Neuroendocrine Regulation of the Hypothalamic Pituitary Adrenal Axis by the nurr1/nur77 Subfamily of Nuclear Receptors

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The present study was designed to examine the role of the nurr1/nur77 subfamily of nuclear receptor transcription factors in the regulation of the hypothalamic/pituitary/adrenal axis at the neuroendocrine level. We demonstrate that this nuclear receptor subfamily can regulate the expression of the CRF and POMC genes by interacting with a specific *cis*-acting sequence in their proximal promoter regions. To examine the physiological significance of this response, we have focused on the POMC gene. We provide evidence that nurr1 and nur77 are rapidly induced by CRF in primary pituitary cells and that this induction is mimicked by forskolin in an anterior pituitary cell line. Further, we demonstrate that both nurr1- and forskolin-dependent induction of a POMC-chloramphenicol acetyltransferase reporter gene are inhibited by mutation of the nurr1-binding site within the POMC promoter and that this site alone can confer cAMP responsiveness to a heterologous promoter. Finally, we provide evidence that the nurr1/nur77 response sequence is pivotal to both nurr1/nur77-dependent positive regulation and glucocorticoid receptor-dependent negative regulation of the POMC gene. These data strongly support the conclusion that the nurr1/nur77 subfamily plays an important coordinate neuroendocrine-regulatory role at all levels of the hypothalamic/pituitary/adrenal axis. (Molecular Endocrinology 11: 39–47, 1997)

INTRODUCTION

Nuclear receptors comprise a superfamily of structurally related transcription factors that control a variety of developmental, physiological, and behavioral processes (1–3). The family includes receptors for lipophilic hormones and vitamins as well as a majority of orphan members whose physiological function is poorly understood (4). Nurr1 (also called rnr-1 and NOT) (5, 6) is an orphan member of the superfamily (7) that is expressed predominantly in the central nervous system (7, 8). The protein exhibits a close structural relationship to the orphan receptors nur77 (also called NGFIB/N10/NAK) (9–12) and NOR-1 (also called MINOR/TEC) (13–16). All three proteins are members of a nuclear receptor subgroup (hereafter referred to as the nurr1 subfamily) that bind as monomers to the *cis*-acting sequence, AAAGGTCA, to regulate gene expression without a requirement for ligand binding (14, 17–19). Nurr1 and nur77 have also been implicated in the regulation of retinoid-signaling pathways by heterodimerizing with the 9-*cis* retinoic acid receptor, RXR, and binding to the AAAGGTCA motif when arranged as two directly repeated elements (20). Thus, the nurr1 subfamily has the capacity to regulate overlapping gene networks if expressed in the same cells.

Transcripts for the nurr1 subfamily are constitutively expressed in a differentially restricted but partially overlapping temporal and spatial pattern (7, 8). Whereas nurr1 expression appears to be restricted to brain tissue in the developing and adult mouse, the constitutive expression of nur77 and NOR-1 is observed in some peripheral tissues in addition to brain (7, 14). Nur77 mRNA is present in several tissues including testis, ovary, and muscle (7) whereas low NOR-1 expression is detected in the thymus, kidney, and spleen (14). Further, unlike most nuclear receptors, these proteins are products of immediate early genes whose expression can be differentially induced in response to a variety of extracellular stimuli including growth factors (9, 10, 21), neurotransmitters (22, 23), and polypeptide hormones (24, 25).

Several lines of evidence indicate that the members of the nurr1 subfamily may play an important role in the coordinate neuroendocrine regulation of the activity of the hypothalamic/pituitary/adrenal (HPA) axis. This axis is regulated at the level of the hypothalamus by CRF, which is synthesized in the hypothalamic paraventricular nucleus (PVN). In response to stressful stimuli, CRF is released from the PVN and transported to the anterior pituitary causing an increase in synthesis of POMC. POMC is a precursor molecule of several

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neuropeptides including ACTH, which is released from the pituitary and regulates the synthesis of glucocorticoids from the adrenal cortex. To maintain homeostasis, glucocorticoids inhibit CRF and POMC synthesis and secretion at the level of the hypothalamus and anterior pituitary. It has previously been shown that while nurr1 is constitutively expressed in the PVN (8), nur77 mRNA is rapidly induced in this region by stress (26) and interleukin-1β (27), both important regulators of hypothalamic CRF. Also, central administration of CRF to conscious rats significantly increases the expression of nur77 within the PVN (28). We have shown that nurr1 and nur77 are both expressed in the anterior pituitary, the site of POMC synthesis (8). Further, nur77 and nurr1 transcripts are strongly induced by stress in the adrenal cortex (25). The induction of nur77 in this region has been implicated in the transcriptional induction of the steroidogenic enzyme steroid-21α-hydroxylase (24), a rate-limiting enzyme in glucocorticoid synthesis. However, recent reports reveal that nur77 null mutant mice display no abnormal functions of the HPA axis (29). The absence of detectable phenotypic changes in the HPA axis has been proposed to reflect a functional redundancy by nurr1 because levels of this mRNA, after HPA axis stimulation, are compensatorily increased in the adrenal gland of nur77 null mutant mice (29). Finally, our laboratory recently identified specific DNA-binding sites for nurr1 and nur77 in the proximal promoter region of the CRF and POMC genes that may mediate nurr1 subfamily-dependent regulation of these genes in the hypothalamus and pituitary, respectively (19).

