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Pro-Opiomelanocortin Gene: A Model for Negative Regulation of Transcription by Glucocorticoids

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The gene encoding pro-opiomelanocortin (POMC) offers an interesting model system to study negative control of transcription in eucaryotes. Indeed, glucocorticoid hormones specifically inhibit transcription of the POMC gene in the anterior pituitary. The POMC gene is predominantly expressed in the anterior and intermediate lobes of the pituitary. However, only anterior pituitary POMC transcription is inhibited by glucocorticoids and stimulated by corticotropin-releasing hormone (CRH). Rat POMC promoter sequences required for anterior pituitary-specific expression were localized between positions −480 and −34 base pairs (bp) by DNA-mediated gene transfer into the POMC-expressing tumor cells, AtT-20. These POMC promoter sequences also confer glucocorticoid inhibition of transcription. While two of the six in vitro binding sites for purified glucocorticoid receptor identified in the rat POMC gene are within these sequences, only one is required for glucocorticoid inhibition; this binding site is located at position −63 bp in the promoter and overlaps a putative CCAAT box sequence. The DNA sequence of the POMC −63 bp receptor binding site is homologous to receptor binding sites identified in the glucocorticoid responsive element (GRE) of glucocorticoid-inducible genes. However, DNA sequence divergencies between these sites, in particular within the conserved hexanucleotide sequence 5′-TGTYCT-3′, may be involved in their opposite transcriptional activity. Alternatively, binding of the receptor in the promoter proximal region of the POMC gene may inhibit transcription by a hormone-dependent repressor mechanism.

Key words: pro-opiomelanocortin, DNA binding site, glucocorticoid-inhibitory element

Repression is an important mechanism in the control of gene expression in procaryotypes [1]. Bacterial repressors inhibit transcription by direct competition with RNA polymerase for overlapping DNA binding sites. In yeast, *cis*-acting nega-

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tive regulatory elements positioned between "upstream activating sequences" and the proximal promoter have been described [2]. The activity of the yeast negative regulatory elements depends on its position within the promoter, which suggest that binding at these regulatory sites disrupts essential protein:protein interactions [3].

In higher eucaryotes, few examples of negative regulation of transcription have been documented, other than the glucocorticoid inhibition of anterior pituitary pro-opiomelanocortin (POMC) gene transcription [4–6]. In many cases, negative control of gene expression was suggested by the stimulatory effect of protein inhibitors on transcription of specific genes like *c-myc* [7], immunoglobulin κ-light chains [8], cytoskeletal actins, and β-interferon [9], where a *cis*-acting regulatory element was identified [10]. In contrast, positive regulation of transcription was extensively studied [11]; particularly, the detailed analysis of glucocorticoid-dependent induction of transcription [12] has provided the conceptual framework for ongoing work on the molecular mechanism of positive regulation of transcription. In fact, the glucocorticoid receptor which binds directly to regulatory sequences of glucocorticoid-inducible genes [13,14] is the best-characterized eucaryotic *trans*-acting factor.

In view of the extensive knowledge gained regarding the mechanism of induction of transcription by glucocorticoids, studies on the mechanism of glucocorticoid inhibition of transcription may provide a basis for understanding negative control of transcription in eucaryotes. The negative feedback action of glucocorticoids on pituitary adrenocorticotropin (ACTH) release and POMC gene transcription has been recognized for a long time as an important feature in the control of the pituitary-adrenal axis [15]. The demonstration that glucocorticoids inhibit POMC gene expression at the transcriptional level [4–6] indicates that POMC is a useful model system to study glucocorticoid inhibition of transcription; in addition, such studies were facilitated by the availability of cloned genomic sequences for the rat [16,17], human [18–20], mouse [21,22], and bovine [23] POMC genes.

TISSUE-SPECIFIC EXPRESSION AND REGULATION OF POMC

POMC is the protein precursor to a variety of active hormonal peptides, which include adrenocorticotropin (ACTH), β-endorphin, β-lipotropin (β-LPH), and the melanotropins (α-, β-, γ-MSH) [24]. The POMC gene is predominantly expressed in the anterior and intermediate lobes of the pituitary; POMC mRNA is also found in the hypothalamus, the amygdala, the cortex, the testes, the ovaries, and the placenta [25–28]. POMC is differentially processed and regulated in these different tissues as summarized in Figure 1.

