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MOLECULAR MECHANISMS OF GLUCOCORTICOID INHIBITION OF HUMAN PROOPIOMELANOCORTIN GENE TRANSCRIPTION

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Summary—The gene encoding proopiomelanocortin(POMC) offers an interesting model for negative regulation of gene transcription by glucocorticoids. A fragment of human genomic DNA containing the entire POMC gene, together with the neo marker gene, was introduced by transfection into the ACTH-producing mouse pituitary tumor cell line, AtT-20, and the mouse fibroblast L cell line. In the transformed AtT-20 cells the human POMC gene was transcribed correctly and the transcript was spliced faithfully. Furthermore, the addition of dexamethasone to the transformed AtT-20 cells resulted in a 40% reduction of the human POMC mRNA levels. Deletion analysis demonstrated that no more than 417 bp in the 5′-flanking region of the human POMC gene are required for transcriptional repression by glucocorticoid. This region was also responsible for the transcription induction of the human POMC gene by cyclic AMP (cAMP). In the transformed L cells, however, most of the transcripts of the human POMC gene were not correctly initiated. The addition of dexamethasone to the transformed L cells did not significantly affect the content of human POMC mRNA, although these cells expressed glucocorticoid receptor(GR). However, the increase of the transcripts by forskolin, a post-receptor adenylate cyclase-activating agent, was partially but significantly suppressed by dexamethasone in the transformed L cells. These results suggest that binding of GR to the negative glucocorticoid response element (nGRE) could lead to steric occlusion of positive transcription factors, such as cAMP-response element binding protein and tissue specific factors or that GR bound to nGRE could interact with DNA-bound positive factors in such a way as to prevent their transcriptional stimulatory activity.

INTRODUCTION

Steroid hormones modulate the expression of various genes both positively and negatively. Many previous studies have revealed positive regulatory mechanisms of the gene expression by steroid hormones [1, 2]. Transduction of the hormonal signal is mediated through specific binding of a steroid receptor binding element of the target genes [3].

Less is known about the way in which a steroid hormone represses the gene transcription. Selective repression is, however, an important mechanism of the transcriptional control. Recently it has become clear that repression is mediated at the transcriptional level by hormone receptors that function by binding to specific DNA recognition sites [4]. Thus hormone receptors can be transcriptional repressors as well as activators.

Glucocorticoids negatively regulate the transcription of certain genes including collagen, α-fetoprotein, stromelysin, α-subunit of glycoprotein hormone, prolactin and proopiomelanocortin (POMC) genes [5]. The molecular mechanisms of gene repression by glucocorticoids have been analyzed in some negatively regulated genes such as α-subunit of glycoprotein hormone and prolactin genes [6, 7].

In this study, we have defined a regulatory element, located in the proximal region of the POMC promoter that confers glucocorticoid repression in the pituitary.

TISSUE-SPECIFIC EXPRESSION OF THE HUMAN POMC GENE

The POMC gene encodes the common precursor to a variety of biologically active peptides, such as ACTH, β-endorphin and so forth.

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The gene is composed of approx. 7.6 kilobase pairs (kbp), with 3 exons separated by 2 introns of approx. 3.7 and 2.9 kbp [8, 9].

The human POMC gene is transcribed preferentially in ACTH-producing cells in the pituitary giving rise a 1100 bp mRNA. Recent studies, however, revealed that low levels of POMC-related RNA species were detected in non-pituitary tissues, such as certain areas of the brain, gastrointestinal tract, adrenal medulla, testes, ovaries, thyroid, thymus and lung. Northern blot analysis of non-pituitary tissues revealed POMC-like mRNAs of approx. 800 bp.

Primer extension and S1 mapping studies showed that these RNA species lack exon 1 and 2. Their 5′ ends are heterogenous and map within exon 3. Whereas DNA sequences upstream of the transcription initial site in the pituitary contain the usual promoter elements such as TATA box, no such element can be found upstream of the putative site of transcription initiation in non-pituitary tissues. This region only contains GC-rich regions which are reminiscent of the promoters of housekeeping genes [9].

The introduction of cloned eukaryotic genes into the cultured cells provides a useful tool to investigate the molecular mechanisms of the regulation of gene expression.

