated two regions of the rat POMC 5' sequences that are essential for CRF- and forskolin-stimulated and basal reporter activity as assessed by deletion mutants. These regions lie between -323 to -134 and -477 to -323. More specifically, the region from -236 to -133 was able to confer CRF-inducibility on a tk promoter construct which exhibited low basal expression (Lorang, 1991). Furthermore, DNase footprinting demonstrated that this CRF/cAMP responsive region harbors binding sites for several proteins (Lorang, 1991; Roberts, 1992). Several CRE-like elements exist in the POMC promoter, however none of these sequences exactly matches a consensus CRE (Young, 1989). Other potential targets for CRF and cAMP regulated POMC expression exist and one likely candidate is the previously identified AP-1 site that is present in exon 1 at +40 to+50 (Therrien and Drouin, 1991). It is possible that this site may be involved in the CRF-induced c-Fos-mediated stimulation of POMC gene expression described recently (Boutillier, 1991).

Glucocorticoid responsive elements

Regions of the POMC promoter that are responsible for glucocorticoid negative regulation have been identified by two groups. Binding sites for the GR were located at six sites in the rat gene; three in the 5' region centered about -579, -146, and -63 one at +64, and two within intron A (Drouin, 1987). Only one

site, the nGRE from -75 to -51, appears to be important in terms of glucocorticoid regulation. This consensus sequence, however, only loosely matches other GREs, including the previously identified bovine prolactin nGREs (Sakai, 1988). Nevertheless, it was shown that mutagenesis of this binding site which resulted in abolition of GR binding, also diminished glucocorticoid repression of promoter activity (Drouin, 1989). Thus, DNA binding of the GR to the POMC promoter was found to be essential for mediating negative regulation. However, conflicting results were found by another group when they examined the POMC region from -77 to-51. They showed that this region was required for basal gene regulation as well as for transcriptional repression by glucocorticoids *in vivo* (Reigel, 1991). The major conflict was that mutation of the -77 to -51 area resulted in decreased basal gene expression, but still remained glucocorticoid repressible. Thus, the functional significance of the nGRE centered at -63 in the POMC promoter remains unclear. It does, however, appear that the negative elements function in concert with other regions of the POMC gene that also mediate basal and regulated gene expression.

Summary

Our understanding of the complex mechanisms that govern regulation of gene expression has increased with the discovery of the multiple interactions between different classes of transcription factors. It has become apparent that these interactions occur in a tissue-, gene-, and effector-specific manner. This may serve to coordinate the response of a cell or an organism to perturbations in its environment.

CHAPTER 2

EXPERIMENTAL METHODS

CELL CULTURE

ER-1 (Lundblad, 1992) and AtT20$_{D16-16}$ (Sabol, 1980) cells were maintained in Dulbecco's Modified Eagle (DME) medium (Gibco) supplemented with fetal calf serum (5%), horse serum (10%) (JR Scientific), and 2mM L-glutamine (Gibco) in a 10% CO_2 humidified environment. Cells were passaged at weekly intervals. Cells were thawed after 15 passages from frozen stocks (stored at -80° C) of low passage number. Approximately four days before their intended harvest, cells were plated at an initial density of 1.5x 10^6 cells/ml such that at 80% confluence cells reached a density of 5x 10^6 cells/ml. For experiments on 17-ß-estradiol regulation, cells were washed and fed phenol red-free DME to minimize the potential estrogenic effects of the phenol red pH indicator prior to the onset of the experiment (Berthois, 1986; Nunez, 1987). Media was supplemented with steroid stripped sera to eliminate endogenous steroids (dextran-charcoal method; Horwitz and McGuire, 1978).

NUCLEAR STEROID BINDING ASSAY

A nuclear steroid binding assay was performed essentially as described previously (Spelsberg, 1987). ER-1 cells grown in monolayer culture were harvested and resuspended in phenol red-free DME supplemented with stripped serum. Equal volumes of cell suspension (0.8ml) were incubated with varying concentrations of [^{3}H]-17-ß-estradiol (New England Nuclear; 114 Ci/mmole) in a total volume of 1ml for 1 hour at 25° C. Excess unlabelled estradiol (100-fold) was used to define non-specific binding. The binding reaction was terminated by the addition of a 2-fold excess of DME and homogenization of the cell suspension in a Dounce glass/Teflon homogenizer in 1M sucrose, 10% glycerol, 2% Triton-X-100, 10mM KCl, 50mM Tris, pH 7.4. Nuclei were isolated by sucrose gradient centrifugation at 6000x g followed by vacuum filtration through nitrocellulose filters (Schleicher and Schuell; BA 85) in a buffer consisting of

50mM Tris pH 7.4, 10% glycerol. Filters were washed with excess buffer to remove unbound steroid and then oven dried. Radioactivity (dpm) was determined by counting in a Beckman LS counter.

HORMONE TREATMENTS

Dexamethasone (DEX; Sigma) and 17-ß-estradiol (E2; Sigma) were prepared as 10mM stocks in 100% ethanol and stored at -20° C. CRH (Peninsula Laboratory, Inc.) was stored as a 10µM stock in 10mM HCl and 1% ascorbic acid at -80° C. For the time course experiments, cells were treated with E2, DEX, or CRF that was diluted in DME such that the final concentration was 100nM (E2 and DEX), or 10nM (CRF) in a volume that was 1% of the total culture volume. Cells were treated for the indicated times and then harvested as described below. Dose response studies were performed with 20 hr treatment of increasing concentrations (log or half log increments) of E2 from 1nM to 1µM.

ISOLATION OF RNA

Cells were harvested and RNA was isolated by previously described methods (Blum, 1989a). Cells were lysed in cold 0.3 M sucrose AT buffer (0.3M sucrose, 10mM Tris-Cl pH 8.0, 3mM $CaCl_2$, 2mM $MgCl_2$, 0.5mM DTT, 0.15% Triton-X-100) and nuclear and cytoplasmic fractions were separated by centrifugation through a sucrose gradient which resulted in the separation of nuclear and cytoplasmic fractions. The supernatant containing the cytoplasmic portion was treated with 5µl proteinase K (10 mg/ml, Boehringer Mannheim) and 50µl 10x SET (10%SDS, 50mM EDTA, 100mM Tris-Cl, pH 8.0) for 60 min at 45° C. Nuclear pellets were first washed in 500µl 0.4 M sucrose AT buffer in order to ensure no cytoplasmic contamination and then incubated with 300µl of DNase buffer (50mM Tris-Cl, pH 8.0, 5mM $MgCl_2$, 1mM DTT, 0.1mM EDTA) and 1µl DNase I (20 U/µl, Worthington) for 10 min at 37° C. This was followed by the addition of 300µl 1x SET and 3µl proteinase K and incubation of the samples at 45° C for 30 min. Both the nuclear and cytoplasmic samples were subject to extraction with phenol:chloroform. Nuclear samples were divided into 3 equal aliquots (~200µl) and one aliquot was used to determine DNA content (Burton,

1956). The remaining samples, both nuclear and cytoplasmic, were precipitated with 2.5 volumes of 100% ethanol.

DNA QUANTITATION

DNA quantitation was performed essentially as previously described (Burton, 1956). Nuclear samples were incubated with 200µl 2N perchloric acid for 30 min at 68° C. Samples were cooled to room temperature, mixed with 400µl of chromogenic agent (1.5% diphenylamine w/v, 97% glacial acetic acid v/v, 1.5% sulfuric acid v/v 16mg/ml acetaldehyde), and incubated at 25° C for 6-8 hours. DNA content was determined by measuring the absorbance at 595nm in a Beckman spectrophotometer and comparing the sample values to a standard curve generated with known quantities of sonicated salmon sperm DNA (Sigma).

SOLUTION HYBRIDIZATION AND NUCLEASE PROTECTION ASSAY

RNA from either cytoplasmic or nuclear fractions was prepared as described above. Cytoplasmic RNA was resuspended in diethylpyrocarbonate (DEP; Sigma) treated H_2O and aliquots were quantitated spectrophotometrically. A 1µg equivalent of RNA was added per reaction along with 20µl hybridization buffer (80% formamide, 40 mM PIPES pH 6.7, 400 mM NaCl, 1 mM EDTA), and 1ng [^{32}P]-UTP (NEN; 800 Ci/mmole) radiolabelled antisense RNA probe. Samples were heated to 85° C for 5 min and then incubated at 45° C for 16 hours. Nuclear RNA samples were resuspended in 20µl hybridization buffer and incubated with 1ng [^{32}P] radiolabelled probe at 45° C as described above for the cytoplasmic RNA. Excess unhybridized RNA was eliminated by digestion with RNase buffer (10 mM Tris pH7.5, 5 mM EDTA, 300 mM NaCl, 40 µg/ml RNase A (Sigma), 2 µg/ml RNase T1 (Sigma)). The digestion reaction was stopped by the addition of 20µl 10% SDS and 5µl proteinase K (10 mg/ml) followed by extraction with phenol:chloroform. RNA was precipitated with 2 volumes 100% ethanol and 2 µg yeast RNA. Double stranded RNA was resolved on 6% polyacrylamide gels. Bands corresponding to the correct size of duplex RNA were excised from dried gels and counted using Opti-flour scintillant in a Beckman LS counter.

The amount of specific RNA protected in an assay was determined by comparison to a standard curve generated by hybridizing from 1.25 to 250pg of *in vitro* synthesized (+) RNA that corresponds to the sequences in the pMEX3-KS probe. The (+) RNA was synthesized using the T7 promoter and T7 RNA polymerase (Promega) from a Hind III linearized pMEX3-KS plasmid, in a reaction with unlabelled ribonucleotide triphosphates. Hybridization conditions and subsequent processing of the standard curve samples was identical to the methods described for the isolated RNA samples. The cpm were converted to picogram amounts of RNA by linear regression analysis. Quantities of nuclear RNA protected were normalized to DNA content in equivalent sample volumes as determined by a diphenylamine colorimetric assay described above (Burton, 1956).

STATISTICAL ANALYSIS

Statistical analysis was performed using a one factor analysis of variance (ANOVA). Significance ($p< 0.05$) was determined using a Fisher PLSD test. Data points represent the mean ± SEM of triplicate samples unless noted otherwise.

RNA PROBES

The probe pMEX3-KS spans a 500 base portion of a genomic mouse POMC clone (Notake, 1983) that encompasses 338 bases of exon 3 and 162 bases of intron B, including the intron-exon junction. Use of this probe allowed the distinction between primary transcript and mature mRNA in the nucleus as well as detection of mRNA in cytoplasmic samples. The POMC sequence is contained within the pGEM-4 vector (Promega) as a Kpn I-Stu I fragment. To generate an antisense probe, pMEX-3 was linearized with Ava I and ^{32}P-labelled RNA was synthesized as described previously (Blum, 1989a) using 5 units of Sp6 RNA polymerase (Promega). The resulting probe had a specific activity of approximately 5x 10^8 cpm/µg when transcribed using α-[^{32}P]-UTP (NEN; 800 Ci/mmole) mixed with unlabelled 12.5µM UTP and unlabelled ribo ATP, CTP, and GTP (Promega).

The c-fos probe, mcfos4, encompasses a 284 bp portion of a mouse genomic clone (NIH; Van Beveren, 1983) that is inserted as an Eco RI-Hind III fragment into the plasmid pBluescript KS(+) (Stratagene). To generate an antisense RNA probe, an Eco RI linearized plasmid was used as template in a reaction using 150μCi [^{32}P]-UTP (NEN; 800 Ci/mmole), unlabelled ribonucleotide triphosphates, and 5U T3 RNA polymerase as per manufacture's instructions. The resulting probe had a specific activity of approximately 2.6x 10^9 cpm/μg.

CONSTRUCTION OF POMC REPORTER PLASMIDS

Oligonucleotides corresponding to portions of the rat POMC promoter from -180 to -150, -175 to -157, and to the latter region with an insert of 5bp, -175 to -157 (+5) were synthesized (fig 5-2A) with Sal I restriction endonuclease ends. The oligonucleotides were cloned in front of a HSV tk minimal promoter, which was upstream from sequences encoding the CAT protein using a unique Sal I restriction site in the multiple cloning region of the pUSCAT plasmid (fig 5-2B). Positive clones containing the oligonucleotide sequences were identified by colony hybridization screening using ^{32}P-labelled oligonucleotides corresponding to the inserted POMC regions. Dideoxy DNA sequencing was employed to confirm the positive clones and to determine the orientation of the inserts. Sequence analysis showed that the oligonucleotides -175 to -157 and -175 to -157 (+5) inserted in the opposite orientation and the -180 to -150 oligonucleotide was in the correct 5' to 3' direction. The resulting POMC reporter genes were named -180/-150P-tk-CAT, -175/-157P-tk-CAT, and -175/-157 (+5)P-tk-CAT.