Pituitary Gland

The release of POMC-derived peptides from the pituitary gland is under multihormonal control [29]. In the anterior but not in the intermediate pituitary, glucocorticoids inhibit POMC gene transcription [4–6] rapidly (Fig. 2) and by a mechanism which is independent of de novo protein synthesis (Table I). Since these observations are consistent with a glucocorticoid receptor-dependent mechanism of inhibition, it was suggested that the lack of glucocorticoid response in the intermediate pituitary may be due to the absence of glucocorticoid receptors in POMC-expressing cells of this tissue [30]. The in vivo administration of dexamethasone (DEX) to adrenalectomized rats inhibits anterior pituitary POMC transcription rate by about tenfold (Fig.

	MAJOR PROCESSING PRODUCTS	REGULATORY AGENTS	
		STIMULI	INHIBITORS
ANTERIOR PITUITARY	ACTH β-LIPOTROPIN	CRF ARG-VASOPRESSIN α-ADRENERGIC	GLUCOCORTICOIDS
INTERMEDIATE PITUITARY	α-MELANOTROPIN CLIP β-ENDORPHIN γ-LIPOTROPIN	CRF β-ADRENERGIC	DOPAMINE
HYPOTHALAMUS	α-MELANOTROPIN β-ENDORPHIN		ESTROGENS
TESTIS (LEYDIG CELLS)	α-MELANOTROPIN β-ENDORPHIN	LH	
OVARIES	α-MELANOTROPIN β-ENDORPHIN	LH ANDROGENS	

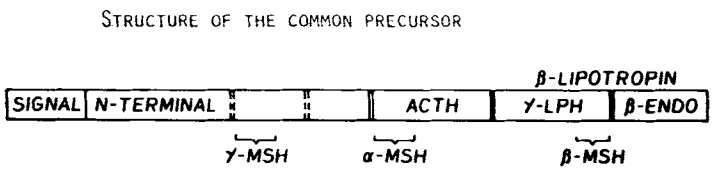

Fig. 1. Regulation of pro-opiomelanocortin (POMC) expression. Tissues in which POMC mRNA was detected are listed together with a list of known POMC processing products and regulatory agents. Original work on the identification of POMC processing products in these various tissues was recently reviewed [24]. Supporting data on the role of various regulatory agents on POMC expression was presented or reviewed in references 27–29, 31–36, 59–61. The structure of the POMC precursor protein is presented at the bottom of the figure.

2). Because the inhibition of transcription observed in vivo could be due to a direct glucocorticoid action at the pituitary level as well as through inhibition of hypothalamic corticotropin-releasing hormone (CRH) release or a combination of both, the effect of glucocorticoids on isolated pituitary cells was assessed to determined the extent of direct pituitary glucocorticoid feedback [6]. The threefold inhibition of POMC transcription rate observed in anterior pituitary cells maintained in primary culture (Table I) suggests that POMC transcription rate is regulated both by a direct pituitary glucocorticoid action and through modulation of other hormone(s) which contribute to control POMC transcription. In keeping with this hypothesis and with the stimulatory effect of CRH on ACTH release, we found that CRH specifically stimulates anterior pituitary POMC transcription rate (Table I). This stimulatory effect is mimicked by increased intracellular cyclic AMP levels and is competed by DEX. As expected [29], DEX also competes CRH- and cyclic AMP-stimulated ACTH release. The control of POMC gene expression is similar in the mouse corticotrophic cell line of pituitary origin AtT-20 [31,32], thus offering a useful host system for studies of POMC promoter function and regulation.