The aim of this study was to examine the neuroendocrine regulation of the HPA axis by the nurr1 subfamily. We report here that, as predicted by our previous DNA-binding studies (19), nurr1 and nur77 interact specifically with the CRF ($-352/-332$) and POMC ($-70/-47$) promoter elements in electrophoretic mobility shift assays (EMSA). Cotransfection experiments in pituitary-derived cells show that nurr1 can increase the transcriptional activity of both promoters. Further, mutational analysis of the nurr1 consensus site within the POMC promoter results in loss of nurr1-stimulated expression. CRF functions through the secondary messenger cAMP to potently stimulate POMC gene transcription within pituitary cells. By increasing cAMP levels within a pituitary cell line, we observe a rapid and robust increase of nurr1 and nur77 mRNAs, suggesting CRF induction of POMC synthesis may be mediated through these transcription factors. Finally, examination of the nurr1 consensus sequence in the POMC promoter reveals that the element overlaps with a well characterized negative glucocorticoid receptor response element (nGRE). We provide evidence to indicate that glucocorticoid repression of the POMC gene may be mediated, at least in part, by glucocorticoid receptor (GR)-dependent inhibition of activation of the POMC gene by nurr1 subfamily members. Our results strongly support the conclusion that the nurr1 subfamily of nuclear receptors plays a coordinate role in neuroendocrine regulation of the activity of the HPA axis.

RESULTS

Specific Binding of nurr1 and nur77 to Proximal Promoter Fragments of the CRF and POMC Genes

In a previous study, we identified three *cis*-acting sequences, GAAGGTCA, AAAGGTCG, and GAAGGTCG (19), in addition to the previously characterized AAAGGTCA (NBRE) site (18), that bind specifically to both nurr1 and nur77. Examination of Genbank for sequences containing one of these sequences, GAAGGTCA, revealed several genes of neuronal and neuroendocrine origin whose proximal promoters contain this *cis*-acting sequence (19). Two identified genes, ovine CRF (oCRF) (30) and rat POMC (rPOMC) (31), were of particular interest since previous analysis of the spatial expression of nurr1 and nur77 within the central nervous system indicated that nurr1 and nur77 are either expressed or induced by HPA-activating signals in the hypothalamic and pituitary structures that express the CRF and POMC genes (8, 26). The GAAGGTCA sequence of the POMC is highly conserved across the rat, human, and mouse species (31–33), suggesting this sequence has important regulatory functions.

To test whether nurr1 and nur77 interact directly with the POMC $-70/-47$ and CRF $-352/-332$ regions, we prepared ^{32}P- labeled oligonucleotides containing these regions and used EMSA to examine their binding to nurr1 and nur77 translated *in vitro* in the reticulolysate system. The results of these assays are shown in Fig. 1. Incubation with nurr1 resulted in a retarded radiolabeled complex that was observed when either the POMC (panel A, lane 2) or CRF (panel C, lane 2) promoter fragments were used. These complexes were nurr1 dependent and were not observed in the absence of nurr1 in the reticulolysate (lane 1, both panels). Furthermore, complex formation on both promoters was specifically inhibited by increasing concentrations of unlabeled homologous oligonucleotide (lanes 3–5) but not by a heterologous oligonucleotide (lane 6), indicating that binding to these DNA fragments was specific and competitive. Finally, mutation of the GAAGGTCA motif to GAACATCA or GTACGTCA within these sequences resulted in loss of ability to competitively inhibit nurr1-dependent binding, indicating that this sequence is essential for the nurr1 interaction (lanes 7–9). Similar results were obtained when nur77 was used in EMSA instead of nurr1 (panels B and D).