ACTH-producing mouse pituitary tumor cell line, AtT-20 cells or the mouse fibroblast cell line, L cells, were co-transfected with two plasmids, pHAL1 and pSV2-neo, by the calcium–phosphate precipitation method [9]. The AtT-20 cells efficiently express the endogenous mouse POMC gene and have been used extensively as a model system for analyzing the expression of the endogenous mouse POMC gene [10]. On the other hand, L cells do not express the endogenous mouse POMC gene, but possess functional glucocorticoid receptor (GR) [11].

The plasmid pHAL1 is carrying the 11.5 kb *Eco* RI fragment of human genomic DNA containing the entire POMC gene, about 7.6 kb in length, with 3 kb of the 5′-flanking sequence and 0.9 kb of the 3′-flanking sequence. The stable cell lines that express the neo gene were selected in medium containing an antibiotic G418 (Geneticin).

To test whether the integrated human POMC gene expressed, RNA isolated from G418 resistant transformants was analyzed by Northern blot analysis. Total RNA extracted from the transformed AtT-20 cells contained two hybridization bands: the larger one is identical in size to mature human POMC mRNA found in the human pituitary and the smaller one apparently represents endogenous mouse mRNA.

Blot analysis using poly(A)-rich RNA from the transformed L cells showed apparently several hybridization bands, approx. 800 to 1500 nucleotides. The amount of mRNA transcribed from integrated human POMC gene in the L transformants was much less than that in the AtT-20 transformants. Poly(A)-rich RNA from non-transformed L cells showed no hybridization with the POMC-specific probe.

Southern blot analysis revealed that the intensity of the 11.5 kb bands, identical in size to the *Eco* RI fragment containing the human POMC gene in pHAL1, was not significantly different among AtT-20 cell clones and L cell clones.

These results suggest the presence of tissue-specific transcription factors or repressors in these tissues [9, 12].

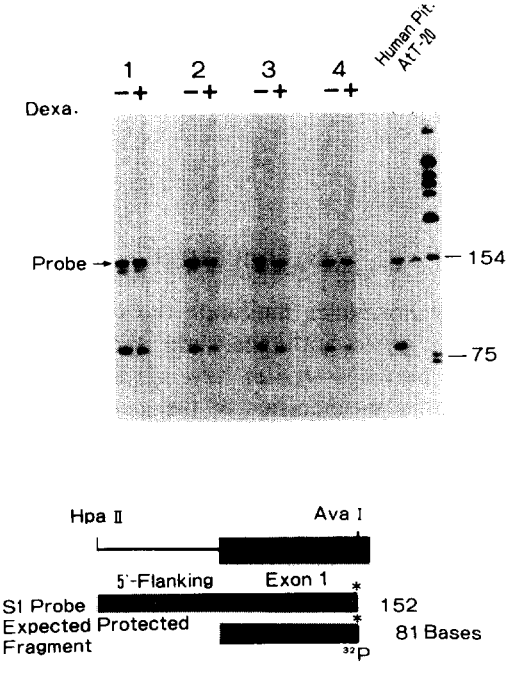

Fig. 1. Effect of dexamethasone on the human POMC gene expression in AtT-20 transformants estimated by S1 mapping analysis. The probe was a 152 bp *Hpa* II–*Ava* I fragment containing exon 1 of the human POMC gene (5′ end-labeled with ^{32}P at *Ava* I site). A fragment of 81 nucleotides was protected by POMC mRNA. Lanes 1–4: total RNA (10 μg) from 4 groups of AtT-20 transformants grown in the absence (−) or presence (+) of dexamethasone. Each group consists of 3 independent clones; lane 5: total RNA (1 μg) from the human pituitary; and lane 6: total RNA (5 μg) from wild-type AtT-20 cells.