Extrapituitary POMC Expression

POMC is expressed in nonpituitary tissues, albeit at much lower levels [25–28]. While hypothalamic and pituitary POMC mRNAs are similar, shorter POMC transcripts are present in gonadal tissues. This size difference appears due to differences

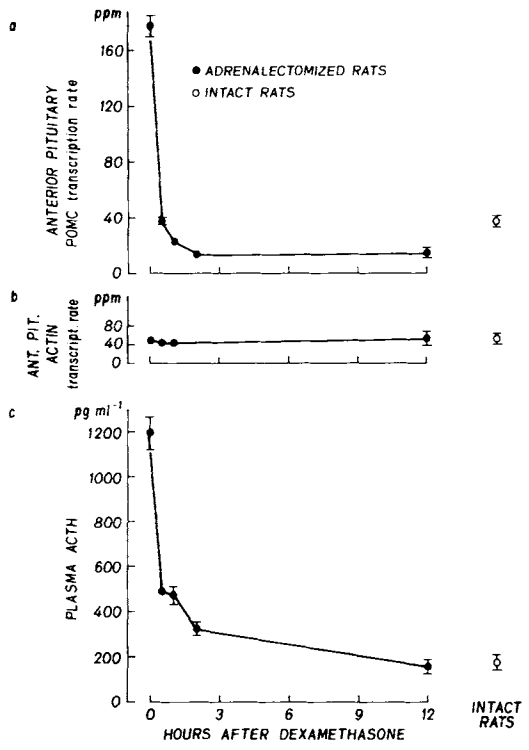

Fig. 2. Effect of a single injection of 75 μg dexamethasone to adrenalectomized male Sprague-Dawley rats on anterior pituitary POMC (**a**) and actin (**b**) gene transcription rates and on plasma ACTH levels (**c**). Adrenalectomy was performed 7–9 days before DEX injection. [^{32}P]-RNA input was 0.5–1.9 $\times$ 10^6 cpm per hybridization. Transcription rates and plasma ACTH were also measured in intact untreated animals of the same group for comparison.

in the 5′-end of the mRNA [28]. However, POMC-derived peptides appear similar in gonadal tissues and in the hypothalamus (Fig. 1). Extrapituitary POMC expression also appears to be regulated by steroid hormones, but not by glucocorticoids [33,34]. Indeed, hypothalamic POMC mRNA is decreased by estrogens [35], while ovarian POMC mRNA is increased by androgens [36]. In both testicular Leydig cells and ovarian granulosa cells, but appearently not in the placenta, gonadotropins increase POMC mRNA levels [28,34,36]. Even if the role of POMC-derived peptides in nonpituitary tissues is still elusive, it may be interesting to compare the mechanism of steroid regulation of nonpituitary POMC with the glucocorticoid inhibition of anterior pituitary POMC transcription.

POMC PROMOTER ACTIVITY

In order to study glucocorticoid inhibition of transcription, we developed an assay for POMC promoter sequences using the AtT-20 pituitary tumor cells as host. To this end, we constructed chimaeric genes containing different rat POMC promoter fragments fused to coding sequences for bacterial neomycin (neo) resistance [37]. The chimaeric POMC neo genes contain different lengths of POMC 5′-flanking sequences down to nucleotide + 63 within the first exon. A short segment (24 bp) of

TABLE I. Regulation of POMC Transcription Rate in Primary Cultures of Rat Pituitary Cells*

	Anterior pituitary cells		Neurointermediate pituitary cells	
	Relative transcription rate	ACTH release	Relative transcription rate	α-MSH release
Control	(26.7 ± 3.7 ppm)	(1.1 ± .2 ng/ml)	(204 ± 20 ppm)	(3.8 ± .8 ng/ml)
Dexamethasone 10^{-7}M (DEX)	0.37 ± .02	1.04 ± .08	ND	ND
CRH 10^{-8}M	1.8 ± .06	5.9 ± .8	1.1 ± .02	2 ± .1
CRH + DEX	0.8 ± .05	1.7 ± .4	1.1 ± .02	1.6 ± .04
Cycloheximide (CYCLO) 3×10^{-5}M	0.73 ± .03	1.08 ± .01	0.8 ± .06	0.8 ± .02
CYCLO + DEX	0.31 ± .02	1.17 ± .04	ND	ND
CYCLO + CRH	1.86 ± .2	8.0 ± .5	1.1 ± .07	1.1 ± .02
8-Bromo-cAMP 3mM	1.95 ± .05	3.8 ± .8	1.55 ± .01	2.3 ± .05
8-Bromo-cAMP + DEX	0.83 ± .07	1.6 ± .1	ND	ND
Forskolin 10μM	1.62 ± .07	2.1 ± .01	1.5 ± .1	3.1 ± .2