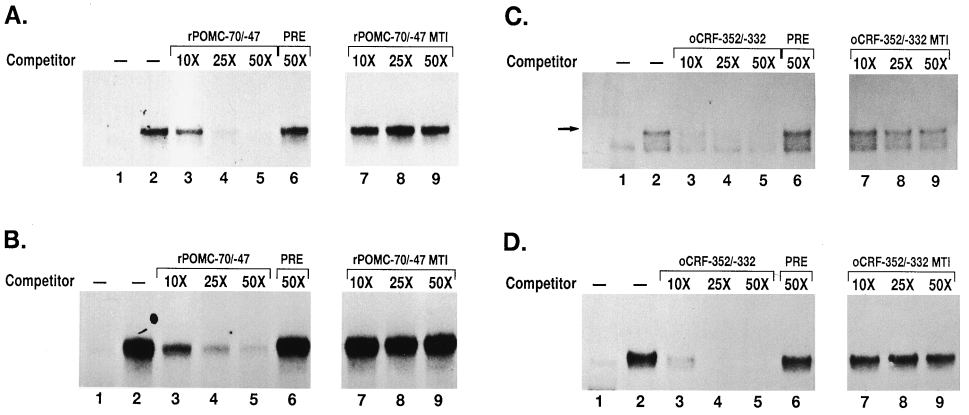

Fig. 1. EMSA of nurr1 and nur77 Binding to the rPOMC and oCRF Promoters

Nurr1 (panels A and C) and nur77 (panels B and D) were transcribed and translated *in vitro* and incubated with α^{32}P-labeled rPOMC (panels A and B) and oCRF (panels C and D) oligonucleotides (lane 2). For competition analysis 10–50× molar excess of homologous oligonucleotide (lanes 3–5), 50× molar excess of heterologous oligonucleotide (lane 6), and 10–50× molar excess of mutant oligonucleotide (lanes 7–9) were used.

Nurr1 Enhances the Transcriptional Activity of the CRF and POMC Promoter Regions

To determine whether nurr1 and nur77 were capable of regulating the expression of the CRF and POMC promoters, we generated target gene constructs containing the proximal promoter regions of both genes and used these in cotransfection experiments with nurr1 or nur77 expression constructs. In these experiments, we constructed target vectors in the promoterless pBL$_3$CAT plasmid (34) using a −483/+1 promoter fragment of the rPOMC gene (31), a −483/+81 fragment of the human POMC promoter (32), and a −372/+11 fragment of the oCRF gene (30). Transcriptional regulation of these target constructs by nurr1 and nur77 was then measured by transfection in the anterior pituitary corticotropic cell line, AtT20/D. As shown in Fig. 2A, cotransfection of these target genes with nurr1 results in a 7- to 12-fold stimulation of the rat, human POMC, and oCRF promoters over that observed when the target genes are cotransfected with the parent expression vector lacking nurr1 (p91023B). To confirm that the nurr1-dependent induction transcription is due to the presence of the GAAGGTCA motif within the promoter, we introduced point mutations within this element in the rPOMC promoter to GAACATCA, which we have shown to result in loss of nurr1 binding *in vitro* (Fig. 1), and we examined the regulation of this promoter construct by nurr1. As shown in Fig. 2B, nurr1 induction of the POMC promoter is lost when the mutated promoter construct (rPOMC MT1) is used, indicating that a functional GAAGGTCA motif is essential for transcriptional induction of the POMC promoter by nurr1. When nurr1 was replaced by nur77 in these assays, similar results were observed (data not shown), indicating that both subfamily members can regulate the expression of the CRF and POMC promoters and may function redundantly to do so in a physiological context if coexpressed.

CRF Rapidly Induces nurr1 and nur77 Expression in Pituitary Cells

To determine whether the nurr1/nur77 subfamily is likely to contribute to the regulation of POMC expression by CRF in a physiological context, we incubated isolated mouse pituitary cells with CRF (10^{-8} M) and examined its ability to induce expression of the nurr1 and nur77 transcripts. As indicated in Fig. 3, both nurr1 and nur77 transcripts are rapidly induced by CRF within 15 min of treatment and are maximal at approximately 30 min. Further, this induction slightly precedes the previously reported time course of POMC transcription by CRF, which is maximal by 30–60 min (35, 36).

Forskolin Induces Expression of the p-483/+1 rPOMC Reporter Construct

While AtT20/D cells are a suitable model to examine the regulation of POMC in corticotropic cells, the induction of POMC by CRF in these cells is very weak and variable and most likely due to loss of membrane CRF receptors in the transformed cell line. However, since CRF-dependent regulation of POMC expression is known to be mediated by the secondary messenger cAMP pathway (37, 38), CRF-dependent POMC induction can be mimicked by incubation of cells with forskolin to activate this pathway. Direct activation of adenylate cyclase with forskolin induced both nurr1 and nur77 mRNAs rapidly (Fig. 4). The time course of this induction correlates with the time course of CRF induction of nurr1 and nur77 in the isolated pituitary cells. Further, we show that forskolin can significantly stimulate the expression of the rPOMC-chloramphenicol

