Identification of a Functional Glucocorticoid Response Element in the Phenylethanolamine *N*-Methyltransferase Promoter Using Fusion Genes Introduced into Chromaffin Cells in Primary Culture

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The rat gene encoding phenylethanolamine *N*-methyltransferase (PNMT) was cloned and a consensus sequence for a glucocorticoid response element (GRE) was found at –513 bp, 5' to the transcriptional start site. In order to define the function of this element, fusion genes containing the PNMT promoter and a chloramphenicol acetyltransferase (CAT) reporter gene were constructed. These constructs did not express after transfection into any of 7 continuous cell lines, none of which endogenously produce PNMT. A system for transfecting chromaffin cells in primary culture was therefore devised using constructs containing 200 bp of the proenkephalin (ENK) promoter, whose expression characteristics are well known. pENK β GAL-1, containing the ENK promoter with a lac Z reporter, was introduced into these cells and β -galactosidase activity was visualized *in situ*. Approximately 90% of cells transfected were chromaffin; transfection efficiency was 5%. High levels of CAT activity were measured in chromaffin cells transfected with pENKAT12, possessing a CAT reporter. In contrast to tumor cell lines, pENKAT12 induction in these cells by forskolin and phorbol esters did not require a phosphodiesterase inhibitor. In this chromaffin system, both basal and regulated expression of the PNMT fusion genes were detected. Dexamethasone (dex) induced expression of pPNMT3000 and pPNMT900, containing the putative GRE and 3000 bp or 863 bp of PNMT promoter sequence, 4- to 10-fold. Expression of pPNMT300 and pPNMT100, which lack the GRE and contain 273 bp or 99 bp of PNMT promoter sequence, was unaffected by dex. Addition of the PNMT region spanning –490 to –863 bp conferred full dex responsiveness to a thymidine kinase promoter. Deletion of the putative GRE sequence by site-directed mutagenesis abolished the dex response. These data identify the sequence at –513 bp in the rat PNMT gene as a

functional, positively acting GRE. Primary cultures of bovine chromaffin cells provide a biologically relevant expression system for transcriptional studies of catecholamine genes and their related neuropeptides.

The catecholamines—dopamine, norepinephrine, and epinephrine—are classical neurotransmitters in the CNS and PNS. Moreover, norepinephrine and epinephrine serve as hormones released from the adrenal medulla. The biosynthetic pathway comprises 4 enzymes: tyrosine hydroxylase (TH), dopa decarboxylase (DDC), dopamine β -hydroxylase (DBH), and phenylethanolamine *N*-methyltransferase (PNMT). Transcriptional control of both the appearance and levels of these enzymes is of particular interest because of the coordinate nature of their tissue-specific expression (Teitelman et al., 1979; Foster et al., 1985), transsynaptic induction (Thoenen et al., 1969, 1970; Reis et al., 1975), and hormonal induction (Wurtman et al., 1972; Bohn, 1983). In addition, these amines are coexpressed in catecholaminergic cells with a variety of neuropeptides, among them, enkephalins (for review, see Chan-Palay and Palay, 1984). The mechanisms of transcriptional regulation of several of these genes, notably TH (Lewis et al., 1987) and proenkephalin (Comb et al., 1986, 1988; Hyman et al., 1989), have been studied by mutational analyses using fusion gene constructs. Although PNMT promoter constructs have been expressed in transgenic animals and have proven to be highly tissue specific (Baetge et al., 1988), mechanistic studies of PNMT transcription have not been possible because a continuous cell line that endogenously produces this enzyme is not available. In an effort to examine the coordinately regulated gene expression of the catecholamine-synthesizing enzymes and coexpressed neuropeptides, we sought to produce a transient transfection system in which all of their promoters, including PNMT, could function.