*Transcription rates and peptide release were measured as described [6] after 30-min incubations with the indicated substances. The data presented are the average ± SEM of values from 2–4 experiments, each performed in duplicates or triplicates. Absolute values are shown for controls while other values are relative to control taken as one. ND = not determined.

simian virus 40 (SV40) early 5′-untranslated sequences was inserted between POMC and neo sequences to serve as a tag in RNase mapping experiments [38]; this tag is required in order to differentiate endogenous mouse POMC mRNA present in AtT-20 cells from ratPOMCneo transcripts [39]. The efficiency of promoter utilization was assessed by the number of G418-resistant colonies after electroporation [40] of plasmids into AtT-20 cells. Electroporation of the plasmids, $pPOMC_{4500}neo$ and $pPOMC_{706}neo$, yielded on average 200 G418-resistant colonies per 5×10^6 electroporated AtT-20 cells; the same plasmids were as inefficient in L cells (five G418-resistant foci per 5×10^6 electroporated cells) as a negative control plasmid devoid of any eucaryotic promoter sequences (Fig. 3), indicating that the POMC promoter is only efficiently utilized in the pituitary cell line [39]. In order to minimize the effects of chromosomal integration site on transcription of the POMCneo gene, we analyzed transcripts isolated from pools of G418-resistant AtT-20 colonies propagated in mass culture.

The specificity of POMC transcription initiation was assessed using an RNase protection assay as described [38]; the structure of the RNA probe used in these assays is illustrated at the bottom of Figure 3B; the same probe allows simultaneous detection of the rat POMCneo (rPOMCneo) transcripts (protected fragment of 87 nt) and of endogenous mouse POMC (mPOMC) transcripts (protected fragment of 63 nt). As shown in Figure 3B, initiation of transcription from the electroporated POMC promoter is extremely specific in AtT-20 cells, while it is completely artefactual in L cells. This pattern of specifity is in agreement with a tissue-specific recognition of the POMC promoter. The similar activities of the two plasmids containing either 4,500 bp or 706 bp upstream POMC sequences indicates that no more than 706 bp 5′-flanking sequences are required to mimick anterior pituitary-specific expression. Deletion of POMC sequences up to position −480 bp does not affect promoter activity; however, promoter activity is progressively reduced when sequences beyond position −480 bp are deleted [39].