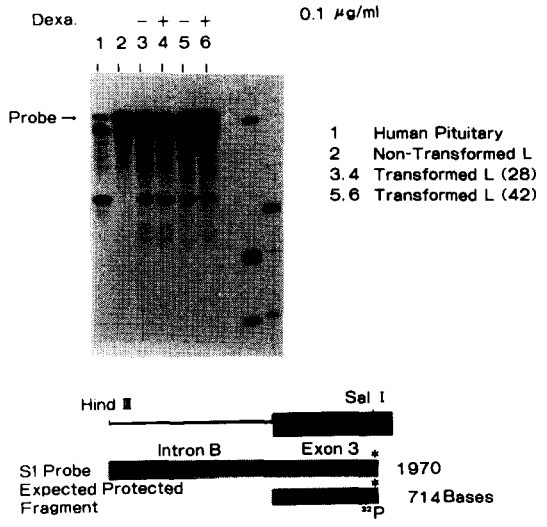

Fig. 2. Effect of dexamethasone on the human POMC gene expression in L transformants estimated by S1 mapping analysis. The probe was a 1970 bp *Hind* III–*Sal* I fragment containing exon 3 of the human POMC gene (5′ end-labeled with ^{32}P at *Sal* I site). A fragment of 714 nucleotides was protected by POMC mRNA. Lane 1: total RNA (1 μg) from human pituitary; lane 2: total RNA (100 μg) from wild-type L cells, and lanes 3–6: total RNA (100 μg) from two clones of L transformants grown in the absence (−) or presence (+) of dexamethasone.

REGULATION OF HUMAN POMC GENE EXPRESSION

Corticotropin-releasing hormone (CRH) stimulates both ACTH release and POMC mRNA levels in the human pituitary, while glucocorticoids inhibit both [8, 13, 14]. The cyclic AMP (cAMP) mimics the stimulatory effect of CRH on the pituitary POMC transcription rate [8]. Very little is known, however, about the regulation of POMC production in non-pituitary tissues in human.

To test the effect of glucocorticoid on the expression of the introduced human POMC gene, the transformed AtT-20 cells were grown in dexamethasone-supplemented medium for four days. Quantitation by S1 nuclease analysis indicated that the level of human POMC mRNA was suppressed to approx. 40% of basal levels by the addition of 2.6×10^{-7} M dexamethasone to the medium (Fig. 1).

On the other hand, the amount of human POMC-related mRNA in L transformants was not significantly affected by the addition of dexamethasone to the medium (Fig. 2).

HUMAN POMC GENE PROMOTER STRUCTURE

In order to localize the POMC promoter sequences responsible for tissue-specific expression and glucocorticoid inhibition of transcription, different portions of the 5′-flanking region of the human POMC gene were fused to the bacterial chloramphenicol acetyltransferase (CAT) gene and stably transfected into AtT-20 cells, L cells and rat glial cell line, C6 cells, together with the neo marker gene, resulting in APCAT, LPCAT and CPCAT, respectively. Plasmid pPOMC–CAT1 contains 2.9 kb of the 5′-flanking sequence of human POMC gene including TATA box and 63 nucleotides of exon 1. The plasmids pPOMC–CAT2, 3 and 4 contain 417, 97 and 40 bp of the 5′-flanking sequence of human POMC gene, respectively.

Chimeric genes containing various fragments in the 5′-flanking POMC gene were tested for transcriptional activity and tissue specificity by introduction into AtT-20 cells. As shown in Fig. 3, no more than 1600 bp 5′-flanking POMC sequences are required for tissue-specific expression.

Dexamethasone inhibited CAT activity in a dose-dependent manner up to 10^{-6} M in APCAT1 cells. The time course of inhibition of CAT activity was determined in APCAT1 cells with 10^{-6} M dexamethasone for 12, 24, 48 and 72 h. The CAT activity was inhibited in every time period (Fig. 4). Thus, this promoter region

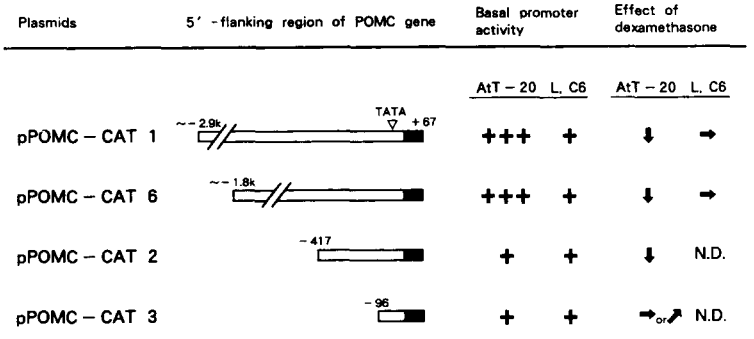

Fig. 3. Localization of POMC promoter sequences and of negative glucocorticoid response elements. For more details see text.