TRANSFECTIONS AND CAT ASSAY

AtT20 cells, grown in 6-well plates, were transfected with 5μg/well of the POMC reporter genes, -180/-150P-tk-CAT, -175/-157P-tk-CAT, -175/-157(+5)P-tk-CAT, or pUSCAT using a lipopolyamine reagent (Gibco/BRL; Behr, 1989) in a 2:1 Lipofectin:DNA ratio. Cells were fed serum-free DME for 2 hours prior to transfection which was timed for ~50-70% cell confluence. Following an 8 hour incubation period, the transfection solution was removed. Cells were fed with serum-free DME and treated with 10nM CRF for 16 hours.

Following transfection, cells were washed in 1x PBS and isolated by centrifugation. The cell pellet was resuspended in 0.25M Tris pH 7.8 and whole cell extracts were prepared by three cycles of freeze-thaw lysis as described previously (Sambrook, 1989). Endogenous deacetylase activity present in whole cell extract (Sambrook, 1989; Nordeen, 1987) was inactivated by 10 min incubation at 65° C. CAT activity was measured using the [^{14}C]-chloramphenicol (NEN; 40 Ci/mmole)/TLC method (Gorman, 1985). CAT activity was normalized to protein content in each extract tested as measured spectrophotometrically using a standard colorimetric assay (Biorad; Bradford, 1976) and comparison of sample values to a standard curve generated using known quantities of bovine serum albumin.

NUCLEAR PROTEIN EXTRACT

AtT20 were used as the source of nuclear protein extract for gel mobility shift assays. AtT20 cells were grown in monolayer culture until confluence. Two hours prior to treatment cells were washed and fed serum-free DME and then they were treated with 10nM CRF for 30 min or left untreated (control). Cells were harvested in 1x PBS and nuclear protein was isolated essentially as described previously (Dignam, 1990). Briefly, cells were resuspended in 5 packed volumes of 1x PBS and centrifuged at 2000 rpm at 4° C for 10 min. Cells were next allowed to swell on ice in Buffer A (10mM HEPES pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.5mM PMSF) for 15 min, centrifuged, and homogenized using a Dounce all glass homogenizer and pestle B. The homogenate was centrifuged at 2000 rpm and the resulting nuclear pellet was centrifuged at 25000x g for 20 min to yield a crude nuclear extract. The crude extract was further purified by homogenization followed by centrifugation and then the samples were dialyzed in Buffer D (20mM HEPES pH 7.9, 20% glycerol, 100mM KCl, 0.2mM EDTA, 0.5mM DTT, 0.5mM PMSF). Protein concentrations were determined using a standard colorimetric assay (Biorad; Bradford, 1976). The resulting extracts were stored in 50µl aliquots at -70° C.

OLIGONUCLEOTIDE PROBES

Single stranded oligonucleotide DNA corresponding to the region of the rat POMC gene between -175 and -157 (5'-TCGACCTGCTGTGCGCGCAGCCCG-3'), was synthesized such that it possessed Sal I restriction endonuclease ends. The palindromic element identified in the rat POMC promoter area is underlined. An oligonucleotide (5'-TCGACATGAGTCAGCCGTTATGAGTCAGG-3') whose sequence contains two copies of the phorbol ester (TPA) responsive element (TRE) consensus was also synthesized. Complementary oligonucleotides were annealed by incubation at 85° C for 10 min followed by cooling to room temperature over a 30 min period of time. Oligonucleotides were labelled using α-[^{32}P] (NEN; 3000Ci/mmole) dATP, dCTP, dGTP, TTP and the Klenow fragment (NE Biolabs) of DNA polymerase such that the Sal I overhangs were filled in. Unincorporated ^{32}P nucleotides were separated from the labelled oligonucleotides using a Nuctrap push column (Stratagene).

GEL SHIFT ANALYSIS

Protein extract (8μg) from control and CRF-treated AtT20 cells was incubated with 2μg poly dI-dC (non-specific DNA) for 15 min at 25° C. Binding was initiated by the addition of 1ng ^{32}P-labelled oligonucleotide probe for 15 min at 25° C. Excess (100-fold) unlabelled oligonucleotide was added to define non-specific binding. The resulting complexes were resolved on a 5% polyacrylamide gel in low ionic strength buffer (0.5x TBE) and run at 200 V for 3 hours at 4° C. The dried gel was exposed to X-ray film for 16 hours with a screen.

CHAPTER 3

ACUTE AND LONG TERM ESTROGEN REGULATION OF POMC GENE EXPRESSION

Characterization of ER-1 cells

A model POMC-expressing cell culture system derived from hypothalamic neurons does not exist . Thus, ER-1 cells (Lundblad, 1992), a derivative of the AtT20 $_{D16\text{-}16}$ mouse anterior pituitary cell line, (Sabol, 1980) were employed for the study of estrogen regulation of an endogenously expressed POMC gene. ER-1 cells were created by stable transfection using the calcium phosphate precipitate method (van der Eb, 1980) of an expression vector pMThERGH, (fig 3-1) encoding a human estrogen receptor (Greene, 1986) into AtT20 cells. ER-1 cells produce full length ER mRNA when compared to MCF-7 breast carcinoma cells; they express functional ER protein capable of nuclear steroid binding (Lundblad, 1992). The cDNA encoding the ER in pMThERGH contains a previously identified point mutation that results in an amino acid substitution (gly^{400} to val^{400}) in the ligand binding region (Tora, 1989a). The mutation reduces the Kd of the receptor for its ligand, 17-ß-estradiol, from ~0.14nM to ~0.5nM at 25° C as demonstrated by *in vitro* binding studies performed with cells that were transfected with either the wild type or mutant ER, respectively. The effects of this mutation on binding at 37° C were not investigated. Preliminary data, from this laboratory (Lundblad, 1992), characterizing the ER-1 cells suggested that the endogenous mouse POMC gene is negatively regulated by estrogen.

Prior to studies characterizing estrogen-mediated regulation of POMC gene expression, ER-1 cells which had been frozen and subsequently thawed were assessed for the presence of estrogen receptors that were able to exhibit nuclear 17-ß-estradiol binding. Cells grown in monolayer culture were harvested, resuspended in phenol-red free DME, and incubated in the presence of varying concentrations of [^{3}H]-17-ß-estradiol either with or without 100-fold excess unlabelled steroid to determine non-specific binding. Receptor-ligand complexes were collected on nitrocellulose filters as described previously (Spelsberg, 1987). ER-1 cells exhibited specific 17-ß-estradiol binding at concentrations of 1nM and 10nM (fig 3-2). It can be concluded that the [^{3}H]-17-ß-estradiol binding occurred

via an ER, and not another steroid receptor species because research has demonstrated that AtT20 cells possess binding sites for only the type II glucocorticoid receptor and not for other steroid receptors (Gannon, 1990).

Estrogen decreases POMC mRNA

Previous data from this laboratory measuring estrogen regulation of the POMC gene with a single treatment time suggested that negative regulation of the POMC gene in ER-1 cells is exhibited at the level of a reduction in mRNA (Lundblad, 1992). Using a solution hybridization/nuclease protection assay the extent of E2-mediated inhibition of the endogenous mouse POMC gene was thoroughly assessed in ER-1 cells. Cytoplasmic RNA from ER-1 cells treated for 24 hours with 17-ß-estradiol was hybridized for 16 hours with a mouse antisense probe, pMEX-3 (fig 3-3), that was uniformly labelled with [^{32}P]-UTP. Excess unhybridized RNA was eliminated by digestion with RNase T1 and RNase A and the resultant duplex species were resolved on a 6% polyacrylamide gel as described in Chapter 2. It was found that E2 treatment for 24 hours resulted in a significant inhibition of expression of POMC mRNA (data not shown).

The time course of E2-mediated inhibition of POMC gene expression was investigated using solution hybridization/nuclease protection assay. ER-1 cells fed with phenol red-free DME supplemented with 10% steroid-stripped (dextran-charcoal method, Horwitz and McGuire, 1978) horse serum and 5% stripped fetal calf serum were treated for 12 and 24 hours with 100nM E2 or untreated (control). Cells were harvested and cytoplasmic RNA was isolated using sucrose step-gradient centrifugation. One microgram aliquots of RNA were hybridized with ^{32}P-labelled pMEX-3. Levels of POMC mRNA were significantly reduced following treatment with E2 for 12 hours as compared to control levels (fig 3-4). The observed inhibition was sustained following 24 hours of E2 treatment, resulting in a maximal 62% reduction in POMC mRNA levels. Reduction of POMC mRNA levels following 24 hour estrogen treatment was confirmed in two other separate experiments. Thus it appears clear that E2 causes a sustained inhibition of POMC gene expression in ER-1 cells.

Dose response of estrogen inhibition

Measurements of the dose response of estradiol inhibition of endogenous POMC mRNA were made using solution hybridization/nuclease protection assays. ER-1 cells were treated for 20 hours with varying concentrations of 17-ß-estradiol in phenol red-free media. The cells were harvested and cytoplasmic RNA was prepared. RNA was hybridized with the pMEX-3 probe as described above. Excess unhybridized RNA was eliminated by RNase digestion. The resultant duplex species were resolved on a 6% polyacrylamide gel, the protected RNA was excised from the dried gel, and radioactivity was determined by scintillation counting.

It was found that E2 treatment resulted in a dose-dependent, and significant, 50% reduction in POMC mRNA levels with an ED_{50} of about 3nM (fig 3-5). It was noted that 20 hour treatment with 100nM E2 resulted in floating cells perhaps indicating some cell death. The maximal inhibition of POMC mRNA in this study agrees with data obtained in the time course studies presented above. Furthermore, these results suggest that half maximal inhibition of POMC mRNA levels occurs at an E2 concentration which is close to the reported Kd (~.5nM) of the variant ER that is present in ER-1 cells. The ED_{50} also agrees with the Kd (~3.6nM) obtained in two nuclear 17-ß-estradiol binding studies in the ER-1 cells (data not shown). Moreover in a control experiment with AtT20 cells which lack functional estrogen receptor protein (Lundblad, 1992; Gannon, 1990), treatment with 100nM E2 resulted in no alterations of POMC gene expression as measured by levels of primary transcript (fig 3-6). It can be concluded that the observed E2-dependent down-regulation of POMC mRNA occurs via an ER-mediated mechanism.

Nuclear events leading to estrogen negative regulation

The overall expression of a gene is accounted for by a balance between its transcriptional rate and processes that result in degradation of its RNA products. To date, many investigators have shown hormonal modulation of POMC gene transcription (reviewed in Lundblad and Roberts, 1988), while none have reported any effects on mRNA turnover. It has been demonstrated that changes in steady state levels of the POMC primary transcript accurately reflect

alterations in transcription rate as measured by transcription run-on assay with both glucocorticoid and CRF treatment in AtT20 cells (Lorang, 1992b). The involvement of decreased POMC biosynthesis in estrogen negative regulation of the POMC gene was investigated by measuring levels of primary transcript as an index of mRNA synthesis.

Levels of POMC primary transcript were measured using solution hybridization/nuclease protection assay methodologies and the pMEX-3 RNA probe (fig 3-3). The probe spans a portion of a mouse genomic POMC clone encompassing part of exon 3 and intron B, including the intron-exon junction. Use of this probe permitted the detection of both primary transcript which results in a 500 base pair RNA-RNA duplex (fig 3-7A, upper band) and nuclear mRNA which is a 338 base pair protected species (fig 3-7A, lower band) in nuclear samples. A standard curve of *in vitro* transcribed pMEX-3 (+) RNA (fig 3-7B) was utilized as described in Chapter 2 to quantify levels of POMC primary transcript protected in this assay. It was found that treatment with E2 for 12 hours resulted in a 36% reduction in primary transcript levels (fig 3-7C), which was sustained for 24 hours. These observations suggest that E2 treatment results in prolonged inhibition of POMC transcription and that the decrease in cytoplasmic mRNA is due to a decrease in POMC mRNA biosynthesis.

Effects of acute estrogen treatment on POMC gene expression

It has been shown that acute treatment with the potent synthetic glucocorticoid, dexamethasone (100nM) results in a rapid (30 min) 2- to 3-fold suppression of POMC primary transcript, heteronuclear RNA and transcription in AtT20 cells (Lorang, 1992b). Hence to further investigate the mechanism of estrogen-mediated down-regulation of POMC mRNA, acute time course studies were employed to examine whether there was rapid inhibition of primary transcript levels in ER-1 cells. Solution hybridization/nuclease protection assays using the pMEX-3 probe were utilized as described above to measure levels of POMC primary transcript.