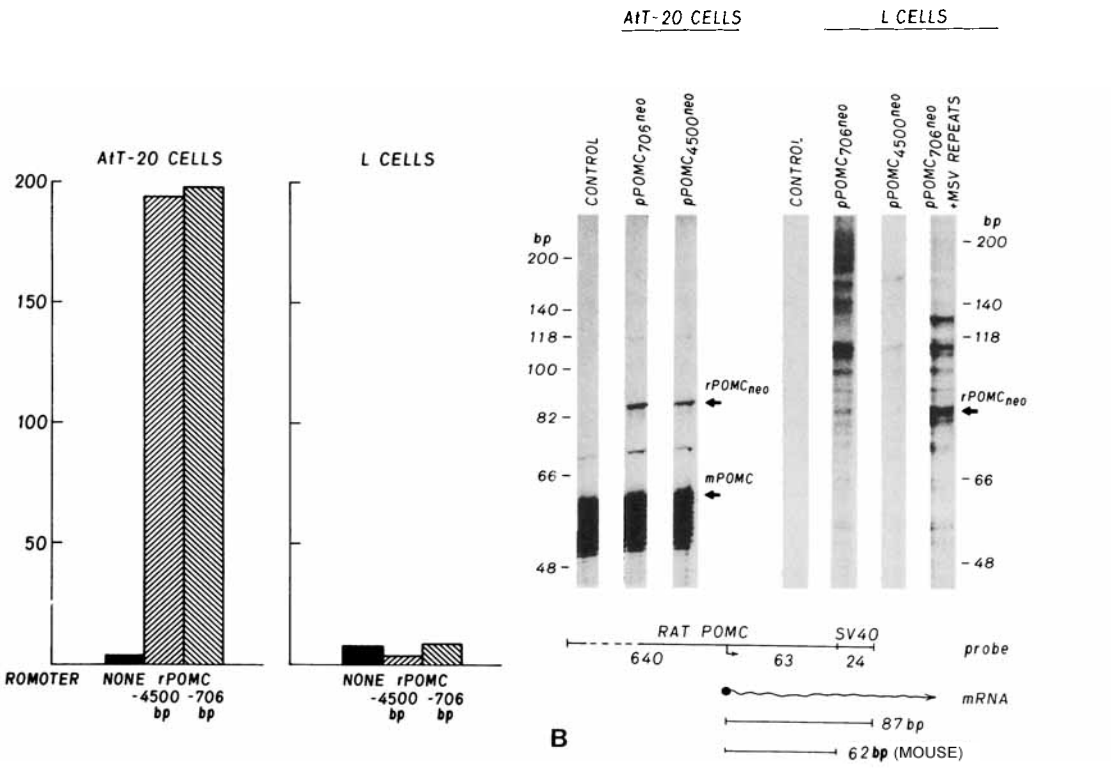

Fig. 3. Specific expression of the rat POMC promoter in AtT-20 cells. **A:** POMC promoter utilization is assessed by the number of G418-resistant colonies produced by electroporation of 5×10^6 AtT-20 or L cells with plasmids pPLneo which does not contain any eucaryotic promoter sequences or with plasmids $pPOMC_{4500}neo$ and $pPOMC_{706}neo$ which contain 4,500 bp or 706 bp 5′-flanking POMC sequences, respectively. The data represent the average of 8–18 determinations. An average of 5–10 copies of integrated plasmid DNA per cell was measured by Southern blot performed on genomic DNA extracted from pools of G418-resistant colonies [39]. **B:** Analysis of POMCneo transcripts and of endogenous POMC mRNA (AtT-20 cells) by RNase mapping [39]. As illustrated at the bottom of the figure, the RNA probe contains the first 63 nucleotides of the rat POMC mRNA and 24 nucleotides of SV40 early 5′-untranslated sequences. Hybridization of this probe to endogenous AtT-20 cells POMC mRNA protects a 63-nucleotide fragment (arrow mPOMC), while hybridization of the same probe to POMCneo transcripts protects an 87-nucleotide fragment (arrow, rPOMCneo). The position of single-stranded DNA markers is indicated on the sides of the figure.

POMC PROMOTER SEQUENCES CONFER GLUCOCORTICOID INHIBITION

The presence of DNA sequences responsible for glucocorticoid inhibition of transcription within the POMC promoter was first demonstrated in expression systems which depend entirely on viral DNA sequences for expression [38,41]. For example, we showed that a hybrid transcription unit constituted of a rat POMC promoter fragment linked to simian virus 40 (SV40) tumor antigen (Tag) coding sequences is transcribed from the correct site of initiation when present on bovine papilloma virus (BPV) episomes [38]. This system was also used to study glucocorticoid induction of mouse mammary tumor virus (MTV) transcription [42]. As illustrated in Figure 4, treatment of three different cellular clones each containing a different BPV episomal