In addition to tissue-specific regulation, levels of PNMT expression are governed by neural (Molinoff et al., 1970; Thoenen et al., 1970) and hormonal signals. It has been demonstrated (Wurtman and Axelrod, 1965) that dramatic decreases in adrenal PNMT occur after hypophysectomy, and these can be reversed with administration of glucocorticoids. That this is largely a transcriptional effect, and not a phenomenon of message stability, has been supported by nuclear runoff data from hypophysectomized rat tissue (Evinger and Joh, 1989). However, earlier isotopic labeling studies (Ciaranello, 1978) suggested that the major effect of glucocorticoids on PNMT is one

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of decreasing the rate of PNMT degradation. In agreement with the nuclear runoff data, other studies (Wurtman and Axelrod, 1966; Sabban et al., 1982; Burke et al., 1983) have indicated that glucocorticoids increase *de novo* PNMT synthesis. Although the presence of several glucocorticoid response elements (GREs) have been proposed in human (Baetge et al., 1988) and bovine (Batter et al., 1988) PNMT genes based on sequence similarities with known GREs, demonstration of the function of these consensus sequences in PNMT, or indeed of the glucocorticoid responsiveness of these promoter fragments, is as yet lacking.

Clearly, analyses of the promoter using a transient transfection assay would be helpful in establishing the transcriptional role of glucocorticoids in PNMT regulation. Therefore, a procedure for transfection of PNMT constructs directly into bovine adrenal chromaffin cells in primary culture was developed. To establish this method we have used a proenkephalin promoter fusion gene, pENKAT12 (Comb et al., 1986, 1988), whose expression properties in continuous cell lines are well known and whose endogenous gene products, enkephalins, are coproduced with catecholamines in these primary cells.

We report here (1) the cloning of the rat PNMT gene and the sequence of its promoter; (2) production of a deletion series of constructs fusing various lengths of the PNMT promoter to a chloramphenicol acetyltransferase (CAT) reporter; (3) a procedure for transfecting bovine adrenal primary chromaffin cell cultures and a detailed characterization of this system for expressing proenkephalin and PNMT promoter constructs; (4) the demonstration of the glucocorticoid-regulated expression of the PNMT constructs; and (5) the functional mapping of a GRE in the 5' flanking region of the rat PNMT gene.

Materials and Methods

Cloning of rat PNMT. A full-length bovine PNMT cDNA (Baetge et al., 1986) was used to screen a rat genomic library, prepared in EMBL3 phage (gift of G. Scherer). Of 5×10^5 plaques screened, 3 hybridized to the bovine probe. The entire gene was isolated on a single phage containing an approximately 20 kb insert.

Sequencing and primer extension. The 5' end of the gene was localized by Southern analysis to a 1.1 kb HincII to HindIII fragment, which was subcloned into m13 for dideoxy chain termination sequencing. In addition, Bal31 deletions of the fragment were subcloned and overlapping sequences were assembled. Primer extension analysis was performed as previously described (Comb et al., 1982).

Plasmids. The expression plasmid, pENKAT12 (Comb et al., 1986), contains 403 bp of the human proenkephalin gene encompassing -192 to +210 bp, relative to the transcription start site, fused to the CAT encoding region of p0CAT (Prost and Moore, 1986). The 3' polyadenylation signals of pENKAT12 were provided by a 1.2 kb HincII to PstI fragment from the proenkephalin gene. The pENK β GAL-1 plasmid was derived by replacing the CAT portion of pENKAT12 with the lac Z region of pCH126 (Hall et al., 1983), preserving the human proenkephalin 5' promoter and 3' polyadenylation sequences. This was accomplished by destruction of an internal HindIII site in pENKAT12, then partial digestion of the plasmid with HincII, followed by addition of HindIII linkers. The appropriate HindIII to Bam HI fragment encompassing the CAT coding sequence was then excised, and the HindIII to Bam HI fragment encompassing the β -galactosidase coding region was ligated into the modified pENKAT12 plasmid.