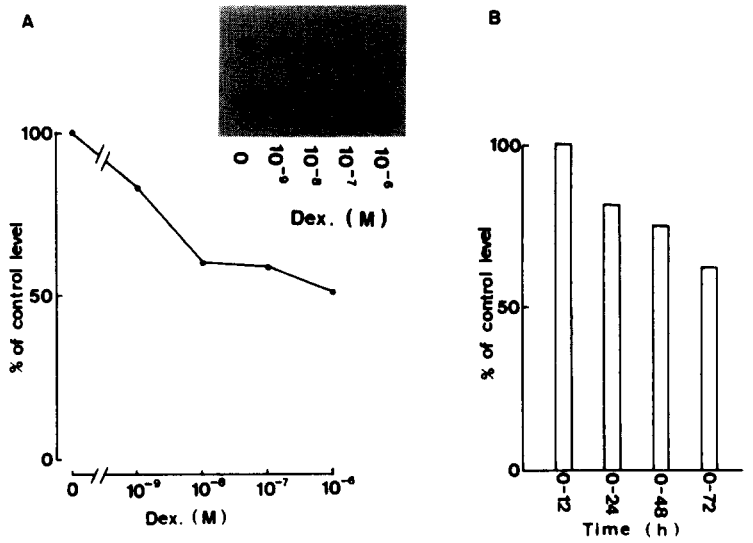

Fig. 4. The effect of dexamethasone on CAT activity in APCAT1-21. A, APCAT1-21 cells were incubated for 24 h in the presence of various concentrations of dexamethasone. Cell extracts were prepared and assayed for CAT activity. The percent ratio of dexamethasone- vs vehicle-treated cells was calculated for each concentration of dexamethasone; and B, APCAT1-21 cells were treated with ethanol vehicle or 10^{-6} M dexamethasone for 12, 24, 48 and 72 h, and cells were collected. The percent ratio to control level (ethanol vehicle) was calculated for each time period.

of human POMC gene is sufficient to confer negative regulation by glucocorticoids to the fusion gene. On the other hand, AtT-20 cells which were stably transformed with pRSVCAT, resulting in ARCAT cells, showed no response to 10^{-6} M of dexamethasone.

To further delineate the region responsible for glucocorticoid inhibition of the human POMC gene expression, promoter deletion mutants, pPOMC–CAT2 and pPOMC–CAT3, which contain -417 and -97 bp of the 5′-flanking region of POMC gene, respectively, were also stably introduced into AtT-20 cells and tested for glucocorticoid inhibition.

CAT activity was inhibited by dexamethasone in 3 randomly selected clones of APCAT1 and APCAT2 cells. However, CAT activity of APCAT3 cells was not inhibited by dexamethasone in all of 3 randomly selected clones (Fig. 3). In these cells, dexamethasone rather increased CAT activity especially in APCAT3-20 cells.

These results suggest that no more than 417 bp in the 5′-flanking region of the human POMC gene are required for transcriptional repression by glucocorticoid.

THE MECHANISM OF GLUCOCORTICOID REPRESSION

To determine whether the repression by dexamethasone is observed in other cell lines, C6 cells and L cells were stably transformed with the POMC–CAT fusion gene (pPOMC–CAT1). Neither in the transformed C6 cells (CPCAT1) nor the transformed L Cells (LPCAT1) inhibited the CAT activity by dexamethasone at the concentration of 10^{-7}–10^{-5} M (Fig. 3).

GR mRNA was examined in these cells by Northern blot analysis using GR cDNA as a probe. All these cells showed a single hybridization band which corresponds in size to mouse and rat GR mRNA, respectively [16, 17]. These results indicate the presence of GRs in C6 and L cells as well as in AtT-20 cells.