The response to estradiol treatment was biphasic. There was a significant 40-60% transient increase in primary transcript levels that occurred between 10-20 min, followed by a substantial 30% reduction in the primary transcript species below control (untreated) levels, after 60 min (fig 3-8). In some experiments, the

stimulation was sustained for 30-45 min. Nevertheless, the same biphasic effect was observed. The observed biphasic response of ER-1 cells to E2 treatment is in contrast to what has been demonstrated for glucocorticoid treatment in AtT20 cells (Roberts, 1987; Lorang, 1992b). This suggests different mechanisms of action for the two hormones and hormone-receptor complexes.

It was predicted that cytoplasmic POMC mRNA levels would not be altered in cells that were acutely treated with E2 because of the relatively large pool of existing mRNA as compared to newly processed mRNA in the AtT20/ER-1 cells. As expected, levels of mRNA in the short term (15-120 min) E2 treated ER-1 cells did not change to a detectable extent (data not shown). This leads to the conclusion that while acute changes in POMC primary transcript levels are not manifested at the level of mRNA content, sustained decreases in levels of primary transcript result in decreased mRNA levels. This is likely due to an overall decrease in POMC biosynthesis caused by the genomic action of the ER.

Furthermore, a control experiment demonstrated that E2 treatment in AtT20 cells, which lack functional estrogen receptor protein, did not affect levels of POMC primary transcript (fig 3-6). It can be concluded that the observed effects of E2 treatment on POMC gene expression as measured by alterations in primary transcript levels are due to the presence of the estrogen receptor.

Characterization of the effects of estrogen on POMC gene expression in the ER-1 model cell system demonstrates that there is a biphasic response exemplified by a significant transient increase in gene expression, followed by a significant sustained decrease in mRNA synthesis to below control levels. This response appears to be receptor-mediated, similar to what is found for glucocorticoids. However, whereas an acute stimulation of POMC gene expression may be consistent with physiological modulation of POMC gene regulation as it relates to reproductive function, it represents a difference in acute regulation patterns of the POMC gene by two steroid hormones, glucocorticoids and estrogens.

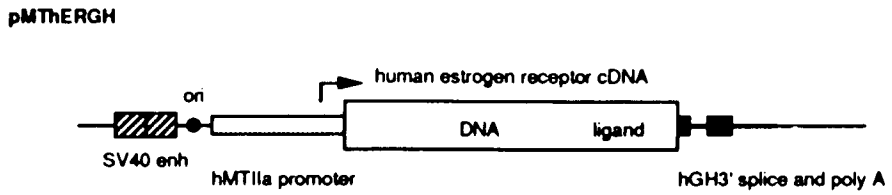


Figure 3-1 The ER expression vector pMThERGH contains an SV40 enhancer and origin of replication cloned upstream from a human metallothionein IIa promoter and a 2.1 kb cDNA encoding a human estrogen receptor. The plasmid also contains 3' splicing and polyadenylation signals from the human growth hormone gene. Relative positions of the DNA binding and ligand binding domains are indicated.

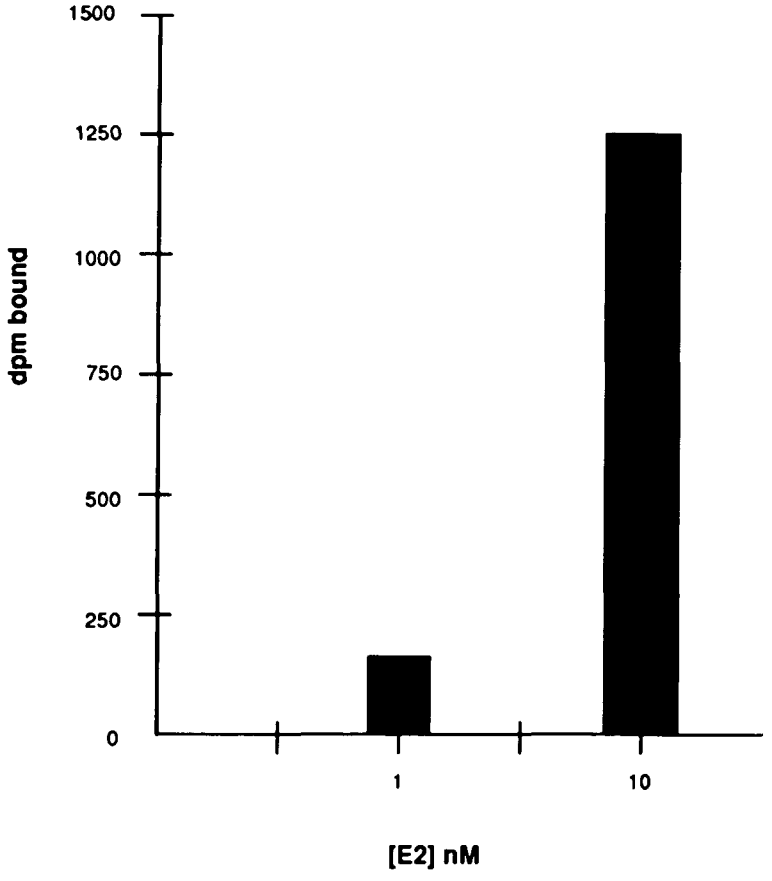


Figure 3-2 [^{3}H]-17-ß-estradiol binding in ER-1 cells was determined using a nuclear steroid binding assay. Excess unlabelled estradiol (100-fold) was used to determine non-specific binding.

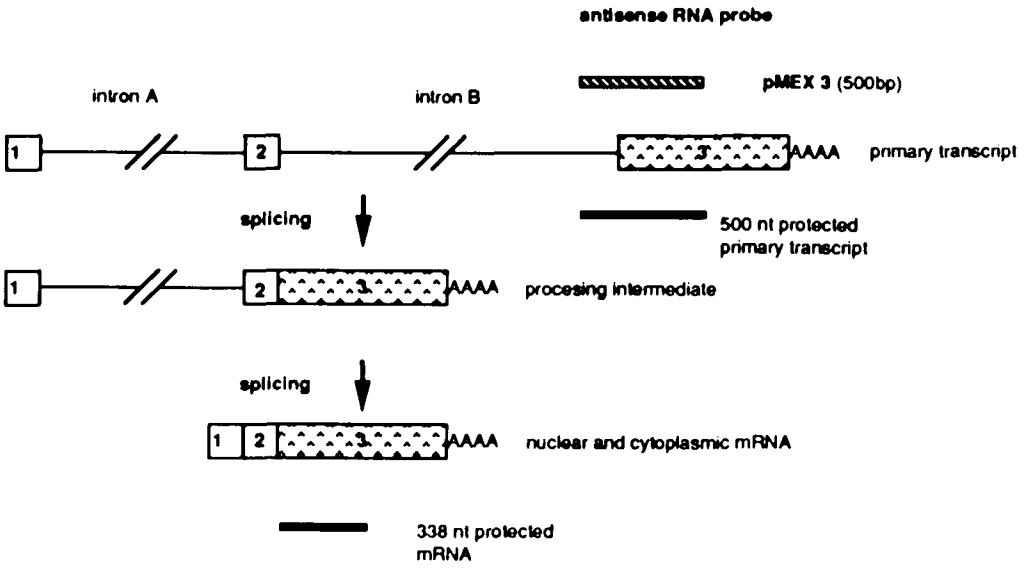


Figure 3-3 The probe pMEX-3 spans a 500 bp portion of a mouse POMC genomic clone that encompasses 338 bases of exon 3 and 162 bases of intron B. The antisense RNA probe protects a 500 bp primary transcript species in nuclear RNA samples and a 338 bp mRNA species in both nuclear and cytoplasmic samples.

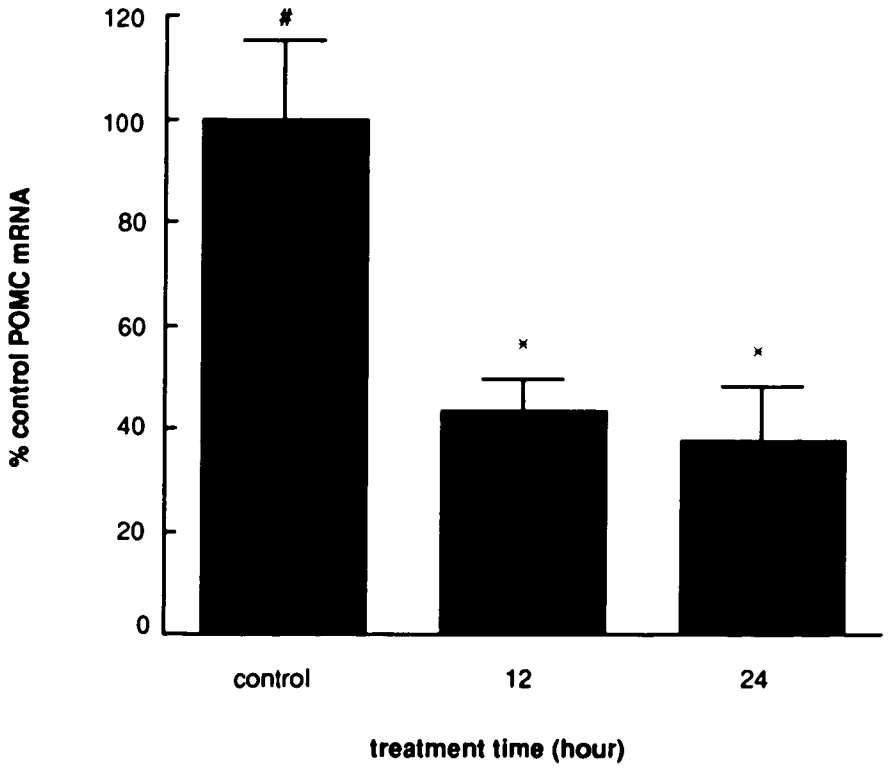


Figure 3-4 ER-1 cells were treated for the indicated times with 100nM 17-ß-estradiol. Cytoplasmic RNA (1µg) was hybridized with ^{32}P-labelled pMEX-3 antisense probe for 16 hours followed by RNase digestion as described in Chapter 2. POMC RNA was quantified by comparison to *in vitro* synthesized (+) pMEX-3 RNA. Levels of POMC mRNA are plotted as % control POMC mRNA/µg cytoplasmic RNA (control~36pg POMC mRNA). Samples were analyzed three separate times and data points represent the mean ± SEM for n=4 samples, except (#) which denotes n=3. Statistical analysis was performed as described in Chapter 2 and values were deemed significant ($p<0.05$) with respect to control (*) using ANOVA (Fisher PLSD).

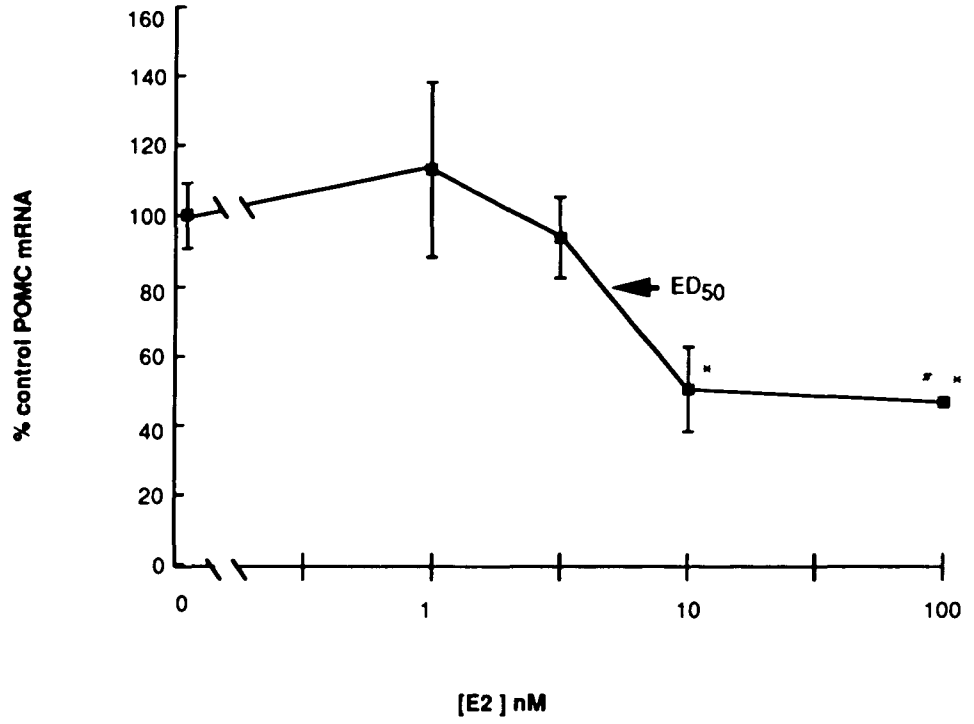


Figure 3-5 Dose response studies were performed with increasing concentrations of 17-ß-estradiol for a 20 hour treatment period. Cytoplasmic RNA (1µg) was hybridized with ^{32}P-labelled pMEX-3 and POMC RNA was quantified using solution hybridization/nuclease protection assay. Data is plotted as % control POMC mRNA/µg cytoplasmic RNA (control~30pg POMC mRNA). Statistical analysis was performed as described in Chapter 2 and values were deemed significant ($p<0.05$) with respect to control (*) using ANOVA (Fisher PLSD). Data points represent the mean ± SEM of triplicate samples and (#) indicates n=2 samples.