The PNMT plasmids, designated pPNMT3000, 900, 300, and 100, were constructed using the rat PNMT promoter fused to CAT in the pBLCAT3 plasmid (Luckow and Schutz, 1987). The pPNMT300 plasmid was made by first isolating a Sau3A to PstI fragment which includes 50 bp of the coding region. After partial digestion with HaeIII, an XhoI linker was added to the 3' end of the fragment, creating a fusion site 7 bp downstream of the start of transcription. The Sau3A to XhoI fragment of appropriate size was then cloned into the BamHI to XhoI site of the polylinker in pBLCAT3, and double-stranded sequencing, using

an appropriate 5' CAT oligomer as primer, was performed to verify the identity of the construct. This pPNMT300 plasmid was then used to generate the other 3 constructs. For pPNMT900, a HindIII linker was attached to the HincII site, and a HindIII to BstXI fragment was used to regenerate the full 863 bp promoter in pBLCAT3. An additional 2.3 kb of upstream sequence was isolated from the PNMT EMBL phage and subcloned into the promoter at the NheI site. The pPNMT100 construct was simply truncated at the most 3' NarI site. The heterologous plasmid, pPTK500, was constructed by subcloning the HindIII to NheI fragment of pPNMT900 into the HindIII to XbaI site of pBLCAT2 (Luckow and Schutz, 1987), which contains the minimal TK promoter.

Oligonucleotide-directed site-specific mutations were produced in the PNMT promoter using the method of Kunkel (1985). The uracil-incorporated template was produced by transformation of the PNMT promoter in an m13 vector into the dut⁻ ung⁻ bacterial strain, CJ236. Oligonucleotides in which the entire GRE motif at -513 bp had been replaced with a PstI sequence were then used as primer to synthesize the second strand on the single-stranded, uracil-containing m13 template. The double-stranded hybrid plasmids were then transformed into the dut⁺ ung⁺ bacterial strain, JM109, and mutants were screened by PstI restriction digestion of miniprep DNA. HincII to NheI fragments containing the new PstI site were excised from several of the selected m13 colonies and substituted into the pPNMT900 expression plasmid. The resulting mutant plasmids, designated p Δ GRE, were sequenced to confirm the position and precise sequence of the altered, GRE-, segment.

Primary chromaffin cultures. Dissociated chromaffin cell cultures were prepared from bovine adrenal glands essentially as described by Wilson and Viveros (1981). Following retrograde perfusion of the glands with collagenase (type I, Worthington), medullae were dissected free of cortex and the minced tissue was further dissociated in collagenase before loading the cell suspension onto a step gradient (Wilson, 1987) of 15 and 7.5% Renografin (Squibb). Chromaffin cells were collected from the gradients, washed, and plated at a density of 2.7×10^7 cells/10 cm dish in DMEM:F12 1:1, supplemented with 10 mM HEPES, 10% fetal calf serum (FCS), and antibiotics (penicillin, streptomycin, and nystatin). Cells used for dex experiments were plated in media containing 10% FCS from which endogenous glucocorticoids had been depleted by adsorption to dextran-coated charcoal (Eberwine et al., 1987). In addition to glucocorticoids, the method also depletes other steroid and thyroid hormones. These chromaffin cells were maintained in the depleted serum throughout the transfection procedure.

Transfections. Primary chromaffin cultures were transfected using a calcium phosphate, DNA coprecipitation method, modified substantially from that previously described (Comb et al., 1986). After the first 6–12 hr of incubation at 7% CO₂, 100% humidity, chromaffin cells were gently triturated off the tissue culture plates, leaving behind fibroblasts and cortical cells which are more adherent to the culture dish. Cells were then recounted in the presence of trypan blue vital stain and plated onto 60 mm, poly-L-lysine-coated dishes at a density of 5×10^6 – 10^7 cells/plate. After 2–4 hr, cells were maximally adherent to the coated plastic and medium was carefully replaced with 4.5 ml of fresh medium in preparation for adding the transfection precipitates. Precipitates for each plate (450 μ l) contained 35 μ g of enkephalin or PNMT plasmid DNA and 8 μ g pRSV β GAL (Edlund et al., 1985). pRSV β GAL contains the Rous sarcoma virus promoter fused with the lac Z, β -galactosidase, coding sequence. For *in situ* expression, the pENK β GAL-1 plasmid was used alone. Following dropwise addition, precipitates were left on the cells overnight and cultures were then shocked for 3 min with 15% glycerol in DMEM. Treated cells were extensively washed in Dulbecco's PBS before adding medium containing the appropriate regulator. For experiments involving pENKAT12, 100 nM 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and 25 μ M forskolin were used singly or in combination; where indicated, 3-isobutyl-1-methylxanthine (IMX) was used at a concentration of 0.5 mM. For experiments involving the PNMT constructs, transfected cultures were subsequently maintained in serum-free medium with or without dex at a concentration of 20 μ M. Cultures treated in parallel and used for mRNA quantitation were transfected with pUC18, containing no expression sequences. For tumor data, rat pheochromocytoma (PC12) cells were transfected with pENKAT12 as previously detailed (Hyman et al., 1988). Most experiments were performed 3–5 times.