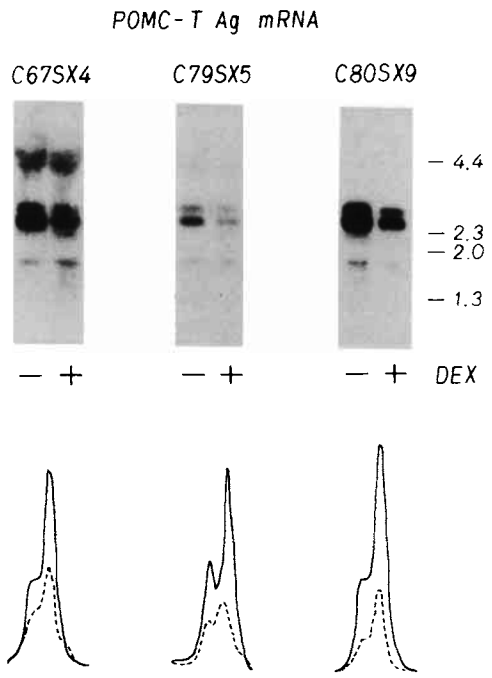

Fig. 4. Northern blot analysis of POMC-Tag transcripts present in three BPV-transformed clones of C127 cells. The three clones contain different episomal BPV constructs which differ in the relative orientation of POMC-Tag and viral transcription. All clones contain the same differentially spliced POMC-Tag transcripts encoding small and large T antigens, despite very different episome copy number and transcript levels [38]. Treatment of cells with 10^{-7}M dexamethasome (DEX, indicated by the plus sign) results in a threefold inhibition of POMC-Tag transcripts as revealed by densitometric scanning. Molecular size markers are indicated in kilobase pairs on the right.

construct with the synthetic glucocorticoid dexamethasone (DEX) decreases the levels of fusion POMC-Tag transcripts about threefold. Thus, the 769-bp rat POMC promoter fragment present in these BPV episomes is sufficient to confer glucocorticoid inhibition. Similarly, Israel and Cohen showed that human POMC sequences extending between positions −670 bp and −38 bp are sufficient to confer glucocorticoid inhibition [41].

Glucocorticoid Receptor Binding Sites

Glucocorticoid induction of transcription is mediated by direct glucocorticoid receptor contact with DNA [12–14,43]. In order to determine whether glucocorticoid inhibition of POMC transcription is associated with binding of the glucocorticoid receptor to specific sequences in the POMC gene, we analyzed the binding of purified rat liver glucocorticoid receptor to rat POMC genomic DNA. Six DNA segments, each 23–26 bp in length, showed glucocorticoid receptor-dependent protection in DNaseI and exonuclease III footprinting experiments and in dimethyl sulfate (DMS) protection experiments [44]. Three binding sites are located in the 5′-flanking region (they are centered on positions −579, −146, and −63 bp), one is within exon 1 (at position +64 bp), and the last two are in the first intron (at positions +1.45 and +1.9

kilobase pairs [Kb]). It was previously shown that exons 2 and 3 and intron B of the rat POMC gene do not contain glucocorticoid receptor binding sites [13]. Comparison of POMC receptor binding sites with those present in glucocorticoid-inducible genes shows that the greatest DNA sequence homology between these sites is within the concensus hexanucleotide 5′-TGTYCT-3′ [12,45]. Although the hexanucleotide sequence is conserved in five POMC receptor binding sites, these hexanucleotide sequences contain bases, in particular at the first and last positions, that were rarely observed before in glucocorticoid-inducible genes [45]. The DNA sequence of the −146-bp binding site is GC-rich and presents an asymmetric strand distribution of purines and pyrimidines. It does not show any homology to the other POMC binding sites or to sites in glucocorticoid-induced genes.

In MTV and in other glucocorticoid-inducible genes, binding of the glucocorticoid receptor results in protection of specific guanine residues from DMS methylation, in particular within the conserved hexanucleotide sequence where guanine residues on both strands are protected [46]. A similar pattern of DMS hypomethylation is found within glucocorticoid receptor binding sites −579, −63, +1,450, and +1,900 of the POMC gene, suggesting that receptor recognition of POMC and glucocorticoid-inducible DNA binding sites is similar.

Glucocorticoid Inhibition of Transcription

In order to localize POMC promoter sequences responsible for glucocorticoid inhibition of transcription, we used AtT-20 cells electroporated with the POMCneo hybrid genes described above and tested them for glucocorticoid sensitivity. The effect of overnight treatment of cells with DEX was assessed using the RNase mapping assay illustrated in Figure 3b. In AtT-20 cells, which contain an average 5–10 copies per cell of plasmid $pPOMC_{706}neo$, DEX treatment decreases by about threefold the levels of both AtT-20 POMC mRNA and rPOMCneo transcripts. The threefold glucocorticoid-dependent inhibition conferred by this 769-bp POMC promoter fragment correlates perfectly with the threefold decrease in POMC gene transcription rate measured after DEX treatment of rat anterior pituitary cells in primary culture (Table I) [6] and with the threefold decreases of POMC mRNA levels observed after DEX treatment of rats [47] and AtT-20 cells [31].