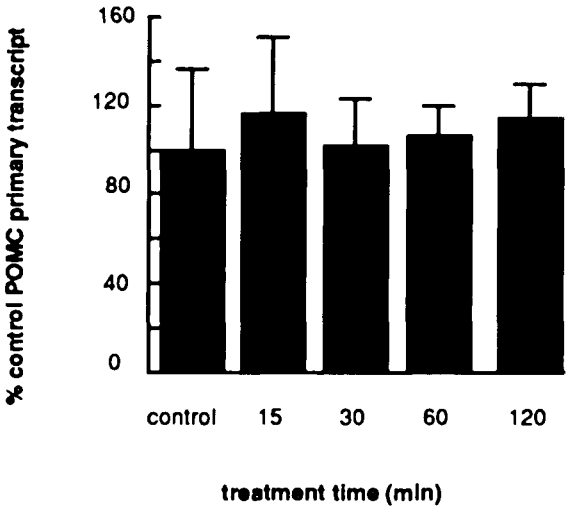


Figure 3-6 Control experiments were performed using AtT20 cells that were treated with 100nM 17-ß-estradiol. Nuclear RNA from AtT20 cells that were treated for the indicated times was hybridized with pMEX-3 and treated as described in Chapter 2. DNA content was determined using a diphenylamine colorimetric assay. Data is graphed as % control POMC primary transcript/µg DNA (control~0.94pg POMC primary transcript). Data points correspond to the mean ± SEM of n=3 samples.

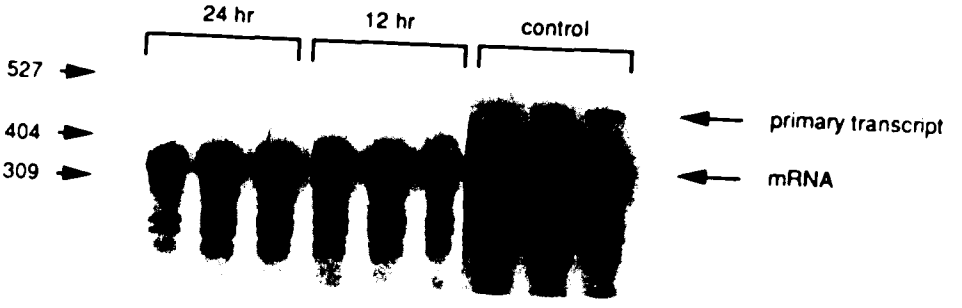
Figure 3-7 The time course of estrogen inhibition of POMC promary transcript was assessed in ER-1 cells using the solution hybridization/nuclease protection assay. ER-1 cells were treated with 100nM 17-ß-estradiol for the times indicated. Nuclear RNA was isolated and hybridized with ^{32}P-labelled pMEX-3 probe as described in Chapter 2.

A. Following RNase digestion the resultant RNA-RNA hybrids were separated on a 6% polyacrylamide gel. Arrows indicate the primary transcript and nuclear mRNA protected RNA species.

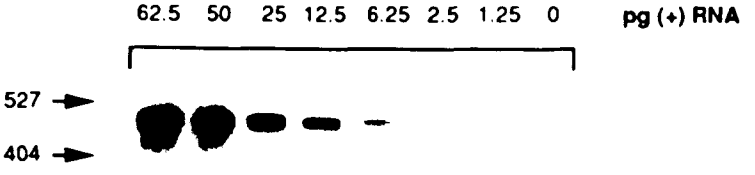
B. A standard curve of *in vitro* synthesized pMEX-3 (+) RNA was hybridized to the antisense probe under the identical conditions as the RNA samples. Use of linear regression analysis permitted the quantification of POMC primary transcript protected in this assay (see Chapter 2).

C. Data from the above nuclease protection assay is plotted as % control primary transcript/µg DNA (control~6pg POMC primary transcript). Data points represent the mean ± SEM of n=3 samples and (*) indicates statistical significance $p<0.05$ vs. control, as described above.

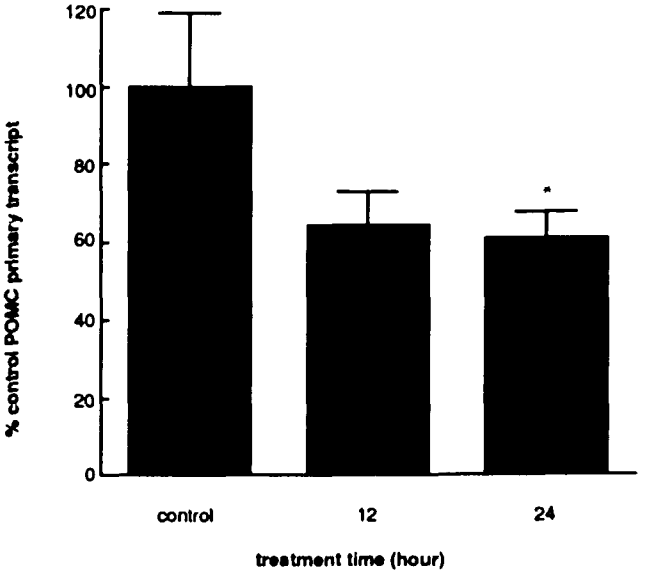
A.


24 hr
12 hr
control
527
404
309
primary transcript
mRNA

B.


62.5 50 25 12.5 6.25 2.5 1.25 0
pg (+) RNA
527
404

C.


120
100
80
60
40
20
0
% control POMC primary transcript
control
12
24
treatment time (hour)

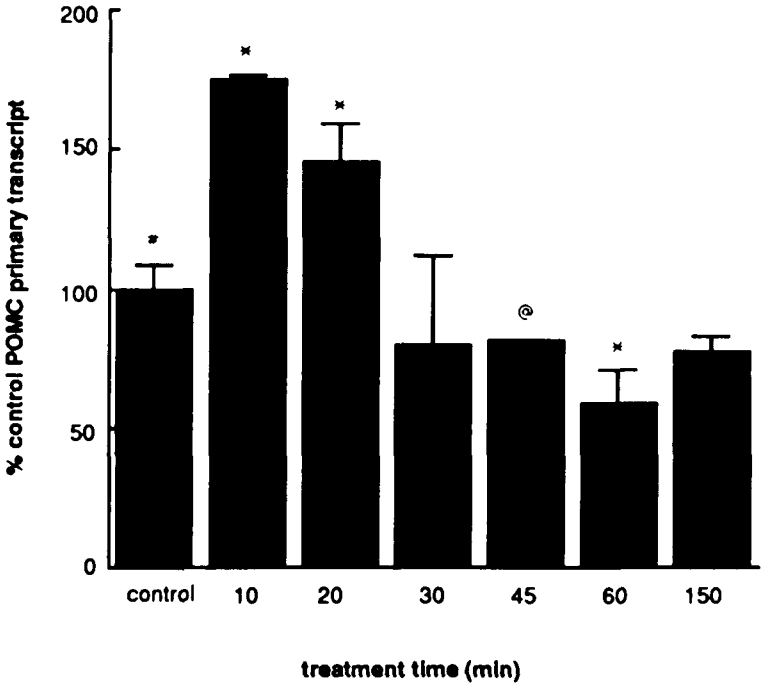


Figure 3-8 Acute time course studies were performed in ER-1 cells that were treated with 100nM 17-ß-estradiol for the times indicated. RNA was quantified as described previously. Data is plotted as % control POMC primary transcript /µg DNA (control~4pg POMC primary transcript). Data points represent the mean ± SEM of n=3 samples, except (#) n=6, and (@) n=2. (*) indicates significance ($p<0.05$) with respect to control.

CHAPTER 4

MECHANISMS OF ESTROGEN REGULATION OF POMC GENE EXPRESSION

Estrogen interferes with CRF stimulation of POMC gene expression

Data presented in Chapter 3 showed that there are two actions of estrogen with regard to expression of the POMC gene in ER-1 cells. One effect of E2 treatment is acute stimulation of gene expression. The other effect of E2, namely sustained inhibition of gene expression, is similar to what is observed for glucocorticoid treatment. Part of the inhibitory actions of glucocorticoids are manifested by inhibition of CRF-stimulated POMC gene expression. When treated with 10nM CRF, AtT20 cells exhibit elevated levels of POMC gene transcription, heteronuclear RNA, and primary transcript within a 60 min period of time (Lorang, 1992b). It has been demonstrated that glucocorticoid pretreatment attenuates CRF induction of POMC gene expression in primary culture of anterior pituitary cells (Eberwine, 1987) and in AtT20 cells (Lorang,1992b). To address the question of whether estrogens and glucocorticoids elicit their effects on POMC gene regulation by a similar mechanism, the interaction between estrogens, glucocorticoids, and CRF was investigated. These studies utilized measurements of primary transcript levels, as determined using solution hybridization/nuclease protection assay, an index of regulated POMC gene expression.

Following a 2 hour prefeeding with DME supplemented with stripped serum, ER-1 cells were treated with either 100nM 17-ß-estradiol (E2), 100nM dexamethasone (DEX), or 10nM CRF for varying time periods (15, 30, or 45 min), or pretreated with one of the hormones for 15 min, followed by cotreatment with CRF and either E2 or DEX for 30 min. Cells were harvested and equivalent samples of nuclear RNA were hybridized with antisense ^{32}P-labelled pMEX-3 probe as described in Chapter 2.

It was found that 10nM CRF treatment significantly elevated POMC primary transcript levels to 250% of control levels when cells were pretreated for 30 or 45 min (fig 4-1). There was attenuation of CRF induction when cells were

treated with either DEX or E2 for 15 min prior to cotreatment with CRF for 30 min (fig 4-1, see @). Moreover, pretreatment with CRF for 15 min followed by cotreatment with either DEX or E2 similarly resulted in reduction of CRF-stimulated induction of POMC primary transcript (fig 4-1, see &). Treatment with 100nM DEX did not result in any significant alterations in POMC primary transcript levels as compared to control levels. Cells treated with 100nM E2 exhibited elevated primary transcript levels with maximal stimulation to levels greater than twice those of control levels occurring at 30 min (fig 4-1).

These results are partly in agreement with what has been demonstrated for the interaction of glucocorticoids and CRF. In primary cultures of anterior pituitaries it was found that DEX pretreatment for 15 or 30 min, but not 10 min, inhibited CRF stimulation of POMC gene transcription (Eberwine, 1987). When 10 min pretreatment with CRF preceded cotreatment with CRF and DEX for 10 min there was attenuation of CRF induction. Whereas, pretreatment for 30 min with CRF, followed by cotreatment for 30 min did not result in reduction of CRF stimulation. Similar results were found in 15 min CRF pretreated AtT20 cells (Lorang, 1992). However, in contrast to what has been previously demonstrated for glucocorticoid treatment in AtT20 cells, pretreatment with CRF for 15 min followed by cotreatment with DEX resulted in attenuation of CRF stimulation in ER-1 cells.

Estrogen attenuates acute CRF induction of POMC gene expression. As exemplified by the studies presented here, both estrogen and glucocorticoids interfere with the acute stimulatory action of CRF, thus suggesting that the two hormones may elicit their effects by a similar mechanism. However, the time course demonstrated a difference in their acute regulation of POMC gene expression. This may be due to the differential ability of these hormones to induce another factor or factors that affect POMC gene expression. Alternatively, this may be due to interactions between the ER or GR and another transcription factor.