The protocols used for transfections of cell lines with the PNMT constructs were carried out as described by Comb et al. (1986), except that 25–35 μ g of plasmid was used to make the precipitates and incubation times in precipitates ranged from 6 hr to overnight. Positive

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-863  GTCGACTGAG GGGTGGGGG TGAAGGATGG CAGGGTCCTC TACCTGTGAA
-813  AAACCAAAAA GTGCGCATGC CGTGCTCTG TAGTGGCTGT GGCAGTACCA
-763  AGAATGTGTT CTGCACTCTC TGTCTTACA CGAGTCCGGT GTCCTGACC
-713  TGGTAGGAAC ATCCTGAACT AACCATGCTT GCCGGACCTC AGATAAGCAG
-663  CGCATAGCCC CAGGGCCCCA AGGGATGCCG GATGTTCTGC TTGCTAAAAG
-613  CATTAGACCC ACTCACTGTA TCTCTGCTGC CATCAATGC TCGGGGCAGA
-563  GACTGCTCAG TACCAGGGCC ACAAGACAGA GGCCAGAACA GAGTGTCTTT
-513  TCTGAAGGAG GATAGAGACG GGGTAAGTCT GAGTGGGGAT GGCATGATAA
-463  AGAAGGGATT TGTAAGGGT ACCCCGAGGA GGGAGGAGGC CGGGAAGGAT
-413  GCTGGGACTG GGAACATGAG CTAGCTCAGA CCTTTGGGAA AGAGAAGGGA
-363  TGGGATGCTG GACGTGGACG GAGTCTGTGG GAGGCTGGAG AGGAGTCAGG
-313  GGGCCACTGG AACTGAGGCT GGGGGAGCAG AGCAGCACTG ATCAAGAGCC
-263  CATAAAGGTG GAGGGTTGTC AAGCAAGAGT AAAGCAGGAG TGGGGCAGGA
-213  GCGATGTTCT AAAGGGCGCC CTCCACATCT CCCCCTCCCC CGCCCCCGCG
-163  CGTCCGTCGG GCGCTCAGAC ACTAAGTGG ATGGATGGGG TGACAGAGAT
-113  GTGCGGCGCT CGGCGCTCTA TCCTCAGCA GCCACCCACC CCTGTGATGG
-63   AGGGGTCTGG GCGGGGGGGA GGGGACCCAG TAGTAGATAA AGGGATGGGG
-13   AGGTTAGCGG AGA tagggcgccctcaacaggagc ATG GAC CGT GGC TCA
      GAC CCG AAG CAC ACT GCA GGG ATG GAC TCT GAC TCC GAC CCT

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Figure 1. Sequence of the 5' end of the rat PNMT gene. The nucleotide sequence of the first 863 bases upstream of the transcriptional start (CAP) site appear in *capital letters*. The CAP site and noncoding sequence of the first exon appear in *lowercase letters*, while the sequence of the first 19 amino acids are arranged in triplets in *uppercase letters*.

controls for these transfections consisted either of parallel transfections with known, expressing CAT plasmids or cotransfection with pRSV β GAL. CAT assays were performed as described for the chromaffin experiments (see below).