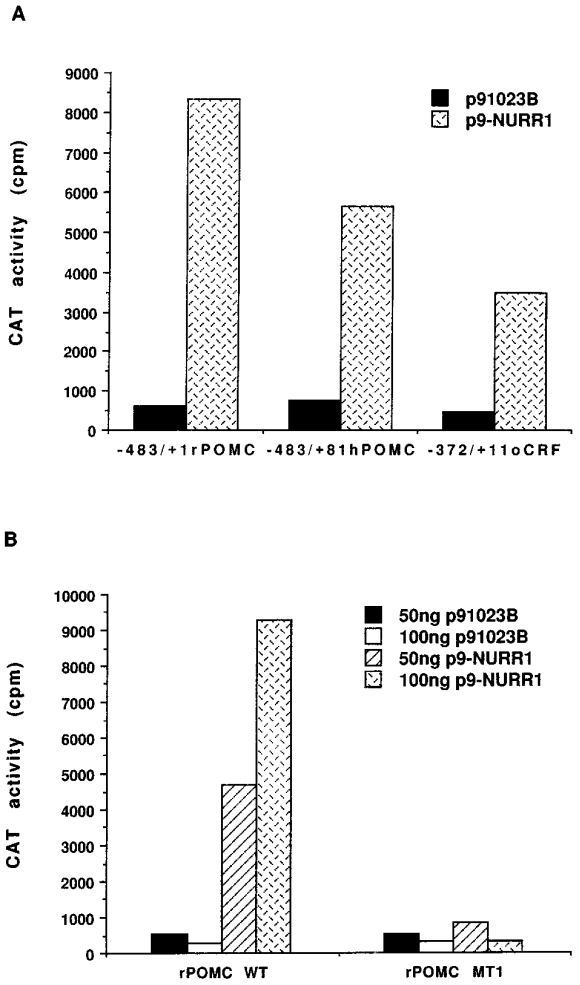

Fig. 2. Activation of Transcription from the rPOMC and oCRF Promoters by nurr1

AtT20/D cells were transfected with 500 ng −483/+1 rPOMC, −483/+81 hPOMC, or −372/+11 CRF reporter plasmids (panel A) and 500 ng −483/+1 rPOMC or −483/+1 rPOMC MT1 reporter plasmids (panel B) together with either p91023B or p91023B-nurr1 expression vector. The results shown are representative of four individual experiments. Each data bar represents two replicates.

acetyltransferase (CAT) reporter gene when transfected into AtT20/D cells. Most importantly, however, the induction of POMC-CAT by forskolin is reduced when the nurr1 binding site is destroyed by point mutation of the GAAGGTCA motif (rPOMC MT1), and the basal activity of the promoter is also decreased. Finally, when the GAAGGTCA alone is placed in front of a heterologous promoter [thymidine kinase (tk) (34)], this enhancer element is sufficient to mediate induction of this promoter by forskolin (Fig. 4). The results confirm that the nurr1-binding site plays an important role in the induction of this promoter by cAMP pathways and also contributes to the basal activity of the POMC promoter in pituitary cells.

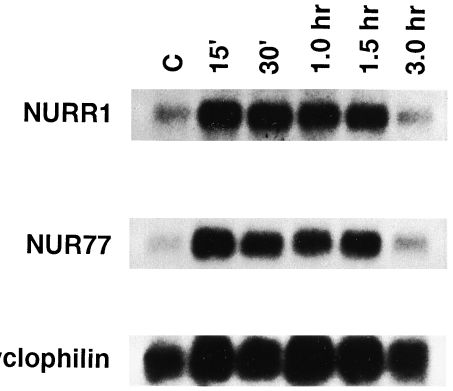

Fig. 3. CRF Treatment of Isolated Pituitary Cells Rapidly Increases nurr1 and nur77 mRNA Levels

After treatment with 10^{-8} M CRF for the indicated times, total RNA was extracted, and Northern blots were performed as described in *Materials and Methods*. The same filter was hybridized with a cDNA probe for nurr1 and nur77 and also with cyclophilin to control for RNA loading and transfer.

The nurr1-Binding Site Mediates Both cAMP-Dependent Up-Regulation and Dexamethasone-Dependent Down-Regulation of the POMC Promoter

Upon identification of the nurr1 enhancer element, we noted that its location overlapped with a previously characterized nGRE that mediates GR-regulated repression of the POMC promoter (39) (Fig. 5A). Mutation of the GAAGGTCA motif to GAACATCA has been shown to convert the nGRE into a positive enhancer element that mediates induction of the enhancer element by GR (40) when placed upstream of a heterologous basal promoter. In preliminary studies to test the hypothesis that the nurr1-binding site is pivotal to both positive regulation by nurr1 and negative feedback by GR, we confirm that forskolin regulation of the POMC promoter is down-regulated by pretreatment with dexamethasone (36) and that rPOMC-MT1, while not responding to forskolin or nurr1, is up-regulated by dexamethasone (Fig. 5B). These data predicted that GR may inhibit expression of POMC in the pituitary, at least in part, by either directly or indirectly inhibiting binding of the nurr1 subfamily to the GAAGGTCA motif. To directly test this predication, we carried out EMSA on nuclear extracts to examine the DNA-binding properties of nurr1 in forskolin- and dexamethasone-treated cells. The results are shown in Fig. 5C. In unstimulated AtT20/D cells, two proteins, presumably nurr1 and nur77, bound to the rPOMC −70/−47 probe (lane 1). Stimulation of the cells with 25 μM forskolin for 1 h resulted in significant increased binding of both proteins to DNA (lane 4). Binding was inhibited by 25× molar excess of homologous oligonucleotide (lanes 2 and 5) but not by the oligonucleotide containing a mutation of the nurr1-binding site rPOMC −70/−47MT1 (lanes 3 and 6). The larger protein complex was selectively blocked by nurr1-specific antiserum