The first 706 bp of the POMC promoter contain three glucocorticoid receptor binding sites (Fig. 5). In order to determine which site(s) is(are) required for glucocorticoid inhibition, we tested the glucocortoid sensitivity of promoter deletion mutants. As illustrated in Figure 5, deletion of POMC sequences up to position −480 bp does not interfere with glucocorticoid inhibition, indicating that the glucocorticoid receptor binding site present at position −579 bp is not essential for glucocorticoid inhibition since its deletion does not affect the magnitude of DEX inhibition. Resection of the promoter beyond the −146 bp receptor binding site affected promoter activity so much that glucocorticoid sensitivity could not be tested.

In order to determine if either or both the −146 and the −63 bp glucocorticoid receptor binding sites contribute to glucocorticoid inhibition, we deleted either site from a POMC promoter fragment extending to position −480 bp. Deletion of sequences between positions −132 and −38, which eliminates the −64 bp binding site, abolishes glucocorticoid sensitivity. Conversely, deletion of sequences between positions −166 and −132 bp does not prevent the threefold glucocorticoid inhibition. These results indicate that the −146-bp glucocorticoid receptor binding site cannot

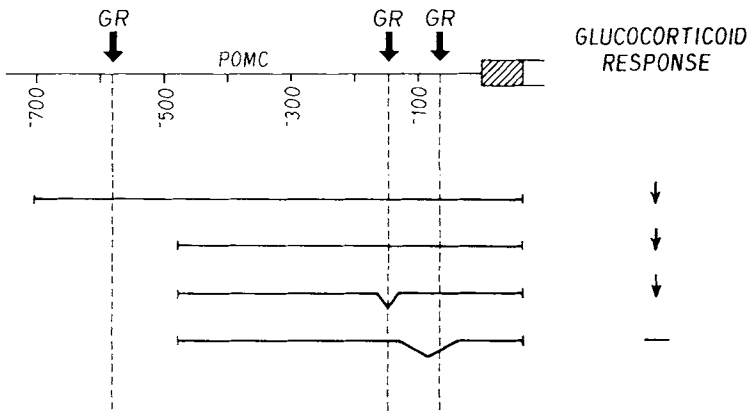

Fig. 5. Localization of POMC glucocorticoid inhibitory element (GIE). The glucocorticoid response of various 5'-deletion and internal deletion mutants is represented schematically below a map of the rat POMC promoter where in vitro glucocorticoid receptor binding sites (GR) are indicated by arrows.

confer glucocorticoid responsiveness; furthermore, it does not exhibit any cooperativity with the active −63-bp glucocorticoid receptor binding site.

Our data show that maximal glucocorticoid inhibition of transcription can be achieved with a promoter fragment which contains only one glucocorticoid receptor binding site (Fig. 5). Thus, we have defined within the POMC promoter a glucocorticoid-inhibitory element (GIE) which contains a single glucocorticoid receptor binding site (−63-bp site) homologous to similar sites in glucocorticoid-inducible genes.

THE POMC GLUCOCORTICOID INHIBITORY ELEMENT (GIE)

It is intriguing to speculate how binding of the glucocorticoid receptor to the −63-bp site of POMC produces an opposite transcriptional effect to binding of the same receptor to the MTV glucocorticoid-responsive element (GRE). Binding of the receptor to glucocorticoid-inducible GREs results in transcriptional activation by a hormone-dependent enhancer mechanism [48,49]. While this enhancerlike mechanism can readily account for transcription stimulation, it is diffiuclt to reconcile with glucocorticoid inhibition of transcription.