Analyses of gene expression. Transfected cultures remained in regulators for 24 hr. Cells were harvested by scraping and rinsed in PBS prior to homogenization in 250 mM Tris, pH 7.5, 0.5% Triton X-100. CAT assays were performed using 3 mM butyryl-CoA (Pharmacia) as cofactor, 14.8 nmol (0.8 μ Ci) per assay of 14 C-chloramphenicol (Amersham) as substrate, and reaction products were extracted with a 2:1 mixture of pristane/xylenes instead of thin-layer chromatography (Seed and Shen, 1988). Reactions were incubated for 1–2 hr for the pENKAT12 experiments, while for the pPNMT experiments, assays were extended to 5 hr with an additional aliquot of butyryl CoA delivered to the samples at 3 hr. Under these conditions, the CAT assay was determined to remain linear for 6 hr (not shown).

β -Galactosidase activities in the cotransfected cultures were measured as described (Edlund et al., 1985), and these values were used to normalize CAT activities within a given experiment. The effect of dex on the endogenous PNMT gene was studied by Northern analysis. Total cytoplasmic RNA was prepared from the mock transfected parallel cultures by lysis of the cells in 0.5% NP-40, removal of nuclei by centrifugation (13,000 rpm in microfuge), repeated phenol/chloroform extractions, and ethanol precipitation. Fifteen micrograms of total RNA were added to each lane of a 1.2% agarose/formaldehyde gel, and electrophoretically separated species were transferred to a nylon (Gene Screen) membrane. A bovine PNMT cDNA probe was labeled by random priming (5×10^8 cpm/ μ g) and hybridized under stringent conditions. Relative levels of message were quantitated by densitometry.

Histochemistry. Chromaffin cells were plated onto poly-L-lysine-coated plastic dual-chamber slides (LabTek) and either cultured for 48 hr in preparation for immunohistochemical staining or transfected with pENK β GAL for visualization of β -galactosidase activity *in situ*. Cells were fixed with 4% paraformaldehyde in 0.1 M PBS, pH 7.3, for 1 hr at room temperature. Immunohistochemical staining was performed by the peroxidase–antiperoxidase method (Sternberger, 1979; Ross et al., 1981) using TH antibody (1:1000 dilution; Eugene Tek), PNMT antibody (1:2000 dilution; Eugene Tek), or met-enkephalin antibody (1:

2000 dilution; Immuno Nuclear Corp.). The histochemical β -galactosidase activity was visualized by the method of Sanes et al. (1986). Fixed slides were incubated in the reaction buffer (15 mM PBS, pH 7.3, 3 mM MgCl₂, 5 mM K ferricyanate, 5 mM K ferrocyanate, 1% X Gal) for 12 hr at room temperature prior to mounting in 50% glycerol for photography.

Results

Cloning of PNMT

The rat PNMT gene was cloned using an EMBL 3 genomic library. The entire gene was located on a single phage containing a 20 kb insert. The 5' flanking region of rat PNMT was localized to a 1.1 kb HincII to HindIII fragment whose sequence appears in Figure 1. The physical map of the PNMT promoter is shown in Figure 2A. The transcriptional start (CAP) site was determined by primer extension analysis (Fig. 2B) to reside 23 bp downstream of the TATA box and 21 bp 5' to the translational start site, in good agreement with published data for the human (Baetge et al., 1988) and bovine (Batter et al., 1988) genes. Consensus sequences for several known regulatory elements are found in the first 863 bp of the rat PNMT promoter. These include a putative GRE matching 12 of 15 bp of the consensus (Jantzen et al., 1987), located at –513 to –528, and several Sp1-like elements matching 6 of 6 bp of the core consensus (Briggs et al., 1986), located at –50 to –55 and –169 to –174.