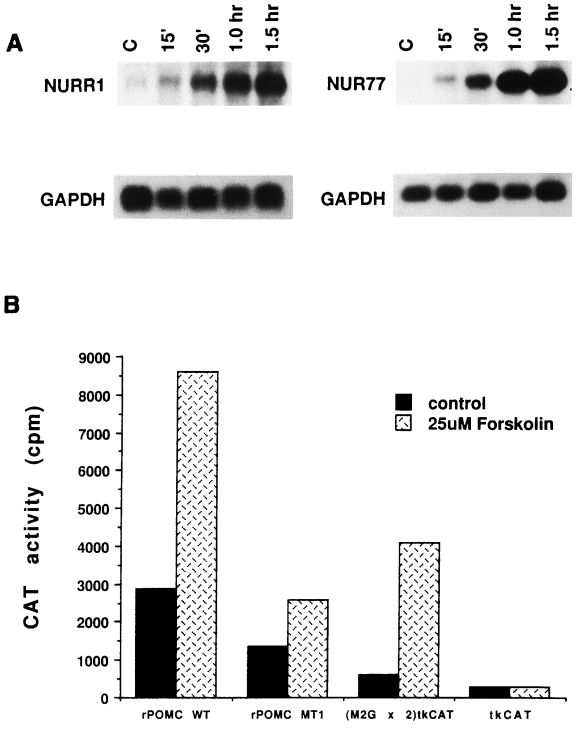

Fig. 4. Forskolin Mimics CRF Induction of nurr1 and nur77 mRNA and Induces rPOMC-CAT in AtT20/D16 Cells

Panel A, AtT20/D cells were treated with 25 μM forskolin for 0–1.5 h. Nurr1 and nur77 mRNA levels were measured using 20 μg total RNA. Each membrane was probed with a glyceraldehyde-3-phosphate dehydrogenase cDNA fragment to control for transfer and loading. Panel B, AtT20/D cells were transfected with −483/+1 rPOMC, −483/+1 rPOMC MT1, or M2G $(GAAGGTCA)_2$-tk reporter plasmids and treated with 25 μM forskolin for 10 h. These results are representative of four individual experiments. Each data bar represents two replicates.

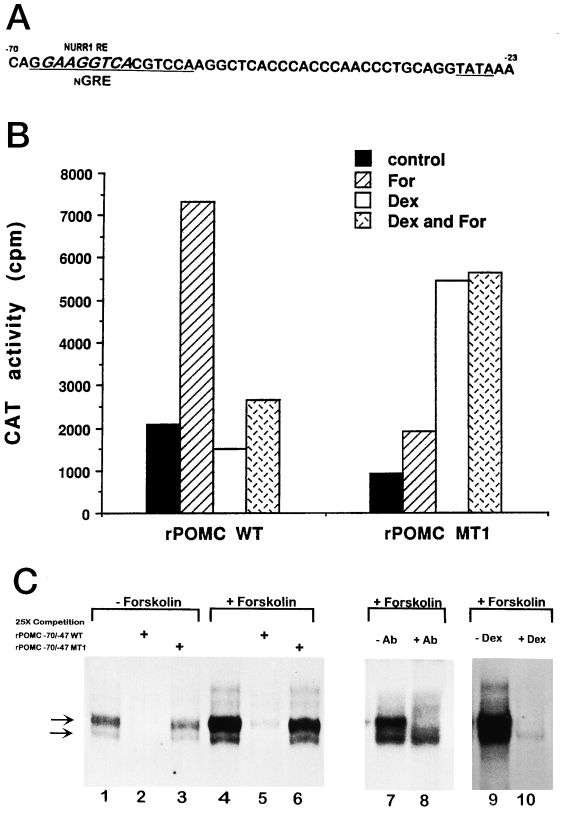

Fig. 5. The nurr1-Binding Site on the POMC Promoter Is Also Required for Glucocorticoid Feedback Inhibition of the POMC Promoter

Panel A, Nucleotide sequence of rPOMC promoter (−70/−23). Positions of the nGRE (*underlined*) and nurr1 response element (nurr1 RE, *italics*) are indicated. Panel B, AtT20/D cells transfected with −483/+1 rPOMC or −483/+1 rPOMC MT1 and cultured in the presence of forskolin (25 μM), dexamethasone (10^{-8} M), or dexamethasone and forskolin (pretreated with dexamethasone and followed by the addition of forskolin). Panel C, Nuclear extracts from AtT20/D cells, untreated (lanes 1–3) or treated with 25 μM forskolin (lanes 4–10) for 1 h, were prepared and used in EMSA with α^{32}P-labeled rPOMC −70/−47 oligonucleotide. For competition analysis 25× molar excess of homologous oligonucleotide (lanes 2 and 5) and mutant oligonucleotide rPOMC −70/−47 MT1 (lanes 3 and 6) was used. Nurr1-specific antiserum was included in the binding reaction (lane 8). AtT20/D cells were pretreated with 10^{-8} M dexamethasone for 2 h before the addition of 25 μM forskolin (lane 10).