Estrogen induces c-fos expression in ER-1 cells

There is a difference between acute regulation of the POMC gene by glucocorticoids and estrogen. It has been shown that estrogen can stimulate the c-fos gene in several systems (Weisz and Bresciani, 1988; Loose-Mitchell, 1988;

Abbud, 1992; Plotsky and Rivest, 1992; Lee, 1990; Kulig and Lloyd, 1992). It was therefore postulated that this may account for differences in acute glucocorticoid and estrogen regulation of POMC gene expression. Identification of an ERE in the c-fos gene (Hyder, 1992), the findings that estrogen, but not dexamethasone stimulate the c-fos gene (Loose-Mitchell, 1988), and the role of c-fos in CRF induction of POMC gene expression (Boutillier, 1991) made this hypothesis attractive. Both the GR and ER have been shown to interact with the AP-1 (Fos/Jun) protein complex in a manner that imparts gene regulatory information (Gaub, 1990; Jonat, 1990; Schule, 1990; Yang-Yen, 1990). To test this theory, induction of c-fos was measured following CRF, E2 and DEX treatment.

A preliminary study was performed to see if CRF treatment in ER-1 cells resulted in induction of c-fos mRNA. ER-1 cells were treated with 10nM CRF for 30 min. Cells were isolated and both nuclear and cytoplasmic RNA samples were prepared. RNA samples were hybridized with a ^{32}P-labelled antisense mouse c-fos probe (specific activity = 2.6 x 10^9cpm/μg), mcfos4. Following RNase digestion the resultant duplex RNA species were run on a 5% polyacrylamide gel (fig 4-2). It was found that CRF treatment in ER-1 cells resulted in stimulation of c-fos mRNA. Furthermore, it was possible to detect the mRNA species in both nuclear and cytoplasmic RNA samples.

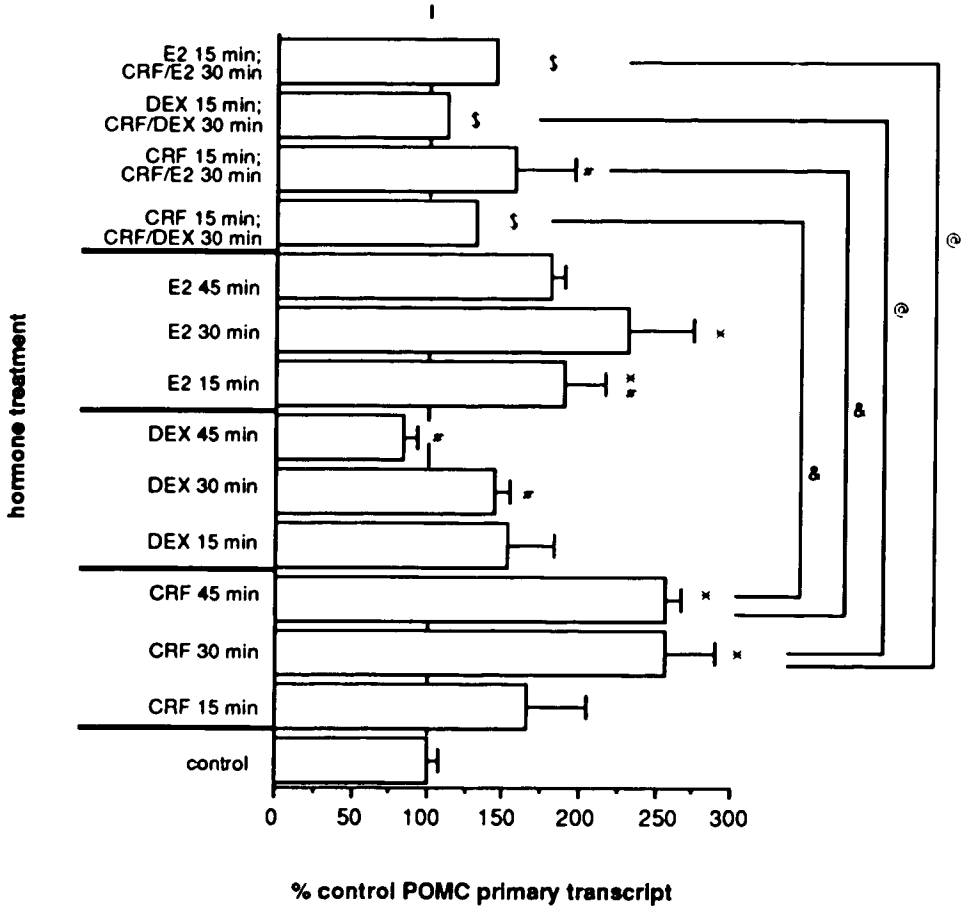
To assess the ability of estrogen and glucocorticoids to induce expression of the c-fos gene, levels of c-fos mRNA were measured in cytoplasmic RNA samples from ER-1 cells that were treated with either 10nM CRF, 100nM Dex, or 100nM E2 for 30 min, or untreated (control). Samples of 5μg cytoplasmic RNA were hybridized with a probe for the mouse c-fos gene in a solution hybridization/nuclease protection assay as described in Chapter 2. The resultant duplex RNA species were run on a 5% polyacrylamide gel (fig 4-3A).

It was found that treatment with 10nM CRF for 30 min resulted in a significant, 5-fold, increase in levels of c-fos mRNA over control (untreated) levels in ER-1 cells (fig 4-3B). Levels of c-fos mRNA were nearly doubled in 100nM E2 treated cells, but there was a minimal increase in c-fos due to 100nM DEX following 30 min hormone administration.

It has been demonstrated that acute treatment with E2 or DEX results in different effects upon c-fos expression in ER-1 cells. These results provide indirect evidence in support of the theory that acute E2-mediated stimulation of POMC gene expression is due to induction of c-fos gene expression by E2 in ER-

1 cells. Moreover, the inability of glucocorticoids to induce expression of the c-fos gene in the same time frame as estrogen suggests that this difference may be significant in terms of POMC gene regulation observed in acute treatment paradigms with E2 or DEX. It is possible that interactions between the ER or GR and the AP-1 protein complex are ultimately responsible for the hormonally-regulated POMC expression observed in these studies. Finally, it can be concluded that while estrogens and glucocorticoids have an overall inhibitory effect on the POMC gene system; estrogen behaves differently in the short term.

Figure 4-1 ER-1 cells were treated with either 10nM CRF, 100nM DEX, 100nM E2, or a combination of CRF and either DEX or E2 for varying times as indicated. Nuclear RNA from these cells was hybridized with a ^{32}P-labelled antisense pMEX-3 probe. POMC primary transcript was measured using solution hybridization/nuclease protection assay and scintillation counting of excised bands corresponding to properly sized duplex RNA. Levels of POMC primary transcript were determined by comparison of sample values to a standard curve generated using *in vitro* synthesized (+) pMEX-3 RNA and they were normalized to cpm nuclear mRNA in the same sample. Data is presented as % control POMC primary transcript/cpm nuclear mRNA. Data points represent the mean ± SEM of n=3 samples, except, (#) n=4 and ($) n=2. Statistical analysis was performed as described in Chapter 2. Values were deemed significant ($p< 0.05$) using ANOVA (Fisher PLSD) with respect to control (*) or with respect to each other (@) and (&).


E2 15 min; CRF/E2 30 min
DEX 15 min; CRF/DEX 30 min
CRF 15 min; CRF/E2 30 min
CRF 15 min; CRF/DEX 30 min
E2 45 min
E2 30 min
E2 15 min
DEX 45 min
DEX 30 min
DEX 15 min
CRF 45 min
CRF 30 min
CRF 15 min
control
hormone treatment
0
50
100
150
200
250
300
% control POMC primary transcript

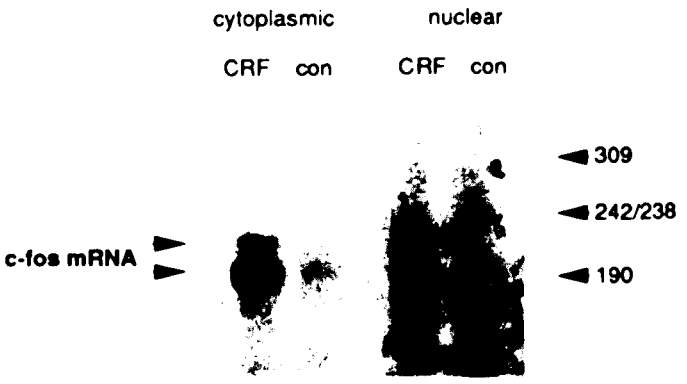


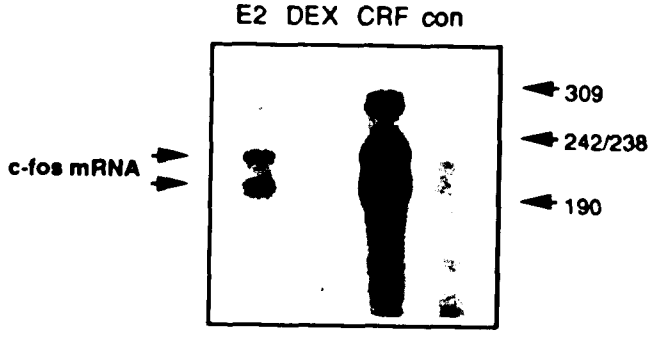
Figure 4-2 Cytoplasmic and nuclear RNA from ER-1 cells treated for 30 min with 10nM CRF or untreated (con) was hybridized with a ^{32}P-labelled antisense mcfos4 probe for 16 hours in solution. Samples were subjected to RNase digestion and RNA-RNA hybrids were resolved on a 5% polyacrylamide gel.

Figure 4-3 ER-1 cells were treated for 30 min with either 10nM CRF, 100nM DEX, or 100nM E2 or untreated (con). Cytoplasmic RNA was isolated and hybridized with ^{32}P-labelled mcfos4 probe as described previously.

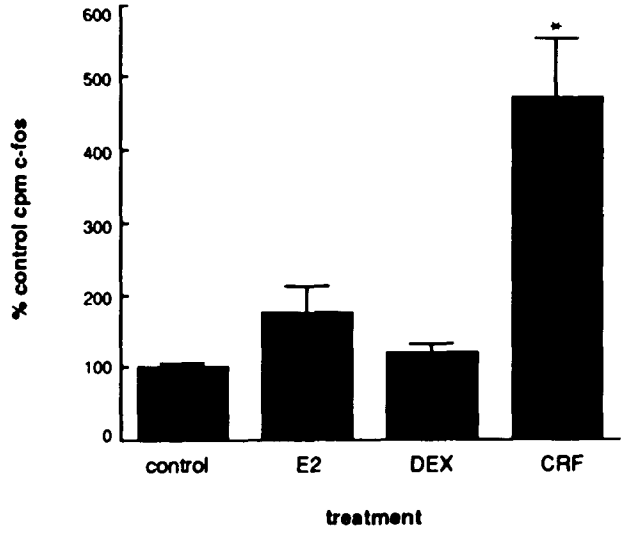
A. Following RNase digestion the resultant RNA species were resolved on a 5% polyacrylamide gel. The protected RNA species corresponding to the c-fos mRNA species are indicated.

4. Bands corresponding to the protected c-fos mRNA were excised from dried gels and counted. Data is presented as % control cpm c-fos and data points represent triplicate samples. Statistical analysis was performed as described previously and (*) denotes significance ($p < 0.05$) compared to control.

A.


E2 DEX CRF con
c-fos mRNA
309
242/238
190

B.


600
500
400
300
200
100
0
% control cpm c-fos
*
control
E2
DEX
CRF
treatment

CHAPTER 5

IDENTIFICATION OF A CRF-RESPONSIVE POMC PROMOTER ELEMENT

Analysis of the POMC promoter

Regulation of the POMC gene by estrogen can occur through a blockade of CRF induction (see Chapter 4). It would be beneficial to delineate sequences of the POMC gene responsible for mediating CRF regulation. Previous studies using a POMC-tk-CAT reporter gene delineated a CRF- and cAMP-responsive region of the rat POMC promoter to the 5' flanking sequences between -234 to -133 (Lorang, 1991; Roberts, 1987; Salton, 1989). When the POMC sequences were compared to known consensus sequences, there did not appear to be any cAMP responsive elements (CREs) in this region of the POMC gene. Sequence analysis of the rat POMC gene from -706 to +63, was carried out using the Mac Vector (Davison, 1985) computer program. It confirmed that this region (fig 5-1A) does not contain any previously identified consensus CREs. However, a sequence that bears similarity (2bp changes) to a consensus CRE has been identified in the POMC promoter region from -175 to -168 (Young, 1989). Footprinting studies disclosed protein binding sites from -214 to -174 and -155 to -115 in this area of the POMC gene (Salton, 1989), suggesting that this promoter region is likely to be involved in control of gene expression. Further examination of sequences contained between -234 and -133 indicated that there is a nearly perfect palindromic element, 5'-CTGTGCGCGCAG-3' present from -171 to -160 in the rat POMC promoter (fig 5-1B). A similar element which contains a 3bp insert between the half palindromic repeat is located from -211 to -197 (fig 5-1B). The functional significance of DNA palindromes in terms of transcriptional regulation by protein factors that bind as dimeric units has been presented extensively in the literature (reviewed in Forman and Samuels, 1990; Ziff, 1990; Jones, 1990).