In addition to that, basal CAT activity in CPCAT1 and LPCAT1 is far less than that of APCAT1. Israel and Cohen [11] reported that negative regulation of human POMC gene transcription by glucocorticoid was observed in transiently transformed L cells when the basal level of the expression was raised by adding a viral enhancer to the gene.

These results suggest that glucocorticoid inhibition of human POMC gene transcription may require some transcriptional factors which may raise the basal level of POMC gene expression. In the α-subunit gene of glycoprotein hormones, cAMP-response elements play an important role in negative regulation by glucocorticoids [6].

The transcriptional expression of human POMC gene in the pituitary is regulated positively by cAMP. We have already reported that

forskolin, a post-receptor adenylate cyclase-activating agent, increased the transcription of POMC–CAT fusion genes, when transfected into C6 cells or L cells [15]. Deletion analysis has shown a cAMP-responsive region between -417 and -97 bp from the transcriptional start site. This region was shown to confer cAMP-responsiveness when placed upstream from a heterologous viral promoter. We have shown in this study that this region may also be responsible for glucocorticoid inhibition of human POMC gene transcription in APCAT cells.

To determine whether the interactions between glucocorticoids and cAMP occurred at the transcriptional level of human POMC gene in the transformed L cells, cultures were exposed to forskolin with or without dexamethasone, at which time CAT activity was analyzed.

Forskolin significantly increased the CAT activity in LPCAT cells. This increase of CAT activity by forskolin was partially but significantly suppressed by the addition of dexamethasone, although the dexamethasone alone did not suppress the CAT activity (Fig. 5).

These results suggest that binding of GR to the negative glucocorticoid response element (nGRE) could lead to steric occlusion of positive transcription factors, such as cAMP-response element binding protein or that GR bound to nGRE could interact with DNA-bound positive factors in such a way as to prevent their transcriptional stimulatory activity.

Recently Drouin *et al.* [18] identified the negative glucocorticoid response element (nGRE) in the rat POMC gene. They suggested that binding of GR to the nGRE could compete with binding of trans-activity factors such as chicken ovalbumin upstream promoter transcription factor.

Steroid-mediated repression may be more complicated than this, however, because in one experimental system, inhibition of the rat prolactin gene in pituitary cells has been shown to be independent of the ability of receptor to bind DNA [7]. The authors suggest that repression is brought about by steroid activated protein–protein interaction between the receptor and a pituitary-specific factor, Pit.1, which prevents Pit-1 from binding DNA.

These results suggest that glucocorticoid inhibition of human POMC gene expression may be brought about by a glucocorticoid activated protein–protein interaction between the receptor and tissue-specific factors.

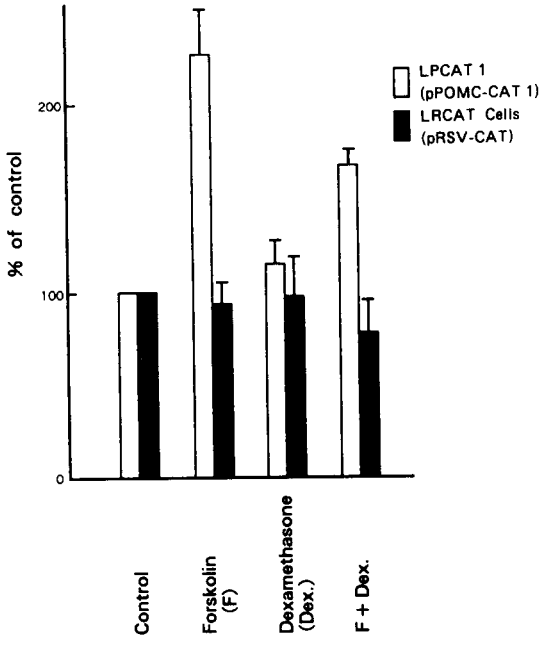

Fig. 5. Effect of forskolin with or without dexamethasone on CAT activity in LPCAT1 and LRCAT cells. LPCAT1 (□) cells or LRCAT (▨) cells were exposed to forskolin (10^{-5} M) with or without dexamethasone (10^{-6} M), at which time CAT activity was analyzed. The percent ratio to control level (ethanol vehicle) was calculated for each treatment.

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