(lane 8) (8). These results confirm that the cAMP-dependent increases in nurr1 mRNA in these cells correlate with an increase in specific binding of nurr1 protein to the POMC promoter sequence. Further, as predicted by our transactivation results, pretreatment with dexamethasone inhibited binding of both proteins to DNA and also diminished the basal DNA-binding activity (lane 10). These results confirm that the nurr1-binding site can play a pivotal role in both basal and cAMP-mediated up-regulation of the POMC promoter and its down-regulation by GRs.

DISCUSSION

We have provided substantial evidence to support the conclusion that members of the nurr1 subfamily of nuclear receptors play an important role in the coordinate neuroendocrine regulation of the activity of the HPA axis as well as its negative feedback inhibition by glucocorticoids. Using *in vitro* DNA binding and cell-based transactivation assays in an anterior pituitary cell line, we have demonstrated that nurr1 and nur77 can bind and regulate the expression of the CRF and POMC promoters. To examine the physiological significance of this transcriptional regulation, we focused on the POMC promoter for several reasons. First, the proximal binding site for the nurr1 subfamily is positionally conserved across species in this promoter and is contained in a region important for both positive and negative regulation of the POMC gene (31, 40). Second, the POMC promoter fragment used in these stud-

ies contains all of the sequences (−323/−34) that have been shown to be necessary and sufficient for the correct spatiotemporal and hormone-inducible expression of a β-galactosidase reporter gene in the anterior pituitary when expressed in transgenic animals (41). Third, the availability of a well characterized anterior pituitary-derived cell line (AtT20/D) (31), which produces endogenous POMC and responds to cAMP activation by induction of POMC expression in a manner that mimics induction by CRF, facilitates examination of the regulation of POMC by the nurr1 subfamily and the promoter elements responsible for this regulation in a physiologically relevant context.

As predicted by the species conservation of the nurr1-binding site, we confirmed that transactivation of the POMC gene by nurr1 is conserved between rat and human species. Further, we showed that mutation of the nurr1-binding site within the POMC promoter results in loss of ability to bind nurr1 and inhibits nurr1-dependent activation of this promoter, confirming that the GAAGGTCA sequence motif is essential for nurr1-dependent induction of POMC expression. To determine whether nurr1 and/or nur77 are likely to mediate CRF- dependent induction of POMC expression in the anterior pituitary, we confirmed that the expression of both nurr1 and nur77 is rapidly induced by stimulation of isolated mouse pituitary cells with CRF. Further, we demonstrated that this induction can be mimicked in AtT20/D cells by activation of cAMP-dependent pathways by forskolin. Forskolin stimulation results in increased binding of endogenous nurr1 to DNA and a functional POMC-CAT transactivation response. These data indicate that cAMP may regulate the POMC promoter, at least in part, by increasing expression of the nurr1 subfamily. However, nur77 has also been shown to be phosphorylated by cAMP (25), and cAMP has also been shown to alter the transcriptional activity of nurr1 and nur77 (42). Thus, both covalent modification of existing pools of nur proteins and *de novo* synthesis are likely to contribute to cAMP-dependent induction of the POMC promoter.

The observation that forskolin induction of the rPOMC-CAT target gene is diminished by mutation of the nurr1-binding site illustrates the importance of this *cis*-acting sequence in mediating cAMP-dependent induction of POMC expression in the anterior pituitary cell line. While CRF is known to induce expression of POMC through a cAMP-dependent pathway, previous studies have not uncovered a recognizable *cis*-acting cAMP response element that may mediate this response (43). The location of cAMP-responsive sequences in the POMC promoter has therefore been controversial. Previous studies have indicated that sequences located upstream (−236/−133) of the nurr1-binding site are responsive to CRF when placed upstream of a heterologous promoter and may contribute to hormonal regulation of the endogenous gene (43). While our data support a major role for the nurr1-binding site located at −60/−70 in mediating cAMP responses in the context of the endogenous POMC promoter, the lack of complete inhibition of POMC induction by mutation of this sequence indicates that additional sequences outside of this region may also contribute to cAMP-mediated induction of expression of this gene.

The nurr1-binding site overlaps with a previously identified nGRE that has been shown to be important for GR-mediated repression of the POMC gene and is also important for basal expression of this promoter (31, 39, 40, 44). Consistent with the reported contribution of this region to basal promoter activity (44), we observed that mutation of the nurr1-binding site also results in decreased basal promoter activity. Two critical nucleotides within the nGRE are critical for both GR repression (40) and nurr1 transactivation. Mutation of these nucleotides, as we have done in our study (POMC-MT1), converted the nGRE into a positive GR response element when placed in front of a heterologous promoter (40). We have demonstrated that although this mutated sequence no longer responds to nurr1 and demonstrates diminished response to forskolin, the mutant target gene is induced by dexamethasone. These data support the conclusion that the nurr1-binding site plays an important role in negative regulation of the POMC gene by glucocorticoids as well as nurr1-mediated basal and cAMP-inducible expression of POMC. Further, we show that GR-mediated inhibition of the POMC gene is accompanied by an inhibition of nurr1 subfamily-dependent DNA binding to the GAAGGTCA response element, demonstrating functional antagonism between these two nuclear receptors. Thus, the nurr1 subfamily may play a pivotal role in regulation of neuroendocrine homeostasis at the pituitary level.