Expression of PNMT fusion genes

In order to examine the transcriptional regulation of the PNMT promoter, a series of constructs were made fusing the PNMT 5' flanking region to a CAT reporter gene. A diagram of the nested set of PNMT expression plasmids, possessing 3000–100 bp of PNMT promoter sequence, is shown in Figure 6A. Each of these plasmids yielded no discernible basal or stimulated CAT activity when transfected into a variety of continuous cell lines, including pheochromocytoma (PC12), glioma (C6), neuroblastoma/glioma hybrid (NG108), pituitary (GH4), kidney (CV1), embryonal carcinoma (P19), and fibroblast (LTK⁺) cell lines. The absence of any detectable expression of these fusion genes when transfected into continuous cell lines suggested that there are either tissue-specific requirements for the proper functioning of the PNMT promoter which are not fulfilled in the available tumor lines or that the constructs themselves did not contain the full complement of PNMT sequences required for expression. These possibilities could only be tested in a cell type that endogenously expresses the PNMT gene and therefore must possess the necessary factors for expression of a correctly constructed PNMT fusion gene. Since there are no continuous cell lines which endogenously express PNMT, a system was devised for transfecting expression plasmids directly into bovine adrenal chromaffin cells in primary culture. The well-characterized proenkephalin promoter plasmid, pENKAT12, was used to establish the ability of these cells to be transfected and evaluate the appropriateness of this system for studying PNMT as well as other catecholamine, and coexpressed neuropeptide, genes.

Primary cultures

In preparation for transfection experiments, primary cultures of bovine adrenal medullary cells were obtained by dissociation in collagenase followed by density gradient separation and differential plating. The appearance of the cultures is shown in Figure 3. After separation on Renografin gradients, the isolates were typically composed of 90–95% chromaffin cells, as judged by Neutral red vital staining. Substantial further purification

Figure 2. Map of the rat PNMT promoter. *A*, Location of the TATA box (shaded), transcriptional and translational start sites, as well as several putative enhancer elements in relation to the unique restriction sites of the fragment. *B*, Autoradiogram of the primer extension product (lane 1) of the rat PNMT message. Lane 2 shows the A reaction of the sequence for the PNMT gene using the same oligonucleotide as primer.

was obtained by differential plating so that the final cell population was composed of more than 95% adrenal medullary chromaffin cells. Immunohistochemical staining for TH revealed that approximately 98% of the cells in culture were catecholaminergic (Fig. 3, *A*, *B*). Immunolabeling of duplicate cultures with PNMT antibody showed that 70–80% of these chromaffin cells are adrenergic (Fig. 3, *C*, *D*). Approximately 50–70% of the chromaffin cells stain for met-enkephalin (not shown).

Characterization of the primary cell transfection system using pENKAT12 and pENKβGAL-1

The bovine chromaffin cells in primary culture, described above, were examined for their usefulness for transcriptional studies by transfecting them with fusion constructs known to yield high levels of expression in continuous cell lines. These proenkephalin promoter constructs were a particularly apt choice since enkephalins are coexpressed with catecholamines in the adrenal medulla. Several transfection methods were explored, including 2 DEAE dextran protocols, electroporation and calcium phosphate coprecipitation. The calcium phosphate method produced by far the most satisfactory results.

In order to ensure that the predominant cell type transfected was chromaffin rather than fibroblast, the pENKβGAL-1 plasmid was introduced into these cultures for labeling *in situ*. The pENKβGAL-1 plasmid contains the proenkephalin promoter, extending 5' to nucleotide -193, fused to a lac Z reporter gene

whose product, β-galactosidase, can be visualized histochemically. Using this analysis, the majority of cells transfected (>90%) were shown to have the morphology of chromaffin cells, not of fibroblasts (Fig. 4). In addition, the efficiency of transfection was estimated to be 5% of cells, based on the histochemical labeling.

The ability of these chromaffin cells to express CAT reporter constructs was examined using the well-characterized pENKAT12 (Comb et al., 1986, 1988) plasmid, containing the proenkephalin promoter. The cultures were simultaneously transfected with pENKAT12 and pRSVβGAL, containing the β-galactosidase transcription unit under the control of the constitutively active RSV promoter, so that CAT values could be normalized, using β-galactosidase activity, for variability in transfection efficiency. In terms of raw data, basal levels of activity for pENKAT12 ranged from 10,000 to 60,000 cpm above blank, while β-galactosidase activities ranged from 0.1 to 0.3 OD units per amount of protein added.