Construction of POMC reporter plasmids

To test the ability of the -171 to -160 POMC palindrome to mediate CRF-inducibility of POMC gene expression, POMC-CAT reporter genes were constructed. Oligonucleotides corresponding to portions of the rat POMC 5' flanking region from -180/-150, -175/-157, and to the latter region with an insert of 5bp, -175 /157 (+5), were synthesized (fig 5-2A) such that they possessed Sal I restriction enzyme compatible ends. The oligonucleotides were cloned in front of a HSV tk minimal promoter that was upstream from a gene encoding the CAT protein using a unique Sal I restriction enzyme site in the multiple cloning site of the pUSCAT plasmid (fig 5-2B). Positive clones were identified using colony hybridization screening and they were confirmed by dideoxy DNA sequencing. Sequence analysis showed that the oligonucleotides -175/-157 and -175/-157 (+5) inserted in the opposite orientation; and the -180/-150 oligonucleotide was in the correct 5' to 3' direction. The resulting POMC reporter plasmids were designated -180/-150P-tk-CAT, -175/-157P-tk-CAT, and -175/-157 (+5)P-tk-CAT.

CRF-induction of POMC-tk-CAT reporter genes in AtT20 cells

The CRF-inducibility of the above POMC-tk-CAT reporter genes was tested by transient transfection of AtT20 cells and use of the CAT assay (Gorman, 1985). AtT20 cells were transfected with plasmid DNA containing the aforementioned POMC-tk-CAT constructs using a polylipoamine reagent (Behr, 1989) and treated for 16 hours with 10nM CRF. Cells were isolated and aliquots of whole cell lysate, prepared by freeze-thaw lysis, were analyzed for CAT activity using [^{14}C]-chloramphenicol and thin layer chromatography.

The -180/-150P-tk-CAT and -175/-157P-tk-CAT POMC reporter genes were significantly CRF-inducible in cells that received CRF as compared to untreated cells (fig 5-2C). Furthermore, cells transfected with the -175/-157 (+5)P-tk-CAT construct did not exhibit CRF-stimulated CAT activity. Similarly, a control plasmid, pUSCAT, that contains only the HSV tk minimal promoter, and no POMC sequences, upstream from CAT encoding sequences, did not yield CRF-induced CAT activity (fig 5-2C).

These results indicate that there are sequences able to mediate CRF induction present from -180 to -150 in the rat POMC promoter. The data also

indicates that these sequences are able to function in an orientation-independent manner. Moreover, the lack of CRF-inducibility of the -175/157 (+5)P-tk-CAT construct is suggestive of a dimeric protein factor activated or induced by CRF treatment whose binding results in transcriptional activation of the POMC gene.

Gel shift analysis

Protein factors that elicit transcriptional modulation in response to hormone treatment usually do so by binding to specific DNA sequences within the regulated genes. It is possible that the data represents the action of a transcription factor whose function is CRF-inducible. To test for a specific protein-DNA interaction that occurs with the POMC palindromic region, gel shift experiments were performed using protein extracts from AtT20 cells.

Nuclear protein extracts were prepared from AtT20 cells that were treated with 10nM CRF for 30 min or untreated (control) using a previously described protocol (Dignam, 1990). Protein extract (8μg) was incubated with ^{32}P-labelled oligonucleotides corresponding to the aforementioned -175/-157 rat POMC sequence. Incubations were carried out in the presence or absence of specific unlabelled competitor DNA which corresponds to the identical POMC sequence. The extract was incubated with another competitor sequence that corresponds to a TPA-responsive element (TRE) consensus sequence in a control reaction. The resulting protein-DNA complexes were resolved on a 5% low ionic strength polyacrylamide gel.

There was a detectable retardation of DNA mobility in mixtures that contained both control and CRF-treated extracts. The mobility shift appears to be more pronounced in extracts from cells treated with CRF than in control extracts (fig 5-3). This study implies that there is binding of a protein or proteins to the -175 to -157 region of the rat POMC promoter. Moreover, the observed protein-DNA interactions appear to be specific for the palindromic sequence as demonstrated by effective competition with the same oligonucleotide sequence (fig 5-3). Concurrent experiments by another student (Wei Dong Jin) in the laboratory, using the same extracts, have demonstrated that an unrelated oligonucleotide (corresponding to a GRE) was not able to compete for binding to the -175/-157 oligonucleotide. Interestingly, the TRE appears to effectively compete for protein binding to the -175/-157 oligonucleotide. This may be due to

disruption of protein-DNA interactions formed with the -175/-157 sequence and a preferential interaction with an AP-1 binding consensus sequence. It is possible that there is involvement of the Fos protein in the binding interactions exhibited. Studies investigating the competition between the POMC palindromic sequence and the TRE are currently being pursued by another student (Wei Dong Jin) in the laboratory.

Results suggest that a region contained within -180 to -150 of the rat POMC promoter are able to direct CRF-inducibility of POMC gene expression. A particular element present in this region from -175 to -157 appears to play a role in the observed regulation of the POMC reporter gene. It is striking that this DNA element is a nearly perfect palindrome. Evidence is suggestive of a role for this palindromic sequence in conferring CRF-regulated POMC gene expression. Firstly, there are functional studies that demonstrate that this region of the POMC promoter directs CRF-induction of a minimal promoter reporter construct. Secondly, there is data to indicate that this region can support *in vitro* binding of a protein or protein complex. Thirdly, results presented here suggest that this protein complex may be composed of either two dimers or a single protein with a bipartite structure. However, the possibility that CRF-induction of this particular element is due to the action of the CREB protein cannot be ruled out at this time. Of relevance to the functional role of any one particular DNA element in mediating hormonally-regulated POMC gene expression, is the observation that the sequences between -234 and -133 of the POMC gene are able to confer 5- to 10-fold CRF inducibility upon the tk promoter (Lorang, unpublished observations). Maximal CRF induction reported here with constructs containing shorter sequences of the POMC promoter is 200% of control. It is possible that the presence of both of the palindromic elements are necessary for efficient CRF induction. Additionally, it is likely that other sequences within the POMC promoter from -234 to -133 working in concert aide in gene regulation. This is consistent with models of cooperation between transcription factors in establishment of a stable transcription complex and in hormonally-regulated control of gene transcription (Maniatis, 1987; Lillie and Green, 1989; Mitchell and Tjian, 1989; Lewin, 1990).

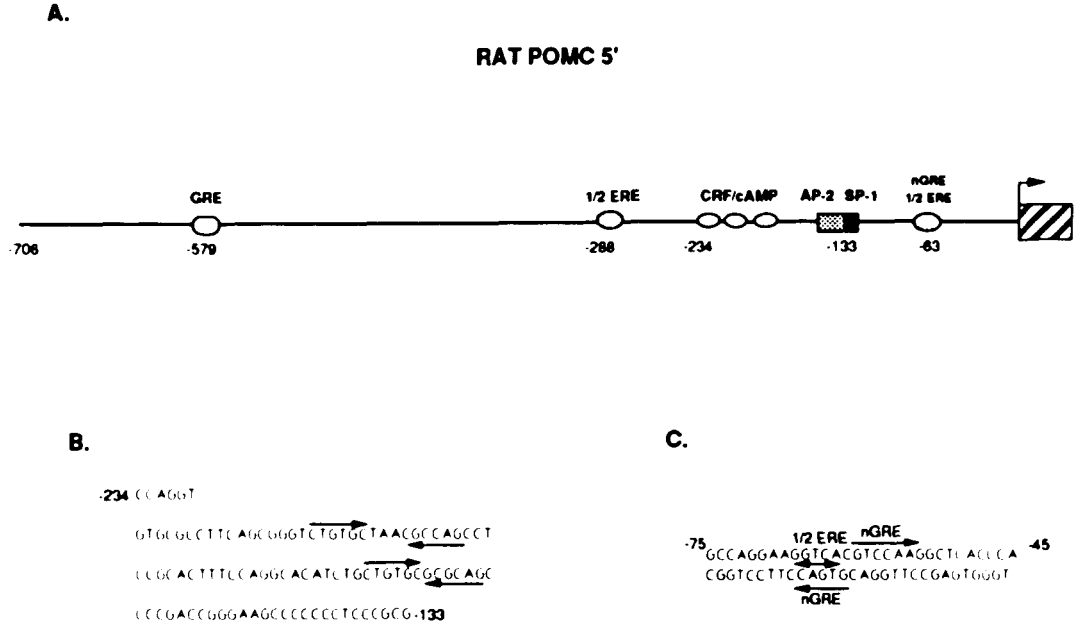


Figure 5-1 Schematic view of the rat POMC promoter including binding sites for several previously identified transcription factors.

A. Rat POMC promoter

B. DNA sequence for the region between -234 and -133 of the rat promoter. The two palindromic sequences are denoted by arrows.

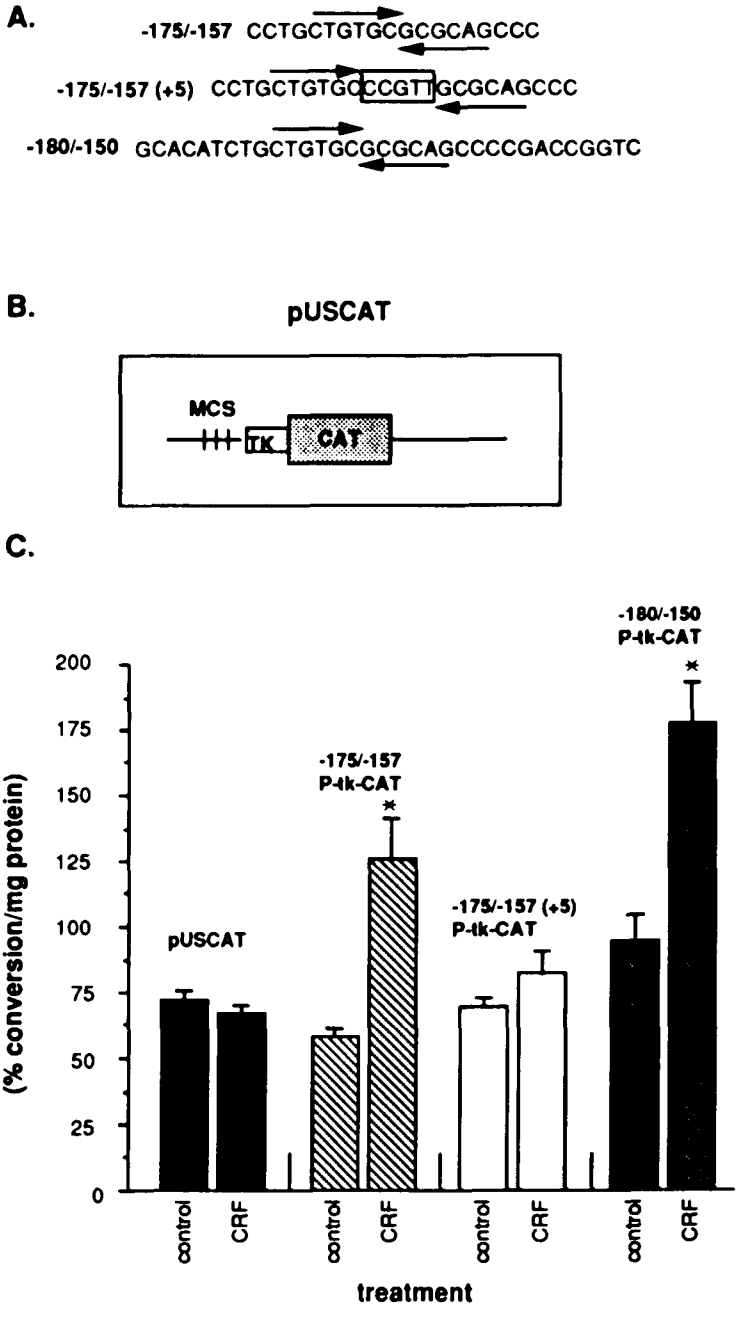
C. DNA sequence for the region surrounding the previously identified nGRE (denoted by arrows) and the half ERE consensus sequence (denoted by a double arrowed line).