The data we have provided in the present study, together with the demonstrated expression (8) and induction (26–28) of nurr1 subfamily members in the hypothalamic PVN, and the demonstration by others (24) that nur77 can mediate the regulation of expression of the steroidogenic enzyme, steroid-21 α-hydroxylase by the POMC processing product, ACTH, indicate that members of the nurr1 subfamily may be important coordinators of the activity of the HPA axis at all levels. Despite these observations, however, recent analysis of HPA activity in homozygous nur77 null mutant mice has detected no disturbance in this neuroendocrine pathway (29). In fact, the only significant difference between wild type and homozygous animals observed in this pathway was a compensatory increase in the induction of nurr1 by stress in nur77 null mutant animals that was not observed in the wild type mice (29). This observation highlights the capacity for redundancy of function between nurr1 subfamily members. Our analysis of the comparative developmental expression of nurr1 and nur77 has indicated that nurr1 is selectively expressed during embryonic development, particularly in the diencephalic regions that give rise to the hypothalamus at a time that coincides with the developmental organization of the HPA axis, whereas nur77 is not expressed until the

postnatal stage (O. Saucedo-Cardenas and O. Conneely, manuscript in preparation). Given the ability of nurr1 to substitute functionally for nur77, it is not surprising that nur77 null mutant mice do not show any detectable aberrant phenotypes in the HPA because nur77 expression does not begin until the postnatal stage of development at a time when nurr1 is already expressed. Thus, nurr1 may play a selective role in the developmental organization and activity of the HPA axis that is not substituted by nur77. Finally, the ontogeny and impact, if any, of the third subfamily member, NOR-1, on this pathway remain to be established. With the use of gene-targeting strategies, null mutation of these genes in mice should provide valuable insights into the selective and collective functions of these proteins *in vivo*, including their essential role, if any, in the neuroendocrine development and activity of the HPA axis.

MATERIALS AND METHODS

Plasmid Construction

The nurr1 and nur77 cDNAs were cloned into the plasmid $pT_7\beta$-6 Sal (45) at the *Nco*I site of the β-Globin linker and the *Sal*I site of the polylinker. This generated $pT_7\beta$-nurr1 and $pT_7\beta$-nur77, which drives the expression of these cDNAs under the control of the T_7 promoter *in vitro.* For expression in tissue culture cells, cDNAs were ligated to the *Eco*RI site of p91023β and expressed under the control of the adenoviral major late promoter (46). The reporter plasmid p-372CRF-CAT was generated by PCR using, as the template, a 5-kb B*gl*II/H*ind*III fragment isolated from the previously identified λ CRF-1 genomic clone (30). p-483POMC-CAT reporters were also generated by PCR using rat and human genomic DNA. The PCR products were subcloned into pBL_3CAT, which lacks the minimal tk promoter (34). All PCR products were sequenced by the dideoxy method (47). The M2G $(GAAGGTCA)_2$ tk-CAT reporter plasmid was made by ligating double-stranded oligonucleotides, containing two inverted copies of the response element oligonucleotide separated by 10 nucleotides, into the *Bam*HI site of pBL_2CAT, upstream of the minimal tk promoter (34).

In Vitro Transcription and Translation

In vitro transcription and translation was accomplished with the TNT kit (Promega, Madison, WI) with the addition of RNAsin (Promega). Nurr1 and nur77 were transcribed with T_7 RNA polymerase from $pT_7\beta$-6 recombinant plasmid (45), a derivative of pGEM 2 in which the β-globin insert of pSP_6 Hβ 166 6 was inserted at the initiation codon to create the sequence CCATGCCTCGACCATGG (48). The translation was carried out in the presence of [^{35}S]methionine and run on an 8.5% denaturing gel or cold methionine for use in the mobility shift assay, according to the manufacturers directions; 1–2.5 μl of a translation mixture was used in each gel shift-binding reaction.

EMSA

EMSA were performed with *in vitro* translated proteins in a rabbit reticulocyte lysate system (TNT, Promega) or AtT20/D nuclear extracts. Proteins were mixed with 100,000 cpm of Klenow-labeled probes in the reaction buffer, 20 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 8% Ficoll, 600 mM KCl, 500 ng/μl poly(deoxyinosinic-deoxycytidylic)acid, and 50 mM dithiothreitol (DTT). The reaction was incubated for 20 min at room temperature and then electrophoresed through a 5.5% nondenaturing polyacrylamide gel in 0.5× Tris-Borate-EDTA (TBE) electrophoresis buffer. Nurr1-specific antiserum (8) was incubated with nuclear extract for 15 min before the addition of probe. For competition studies, the reaction was performed as described with the indicated concentrations of unlabeled probe. The sequences of the oligonucleotides studied are listed as follows:

rPOMC −70/−47
5′-GATCT$_{-70}$CAGGAAGGTCACGTCCAAGGCTCA$_{-47}$
rPOMC −70/−47MT1
5′-GATCT$_{-70}$CAGGAA<u>CA</u>TCACGTCCAAGGCTCA$_{-47}$
oCRF −352/−332
5′-GA$_{-352}$TCTTTCTGACCTTCCCTTTA$_{-332}$
oCRF −352/−332MT1
5′-GA$_{-352}$TCTTTCTGAC<u>G</u>T<u>A</u>CCCTTTA$_{-332}$