Protein domains required for the transcriptional activity of the glucocorticoid receptor were recently identified [50,51]. However, the DNA sequence requirements for receptor binding and transcription activation of glucocorticoid-inducible GREs have not been defined at the single nucleotide level. Binding of the receptor to the −63-bp site of POMC may inhibit (rather than stimulate) transcription because of a different receptor:DNA interaction by comparison to the receptor:DNA interaction taking place in glucocorticoid-inducible GREs. These putative differences in receptor:DNA interactions may result from differences in the primary DNA sequence of the binding sites. The DMS protection pattern of guanine residues within the POMC −63-bp site (Fig. 6) is similar to the pattern observed in receptor binding sites of glucocorticoid-inducible GREs [46]. This suggests that similar receptor:DNA recognition takes place in the major groove of the double helix. However, the intimate interaction between the receptor and DNA sequence may differ as a result of nucleotide changes other than guanosines within the recognition sequence. In this context, it

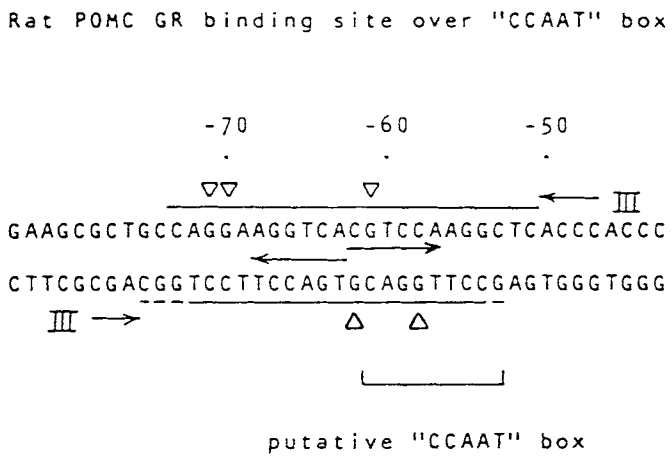

Fig. 6. Structure of the glucocorticoid receptor binding site present in the glucocorticoid inhibitory element (GIE) of POMC. The region protected by the receptor in DNaseI footprinting experiments is indicated by lines above and below the DNA sequence. The boundaries of the receptor binding site revealed in exonuclease III footprinting experiments are shown by arrows labelled III. Guanine residues which are hypomethylated by DMS in the presence of receptor are indicated by triangles. Arrows indicate hexanucleotides which are homologous to the concensus 5′-TGTYCT-3′ present in most glucocorticoid receptor binding sites [45]. The position of a putative "CCAAT" box sequence which overlaps the glucocorticoid receptor binding site is shown.

is noteworthy that the hexanucleotide 5′-TGTYCT-3′ is invariably present in all functional GREs [13,14,52–57]. In contrast, the DNA sequence of the −63-bp site contains notable differences within its homologous hexanucleotide 5′-CGTCCA-3′.

Four out of twenty-two reported receptor binding sites detected in glucocorticoid-inducible genes contain a base other than T in the first position of the hexanucleotide; however, a C was never observed in that position [45]. Furthermore, the only two receptor binding sites which contain a divergent base at the last position of the hexanucleotide were shown to be inactive [53,57]. The significance of the divergent nucleotides within the hexamer of the POMC −63-bp receptor binding sites will have to be tested directly by mutagenesis.

While differences in receptor:DNA interaction could account for opposite transcriptional activities, glucocorticoid inhibition of transcription may result from receptor binding in an essential region of the promoter; thus, the glucocorticoid receptor may behave as a hormone-dependent repressor. This mode of action is compatible with our data, regardless of the nature of the receptor:DNA interaction. Indeed, whether or not the POMC −63-bp binding site could stimulate transcription when tested on a heterologous promoter, the most important property of this site may be its position within the POMC promoter. Within the POMC promoter proximal region, the −63-bp receptor binding site (Fig. 6) overlaps with a putative "CCAAT" box and with a sequence homologous to a cyclic AMP-responsive element [58]. Binding of the glucocorticoid receptor to the −63-bp site could compete with binding of other trans-acting factors to an overlapping DNA sequence, like the "CCAAT" box. Alternatively, binding of the receptor at the −63-bp site may prevent the interaction between two or more trans-acting factors which recognize DNA sequences outside the −63-bp region.

In conclusion, a *cis*-regulatory element which contains a single glucocorticoid receptor binding site and which appears responsible for glucocorticoid inhibition of anterior pituitary POMC transcription was identified in promoter sequences of the rat POMC gene. This glucocorticoid-inhibitory element (GIE) may inhibit transcription by a hormone-dependent repressor mechanism.

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