Figure 5-2 POMC oligonucleotide sequences and CAT assay data from AtT20 cells.

A. Oligonucleotides corresponding to the indicated rat POMC DNA sequences.

B. The tk-CAT plasmid pUSCAT, into which the POMC oligonucleotides were cloned is depicted schematically.

C. AtT20 cells transfected with POMC promoter-tk-CAT reporter genes were assayed for CAT activity. Each data point represents triplicate samples ± SEM and (*) denotes statistical significance ($p < 0.05$) as determined using ANOVA (Fisher, PLSD).


A.
-175/-157 CCTGCTGTGCGCGCAGCCC
-175/-157 (+5) CCTGCTGTGCCCGTTGCGCAGCCC
-180/-150 GCACATCTGCTGTGCGCGCAGCCCCGACCGGTC
B.
pUSCAT
MCS
TK
CAT
C.
-180/-150
P-tk-CAT
-175/-157
P-tk-CAT
-175/-157 (+5)
P-tk-CAT
pUSCAT
CAT Activity
(% conversion/mg protein)
200
175
150
125
100
75
50
25
0
control
CRF
control
CRF
control
CRF
control
CRF
treatment

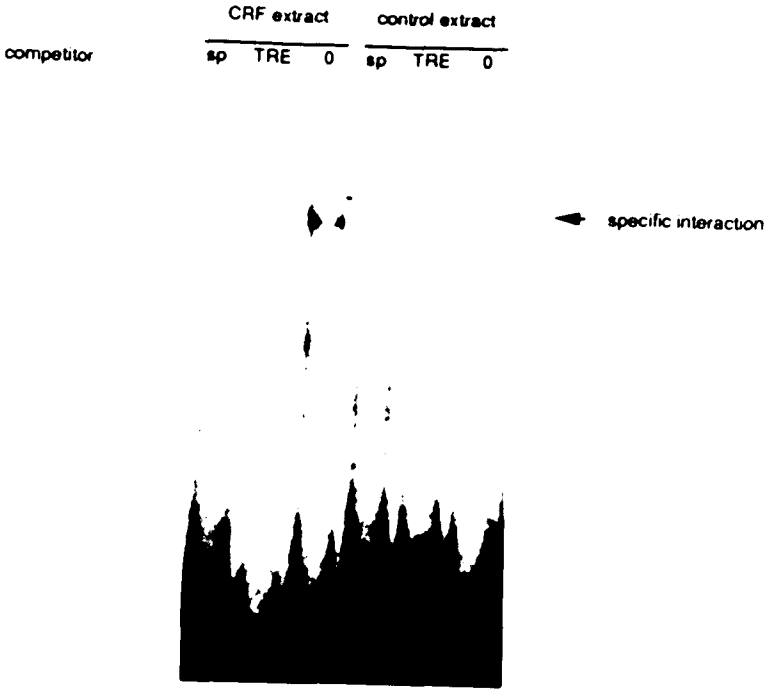


Figure 5-3 Gel shift analysis was performed using rat ^{32}P-labelled POMC-derived oligonucleotides and protein extracts from either CRF treated or untreated (con) AtT20 cells. Excess (100-fold) unlabelled oligonucleotides, either -175/-157 (sp) or TRE, were used as competitors in some reactions. Protein-DNA complexes were resolved on a 5% polyacrylamide gel under low ionic strength conditions. An arrow denotes the specific retarded protein-DNA complex.

CHAPTER 6

DISCUSSION

Estrogen regulation of POMC gene expression

There is extensive evidence for physiological regulation of the POMC gene by estrogen (Weimann, 1989; Wilcox and Roberts, 1985; Chowen-Breed, 1989; Roberts, 1985; Treiser and Wardlaw, 1992). A model to explain estrogen-mediated regulation of POMC gene expression in the hypothalamus does not exist. However, colocalization of ER and POMC-derived peptides in the hypothalamus (Morrell, 1985) suggests a direct ER-mediated mechanism of POMC gene regulation in this system.

Preliminary studies in the ER-1 cell line, a model corticotrope-like cell (AtT20) which has been stably transfected with an expression vector for a human ER demonstrated estrogen-mediated negative regulation of the endogenous mouse POMC gene (Lundblad, 1992). It was proposed that the observed findings were due to ER-mediated events in this system.

To characterize estrogen regulation of POMC gene expression, ER-1 cells were employed in the studies presented as a model cell culture system that represents a homogeneous population of POMC-expressing cells. The studies were aimed at characterizing the effects and mechanisms of estrogen-mediated POMC gene regulation. Parameters such as time-, dose-, and ER-dependence of estrogen-mediated POMC gene regulation were studied. Nuclear events responsible for POMC mRNA expression were investigated, to better understand the mechanism of action of estrogen in modulation of POMC gene expression. The interaction between estrogen and CRF and the involvement of c-fos induction in POMC regulatory processes was explored in ER-1 cells. Lastly, the functional significance of a palindromic DNA sequence in the POMC promoter was tested.

It was found that long term (24 hour) estrogen-mediated inhibition of POMC gene expression occurs in the ER-1 cell line. Inhibition was manifested at the level of POMC mRNA content. Normally neither AtT20 cells nor anterior pituitary corticotropes express ER. This observation strongly suggests that the

presence of an ER and estrogen treatment are sufficient for estrogen-mediated regulation of POMC gene expression. Furthermore, this implies that the POMC gene contains the necessary information to impart this regulatory scheme. This is supported by a lack of observable effect of estrogen treatment in AtT20 cells with respect to POMC mRNA levels. It is important to note the only detectable differences between AtT20 cells and ER-1 cells are 1) the presence of functional ER and 2) the ability of the receptor to mediate estrogen-responsive POMC gene expression. Therefore the possibility that stable integration of DNA encoding the ER expression vector, pMThERGH, into the chromatin may have activated a quiescent gene that affects transcriptional regulation function. If this was the case, it did not result in any changes in cell morphology or growth patterns.

There are some parallels that can be drawn between the observed estrogen-mediated inhibition of POMC mRNA levels in ER-1 cells and physiological modulation of the POMC gene in the hypothalamus. First, there is colocalization of ER and POMC-derived peptides in the hypothalamus (Morrell, 1985), thereby suggesting a direct ER-mediated mechanism of POMC gene regulation. Studies presented suggest that estrogen-mediated regulation of the endogenous mouse POMC gene in ER-1 cells occurs through receptor-dependent events. Second, reduction of arcuate nucleus POMC mRNA by estrogen treatment results in a maximal 40% decrease (Wilcox and Roberts, 1985). It was found that in ER-1 cells inhibition of POMC mRNA expression by estrogen treatment results in an approximate ~60% reduction mRNA levels. However, it appears that the estrogen-mediated effects are more rapid in ER-1 cells than *in vivo*. Third, as measured by *in situ* hybridization, proestrous rats exhibit a diurnal rhythm of POMC mRNA expression in the arcuate nucleus (Wise, 1990). This pattern of POMC gene expression was also found in ovariectomized, estrogen-replaced rats (Wise, 1990). Presumably fluctuations in levels of POMC mRNA are due at least in part to increased synthesis, i.e. increased transcription. Thus, the observation that estrogen exposure in acute treatment paradigms results in stimulation of POMC primary transcript levels in ER-1 cells is not surprising. However, in ER-1 cells the fluctuations in levels of POMC primary transcript were not reflected by detectable alterations in mRNA levels. This brings into question the significance of this observation in light of the net inhibitory effects of estrogen on POMC gene expression.

Negative regulation

Negative gene regulation by estrogen may occur by either direct transcriptional repression as mediated by ER-dependent events or by indirect mechanisms. The POMC gene is one of several genes that are negatively regulated by estrogen. For example, some groups have reported that the GnRH gene is inhibited by estrogen under certain conditions (Wray, 1989; Zoeller, 1988). Similar to the regulation of the POMC gene in the hypothalamus, the genes encoding the α- and ß-subunits of the glycoprotein hormones LH and FSH are both positively and negatively regulated by estrogen *in vivo* depending upon the duration of hormone treatment and the physiological status of the animal (Gharib, 1990). Moreover, negative estrogen regulation of the α-subunit gene in cultured ovine pituitary cells is due to inhibition of transcription (Phillips, 1988).

Estrogen negative regulation of POMC gene expression has been shown to be dependent upon inhibition of transcription in several systems. Firstly, studies performed *in vivo* have clearly demonstrated a decrease in hypothalamic POMC gene transcription following estrogen treatment (Roberts, 1985). Secondly, trans-repression studies performed in a cell culture system suggest that there is direct transcriptional repression of the POMC promoter (Lundblad, 1992). Finally, data presented here shows that POMC primary transcript levels are reduced following prolonged estrogen treatment in ER-1 cells. Changes caused by hormonal regulation of POMC primary transcript have been shown to reflect alterations in POMC gene transcription *in vivo* (Autelitano, 1989), in primary pituitary culture (Levin, 1989), and in AtT20 cells (Lorang, 1992b). Thus, it is concluded that the observed effects of estrogen treatment in ER-1 cells are transcriptional in nature.

Steroid regulation of gene transcription involves selective interactions of receptor proteins with specific cis-acting DNA elements (reviewed in Yamamoto, 1985; Evans, 1988; Beato 1989; O'Malley 1990). While cis-acting DNA elements that are positively estrogen-responsive have been reported (Berg, 1989; Evans, 1988; Beato, 1989), to date there has not been identification of DNA sequences that mediate negative estrogen gene transcriptional regulation. The trans-repression study cited above indicates that regions of the rat POMC gene contained between -706 to +63 with respect to the transcription initiation site are adequate for directing estrogen-regulated POMC gene expression. Furthermore, ER regulation is not likely to occur via binding to any of the previously identified

GREs in the POMC gene (Drouin, 1987) because it has been shown that a human GR does not activate an ERE reporter gene, nor does the converse occur (Green and Chambon, 1987; Green, 1988; Umesono and Evans,1989). However, changing two base pairs in each of the binding half sites converts an ERE to a GRE (Klock, 1987; Martinez, 1987). Hence, it is possible that a loose consensus sequence such as the nGRE from -75 to -51 (Drouin, 1987; Drouin, 1989) may permit promiscuous receptor binding.

The rat POMC gene contains two half ERE consensus sites, one located from -66 to -62 which overlaps with the previously identified nGRE and one further upstream in the opposite orientation from -291 to -287. The functional significance of ERE half sites has been demonstrated for the chicken ovalbumin gene which contains four half palindromic binding sites that are spaced greater than 100 bp apart in the distal promoter region and one in the proximal promoter region (Kato, 1992). In this instance, estrogen-mediated gene activation is thought to occur via the synergistic action of these elements with each other and other promoter elements. ER can bind to a half palindromic ERE motif as demonstrated by footprinting studies (Tora, 1988). Hence, transcriptional activation of the half ERE may take place by cooperative interactions of bound ER molecules as described previously (Martinez and Wahli, 1989; Ponglikitmongkol, 1990). The possibility that widely spaced half EREs can modulate gene transcription exists. Two separate studies have demonstrated that ER dimers are formed in solution (Linstedt, 1986; Skafar 1991) and thus there may be binding to two ERE half sites separated by many bases such that there is formation of a DNA loop structure (Ptashne, 1986). The possibility that the two ERE half consensus sites may function in concert with one another to form a DNA loop remains speculative at this time.

Another mechanism proposed for steroid-mediated negative gene regulation involves steric interference by steroid receptors, which prohibits binding of a positive acting transcription factor. This mechanism of transcriptional inhibition requires binding of receptor to DNA. Examples of genes that are likely to be negatively regulated by glucocorticoids in this fashion are the α-glycoprotein hormone subunit gene which is positively regulated by the CREB protein (Akerblom, 1988) and the proliferin gene which is induced by the AP-1 protein (Mordaq and Linzer, 1989). Both genes contain a GRE that is adjacent to sequences that confer positive regulation by their respective positive effectors.

Moreover, both genes possess complex promoters as exemplified by multiple hormonally regulated sequences.