Preparation of Nuclear Extracts

AtT20/D16V-F2 cells untreated or treated with 25 μM forskolin were washed with cold PBS, resuspended, and incubated for 5 min in 0.25 ml buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCL, 0.5 mM DTT, 0.1 mM EGTA, 0.5 mM phenylmethylsulfonylfluroide, and 2 μg each of the protease inhibitors antipain, pepstatin A, and aprotinin per ml). Then, 1.25 μl of 10% Nonidet P-40 were added, and the cells were incubated for 2 min on ice. The cells were centrifuged at low speed (1,7000 rpm), and the supernatant was removed (cytosolic fraction). To the pellet, 0.125 ml of buffer B (0.4 M NaCL, 10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, and 0.5 mM phenylmethylsulfonylfluoride) was added. The mixture was vortexed at 4 C and left on ice for 5 min. The extracts were then centrifuged, and the supernatant was dialyzed against 50 volumes of buffer C (20 mM HEPES-KOH, pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol, and 0.5 mM phenylmethylsulfonylfluoride) for 4 h at 4 C with one change of buffer C. After 4 h, the materials that precipitated during dialysis were removed by centrifugation, and the supernatant was aliquoted, flash frozen in liquid N_2, and stored at −80°C until further use. The protein concentration was estimated with the Bradford protein assay kit (Bio-Rad, Richmond, CA). One microgram of protein was used in the EMSA.

Cell Culture and Transfection

AtT20/D16V-F2 were grown in DMEM supplemented with 10% FBS, penicillin at 100 μg/ml, and streptomycin at 100 μg/ml in a humidified atmosphere of 5% CO_2 and 95% air. Twenty four hours before transfection, 2×10^5 cells were plated in 3-cm dishes in DMEM supplemented with 10% FBS and were allowed to attach. The cells were then washed with Hanks Balanced Salt Solution (HBSS) lacking calcium and magnesium and incubated in DMEM supplemented with 10% horse serum for AtT20/D cells. Cells treated with forskolin (25 μM) or dexamethasone (10^{-8} M) were grown in DMEM supplemented with 10% stripped serum. DNA (50–100 ng p91023-nurr1/nur77; 0.5–1 μg p-372CRF CAT and p-483POMC CAT; 200 ng M2G-tk CAT) in a volume of 250 μl HEPES-buffered saline was added to 1×10^{10} d1312 adenovirus particles (49) in a volume of 333 μl HEPES-buffered saline and incubated at room temperature for 30 min. Poly-L-lysine was added (the amount required was based on the size of the DNA used) and incubated at room temperature for 30 min. The DNA-modified virus-poly-L-lysine was added to the cells and incubated for 2 h at 37 C. The virus-containing medium was removed, and 3 ml of specific medium were added to the cells. The cells were incubated at 37 C for 24 h

before harvesting. Pituitaries from adult mice (BALB/c) were rapidly isolated intact. Four whole pituitaries were pooled per sample and collected in DMEM containing 10% stripped serum and equilibrated in a 95% air-5% CO_2 mixture.

Northern Blot

Total RNA from cultured cells was isolated at specific times after treatment. RNA was quantitated by UV absorption, and 20 μg of total RNA were electrophoresed on a standard Northern gel and transferred to nylon membrane (50). Nurr1 and nur77 cDNA probes spanned the amino-terminal region to avoid cross-hybridization. All membranes were probed under high stringency conditions.

CAT Assay

Each plate of cells was washed once with PBS without calcium and magnesium, scraped into 1 ml TEN buffer (40 mM Tris, 1 mM EDTA, 150 mM NaCl, pH 8.0) and collected by centrifugation at 13,000 rpm for 30 sec. Cells were resuspended in 250 mM Tris-HCl, pH 7.5, and lysed by four freeze/thaw cycles. Protein concentrations were determined by the micro-plate Bradford assay (51). CAT activity was determined by incubating 5–10 μg protein with 0.2 μCi [^{3}H]chloramphenicol (20 μCi/μmol) and 250 μM butryl-Coenzyme A in 100 μl 250 mM Tris-HCl, pH 7.5, for 3 h at 37 C. Acylated chloramphenicol was extracted using a mixture of 200 μl 2:1 2,6,10,14-tetramethylpentadecane and Xylenes and counted in a scintillation counter (52). The background of the CAT activity ranges from 300–500 cpm and has been subtracted from the assay. Therefore, 1000 cpm represents a low but significant level of basal activity.

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