Table 1 shows the activation of pENKAT12 expression by second messengers after transfection into chromaffin cells. All CAT values have been normalized to β-galactosidase activity. High levels of basal CAT expression were observed. As shown, inductions of CAT activity were virtually equivalent for either the phorbol ester, TPA, or forskolin treatment and were typically on the order of 3- to 5-fold. In combination, these 2 regulators exerted an additive effect on pENKAT12 expression, with inductions as high as 16-fold. In order to ensure that the effect of

Table 1. Effects of TPA and forskolin on pENKAT12 expression in primary chromaffin cell cultures

Control ^a	TPA ^a	TPA fold induction	Forskolin ^a	Forskolin fold induction	TPA + forskolin ^a	TPA + forskolin fold induction
39,222	142,592	3.6	120,474	3.1	216,689	5.5
10,778	76,174	7.1	78,952	7.3	175,199	16.3
10,607	124,251	11.7	53,230	5.0	170,101	16.0
60,025	205,985	3.4			573,676	9.6
51,772	204,420	4.0			617,643	11.9

Bovine chromaffin cells transfected with 35 μ g pENKAT12 and 8 μ g pRSV β GAL were treated with 100 nM TPA and 25 μ M forskolin, singly or in combination for 24 hr, and CAT and β -galactosidase activities were determined as described in Materials and Methods.

^a Activity is expressed as [CATcpm/ β -galactosidase] $\times 10^{-1}$. CAT activities were normalized to the cotransfected β -galactosidase values and fold inductions were determined. Normalization factors for experiments ranged from 0.1 to 0.3.

forskolin was mediated by cAMP, nondegradable analogs of cAMP were also tested. In PC12 cells, 8-(4-chlorophenylthio)-cAMP (cpt-cAMP, 200 μ M) produced inductions equivalent to forskolin plus IMX (not shown). The induction trends for pENKAT12 in chromaffin cells are similar, though not identical, to those observed in transient transfections of tumor cell lines, including PC12 cells. As shown (Fig. 5), in PC12 transfections, TPA or forskolin had an appreciable influence on pENKAT12 transcription only in the presence of a phosphodiesterase inhibitor, IMX. In contrast, IMX was not required to observe substantial TPA and forskolin inductions in the primary cell system.

Identification of a functional GRE in the PNMT promoter

Having established the capability of this system for use in transcription analyses, the PNMT promoter fusion gene constructs described above (Fig. 6A) were transfected into the adrenal medullary cells in primary culture. In contrast to continuous cell lines, when introduced into these chromaffin cells, low but appreciable basal levels of CAT activity were obtained. Basal activity levels were virtually identical for all 4 constructs and ranged from 1000 to 3000 cpm above blank with β -galactosidase activities ranging from 0.1 to 0.3 OD units per amount of protein added. The promoterless pBLCAT3 plasmid yielded 0 to 500 cpm above blank.

The effect of glucocorticoids on the expression of these plasmids is shown in Figure 6B. All values of CAT activity were normalized to cotransfected β -galactosidase activities to correct for differences in transfection efficiencies. In 5 separate experiments, the constructs containing 3000 and 900 bp of PNMT 5' sequence, both of which contain the GRE, showed virtually identical 4- to 6-fold inductions (and, in later series, even 10-fold) with dex treatment. In contrast, the constructs containing only -300 and -100 bp of 5' PNMT flank, which therefore lack the putative GRE sequence, showed no significant response

to dex. In order to ensure that the glucocorticoid depletion-stimulation paradigm used affected the endogenous PNMT gene in a similar fashion, duplicate cultures were sham transfected with pUC18 and treated in parallel with the fusion gene experiments. The endogenous PNMT gene responded to glucocorticoid treatment with a 40-fold increase of PNMT message, as determined by northern analysis (Fig. 6C). Moreover, there was no observed effect of the calcium phosphate treatment alone on the endogenous PNMT mRNA levels (Fig. 6C).