It is possible that glucocorticoid and estrogen inhibition of CRF-induced POMC gene expression occurs through a steric hindrance mechanism. The complex nature of the POMC promoter makes this explanation attractive. A binding site for a factor that mediates CRF induction may reside near the nGRE or the half EREs. This brings into question precisely what factor(s) mediate CRF induction of POMC gene expression. A role for AP-1 involvement in this regulatory scheme has been suggested by studies that demonstrate CRF induction of c-fos expression in AtT20 cells and induction of a POMC reporter gene by Fos protein in these cells (Boutillier, 1991). An AP-1 consensus motif was identified in exon 1 (+40 to +50) of the POMC gene (Therrien and Drouin, 1991), thus further supporting the role of AP-1 in stimulation of POMC gene expression. The location of this site and of those for the nGRE and half EREs, makes it unlikely that the steric hindrance model applies to glucocorticoid or estrogen inhibition of CRF induction as manifested by interference with AP-1 binding to cis-acting sequences in exon 1 of the POMC gene. However, a sequence that is identical to the POMC half EREs (5'-GGTCA-3') mediates phorbol ester induction and is a binding site for the AP-1 protein in another system (Gaub, 1990). It is conceivable that these regions in the POMC gene serve as binding sites for AP-1. Competition for binding at these sequences by AP-1 and the ER is consistent with the steric hindrance model of negative transcriptional regulation.

CRF has been shown to lead to elevated levels of cAMP through activation of adenylate cyclase (Miyazaki, 1984). Hence, cAMP is a possible effector molecule in a signal transduction cascade that affects POMC gene expression through PKA-mediated events. Genes that are regulated by cAMP have been shown to contain binding sites for the CREB transcription factor (Ziff, 1990; Hoeffler and Habener, 1990; Goodman, 1990). Although the only CRE-like elements contained in the POMC promoter diverge from the consensus sequence by 2 base pairs (Young, 1989), there is the chance that part of CRF induction is due to the action of a CREB protein in this system. Moreover, a region of the POMC promoter (-234 to -133) that has been shown to be CRF-responsive (Lorang, 1991; Salton, 1989), is also inducible with forskolin (Lorang, 1991), thereby suggesting that the same POMC sequences may mediate both CRF and cAMP effects. However, interference of positive effectors by binding of

ER or GR to these particular sequences does not appear likely because consensus HREs do not exist here.

Negative steroid-mediated gene regulation may involve protein-protein interactions between steroid-receptor complexes and effectors of positive regulation which results in an overall inhibition of transcription. One model to explain this phenomenon is binding of a negative effector to a positive effector thereby eliminating binding of the positive factor to cis-acting DNA sequences. The collagenase gene is an example of regulation in this fashion. Glucocorticoids inhibit phorbol ester induction of the collagenase gene by a mechanism that suggests DNA binding-independent antagonism between the GR and AP-1 proteins (Schule, 1990). Glucocorticoid inhibition of phorbol ester-induced proliferin transcription appears to have a mechanistic component that is similar to the model presented for the collagenase gene. However, in this system in addition to the juxtaposition of DNA binding elements for the GR and AP-1 protein, and the proposed steric interference, there are also protein-protein interactions between the transcription factors such that the relative amount of each factor determines the regulatory pattern observed (Diamond, 1990). This may represent squelching of a limiting protein that is needed for transcriptional enhancement (Gill and Ptashne, 1988; Levine and Manley 1989).

Analogous to the examples presented above, inhibition of CRF induction of POMC transcription by glucocorticoids and estrogen may also involve protein-protein interactions. This model could apply to the POMC gene since CRF enhancement is propagated at least in part via AP-1 action. It has been clearly established that there is interaction between AP-1 and the GR. Interaction of AP-1 and the ER has also been demonstrated (Gaub, 1990; Tzukerman, 1991). However, in the case of ovalbumin gene regulation both proteins are involved in transcriptional enhancement (Gaub, 1990). Moreover, interaction of AP-1 and ER resulted in repression of ER activity and not in inhibition of phorbol ester induction in several cell culture systems (Tzukerman, 1991). Neither example is completely in agreement with the observed estrogen inhibition of CRF stimulation of the POMC gene. Nevertheless, it appears that the different genes (promoters), cell types, steroid receptors, and oncoprotein components specify the type of gene regulation that is manifested by steroid receptor-AP-1 interactions (Shemshedini, 1991; Lucibello, 1990).

It is important to point out that the model presented above does not fully account for inhibition of CRF induction because it fails to explain CRF stimulation

that is conferred by the -234 to -133 region of the POMC promoter. This suggests that there are multiple elements contained within the POMC promoter that are responsive to CRF. Furthermore, it supports the notion that CRF enhancement of POMC gene expression is only partly due to the action of the AP-1 transcription factor.

Positive regulation

Hormonally-mediated transcriptional enhancement occurs by mechanisms that require binding of protein factors to specific regions of genes subject to regulated expression. These protein factors are thought to interact with components of the transcription initiation complex such that its formation is facilitated and transcription processes ensue (Maniatis, 1987; Lillie and Green, 1989; Lewin 1990). DNA recognition sites for numerous transcription factors have been identified. It is frequently the case that these sites are comprised of palindromic motifs of DNA that exhibit a dyad axis of symmetry. This is true of steroid receptor HREs (Beato, 1989 and references therein), the TPA responsive element (TRE) or AP-1 site (Kouzarides and Ziff, 1989), the CRE (Goodman, 1990), and the related activator transcription factor (ATF) site (Lin and Green, 1988). The functional significance of this arrangement of recognition sites is that it has become apparent that protein factors bind to these sites as either heterodimers or homodimers (reviewed in Jones, 1990; Forman and Samuels, 1990; Ziff, 1990) that are formed by virtue of a leucine zipper interaction (Landschultz, 1988).

The finding that there is a palindromic motif in the POMC promoter (-171 to -160) that confers CRF-stimulated gene expression is consistent with what has been demonstrated for other hormonally regulated genes as discussed above. That this sequence lies in a region (-234 to -133) that also confers cAMP-regulated POMC gene expression, supports the idea that in a complex promoter there are likely to be multiple elements that mediate transcriptional regulation. It is possible that the observed CRF and/or cAMP regulation of the POMC gene is mediated by an as of yet unidentified member of the ATF/CREB family of proteins. However, this would require that binding of the protein would occur to a novel recognition sequence. Thus, whether an ATF/CREB protein mediates the CRF response imparted by the palindromic motif remains unclear. Nevertheless,

it is clear that there is specific interaction of a protein of proteins to this region of the POMC promoter. Moreover, that binding appears to be enhanced in protein extracts from CRF treated cells, suggests that CRF treatment may work by activating a transcription factor that is already present in AtT20 cells by a post-translational modification. A likely mechanism for this is phosphorylation, which has been found to promote CREB protein activation (Gonzalez and Montminy, 1989; Lee, 1990).

The findings in Chapter 3 demonstrate a transient stimulation of POMC gene expression with acute estrogen treatment. As discussed earlier, this estrogen effect is thought to be transcriptional in basis, and it is presumably occurring through ER-dependent events. Rapid gene induction is reminiscent of the pattern of regulation of cellular immediate response genes (reviewed in Sheng and Greenberg, 1990; Morgan and Curran 1991). Transcriptional activation of the c-fos gene occurs within 5 min (Greenberg, 1985) and results in accumulation of c-fos mRNA that reaches maximal levels after 30-45 min (Muller, 1984). In several neuronal and endocrine systems the induction of c-fos gene expression has been associated with implementing responses to neurotransmitters, neuropeptides, and growth hormones.

Induction of c-fos gene expression by estrogen has been demonstrated in both neuronal and endocrine cells and in a reproductive organ. For example, in luteinizing hormone releasing hormone (GnRH) containing neurons, estrogen treatment increases Fos immunoreactivity on proestrous (Abbud, 1992; Plotsky and Rivest, 1992), thereby implying a connection between c-fos gene activation and reproductive function. Estrogen treatment in pituitary tumors has been shown to increase c-fos mRNA levels and this has been implicated in the formation of these estrogen-induced tumors (Kulig and Lloyd, 1992). In rat uterus it was shown that estrogen treatment resulted in rapid transcriptional induction of the c-fos gene and increased levels of c-fos mRNA (Weisz and Bresciani, 1988). Another study demonstrated that induction of c-fos mRNA in the uterus did not occur with progesterone, α-dihydrotestosterone, or dexamethasone treatment (Loose-Mitchell, 1988). Identification of a DNA element that is nearly identical to the ERE present in the Xenopus vitellogenin AII gene in the 3' flanking region of the mouse c-fos gene (Hyder, 1992), suggests that activation of c-fos by estrogen is receptor mediated.

It was initially postulated that induction of c-fos may play a role in the observed transient estrogen stimulation of POMC gene expression in ER-1 cells.

As presented in chapter 4, estrogen treatment, but not dexamethasone treatment induces expression of the c-fos gene in this system. Additionally, the high level of expression of the c-Jun protein in AtT20 cells (Boutillier, 1991) would explain the formation of an AP-1 complex upon estrogen treatment in ER-1 cells. Moreover, half-palindromic ERE consensus motifs have been shown to bind the AP-1 protein complex (Gaub, 1990). This brings into question a mechanism whereby AP-1 transactivation is mediated by either of the half EREs in the POMC promoter. The extremely rapid effects (within 20 min) of estrogen on POMC gene expression leave speculative a mechanism that would require new mRNA and protein synthesis. However, the time course of estrogen-induced c-fos expression in ER-1 cells is currently unknown and is presently under investigation. Nevertheless, induction of c-fos by estrogen but not glucocorticoids in ER-1 cells may explain the observed differences in POMC gene expression obtained with acute hormone treatment.

One possible alternate mechanism of acute estrogen stimulation of POMC gene expression involves fluctuations in levels of intracellular calcium. A role for calcium in POMC gene expression has already been established. The importance of intracellular calcium in positive regulation of POMC gene transcription has been demonstrated in anterior pituitary cultures (Eberwine, 1987) and in AtT20 cells (Lorang, 1992a). Moreover, accumulation of intracellular calcium and POMC mRNA in response to CRF treatment in AtT20 cells is inhibited by blockade of extracellular calcium entry (von Dreden, 1988). In an endometrial cell culture system, estrogen treatment has been shown to result in increased rates of calcium exchange following a minimum of 2.5 min treatment (Pietras and Szego, 1975). Hence, it is conceivable that in ER-1 cells, estrogen treatment results in alterations of calcium levels. Calcium is a second messenger in a signal transduction cascade which results in activation of calcium calmodulin kinase, and thus this offers an explanation of a rapid effect that could lead to post-translational modification of a previously existing transcription factor. This hypothesis remains speculative because to date, measurements of calcium levels in estrogen-treated ER-1 cells have not been made.

Data presented demonstrating a biphasic response of POMC gene expression to estrogen treatment in ER-1 cells, is not unusual in light of the rhythmic physiological modulation of POMC mRNA in the hypothalamus (Wise, 1990). This pattern of regulation relates to reproductive function and timing of the proestrous surge. Interestingly, ovariectomy, and hence removal of most of

the circulating estrogen, destroys the diurnal rhythm of POMC gene expression in rats (Wise, 1990). Thus, there is a physiologically relevant explanation for estrogen-stimulated POMC gene regulation. Moreover, this is concurrent with exposure of POMC-containing neurons to varying levels of estrogen and its positive and negative effects on POMC gene expression in the hypothalamus.

That the acute stimulation of POMC gene expression does not result in concomitant increases in mRNA content in ER-1 cells may be due to the large mRNA pool that exists in these cells. It has been estimated that there are approximately 20,000 to 50,000 POMC mRNA molecules in a single corticotrope (Lundblad and Roberts, 1988). Under basal conditions, there are between 100 to 200 new POMC mRNA molecules synthesized per hour (Eberwine and Roberts, 1984). This correlates to approximately 40 mRNA molecules in a 10 min period in which estrogen treatment results in primary transcript levels that are elevated by 50% (such as in ER-1 cells). Hence, a modest stimulation for a short time period is a minute percentage of the total mRNA pool. There may be differences in the basal level of POMC gene transcription in corticotropes and the corticotrope-derived AtT20/ER-1 cell line versus POMC-containing neurons in the hypothalamus that can account for the detectable changes in POMC mRNA levels *in vivo*. Alternatively, hypothalamic POMC-neurons may have a smaller POMC mRNA pool. Thus, a small stimulation in POMC biosynthesis may cause a greater change in POMC mRNA content.

The POMC gene represents an example of a gene whose regulation is intricate. Exposure of cells that express the POMC gene to numerous different hormones and neurotransmitters further complicates our understanding of the molecular mechanisms responsible for the action of a single effector. Furthermore, the complexity of the POMC promoter has made precise delineation of elements that mediate specific hormonal responses difficult. Nevertheless, it is apparent that as is true of many other genes that possess complex promoters, regions of the POMC promoter function in concert with one another within the context of the entire promoter to modulate gene expression.

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