The functional activity of this GRE consensus sequence was further investigated using a heterologous promoter construct with an enhancerless thymidine kinase (TK) promoter fused to PNMT sequences spanning -391 to -863 (pPTK500) (Fig. 7A). Compared with the PNMT promoter plasmids, this heterologous construct yielded several-fold higher levels of basal activity but showed virtually the same 5- to 8-fold inductions (Fig. 7B) seen for the intact PNMT promoter. The parent plasmid lacking PNMT sequences, pBLCAT2, does not respond to glucocorticoid stimulation. These results narrowed the region responsible for the observed dex response to a 470 bp PNMT fragment containing the GRE consensus sequence.

In order to identify this GRE consensus sequence unequivocally as a functional enhancer, site-directed mutagenesis was used to specifically excise the region spanning -513 to -528 bp, comprising the putative GRE motif (Fig. 7A). In parallel transfections (Fig. 7B), the wild-type PNMT promoter construct, pPNMT900, produced a 6- to 10-fold increase in CAT activity in response to dex while removal of the 15 bp GRE sequence from the promoter (p Δ GRE) abolished its response to dex. These results confirmed this region to be a functional GRE regulating PNMT expression.

Discussion

Though the approach has been fruitful, there are obvious limitations to examining transcriptional activity through the intro-

Figure 3. Immunohistochemical characterization of bovine chromaffin primary cultures used for transfections. *A* and *B*, Cultures are stained using antibody to TH. After purification, virtually all ($\geq 98\%$) of the cells present in culture are immunopositive for TH and are therefore catecholaminergic. *C* and *D*, Duplicate cultures to those in *A* and *B*, stained instead using antibody to PNMT. Roughly 70–80% of the cells present in culture are immunopositive for PNMT and are therefore adrenergic. Unstained cells (arrows) are presumably noradrenergic chromaffin cells. Scale bars: low magnifications (*A* and *C*), 200 μ m; high magnifications (*B* and *D*), 50 μ m.

duction of fusion constructs into a tumor cell. First, as is the case for PNMT, a continuous cell line may not exist that endogenously expresses the gene and therefore can surely fulfill the tissue-specific requirements necessary for its promoter constructs to direct transcription. Second, even if a given promoter fragment can function within a tumor cell, as is the case for pENKAT12, the regulation of the promoter cannot be assumed to be the same as in the normal cell types that express the gene. Third, the array of receptors in the cell line may not be identical to those in the cells normally expressing that gene, so that the transmembrane signals occurring *in vivo* cannot be examined.

In our studies of transcriptional regulation of the catecholamine genes and their coexpressed neuropeptides, we have sought to circumvent these limitations by establishing a method for transfecting primary cultures of dissociated bovine adrenal medullary cells. As demonstrated by immunohistochemical staining, rather pure chromaffin cell cultures can be obtained from bovine adrenal glands when combined methods of density gradient separation and differential plating are used. This obviates the requirement for addition of mitotic inhibitors, which are used to limit the growth of fibroblasts in culture but which could introduce artifacts into studies of transcription. Fusion gene constructs can be transfected into these catecholaminergic and enkephalinergic cells by calcium phosphate–DNA coprecipitation with good efficiency, resulting in excellent levels of regulated expression of introduced plasmids. The predominating cell type (>90%) transfected in these cultures is chromaffin, not fibroblast, in morphology, as demonstrated by *in situ* labeling of β -galactosidase activity generated from pENK β GAL-1.

Expression of pENKAT12

The pENKAT12 plasmid has previously been used in tumor cell transfection experiments to map a cAMP and phorbol ester-inducible enhancer (Comb et al., 1986) which is composed of 2 functionally distinct elements, ENKCRE-1 and ENKCRE-2 (Comb et al., 1988), as well as an AP2 element (Hyman et al., 1989). Under the conditions selected, this plasmid can be introduced into chromaffin cells, resulting in high levels of CAT expression. We have examined in this transfection system the regulation of pENKAT12 transcription by the phorbol ester TPA, presumably acting through protein kinase C, and by the adenylate cyclase activator, forskolin, exerting its influence through cAMP-dependent protein kinase (protein kinase A).

The chosen promoter fragment in pENKAT12 is not tissue specific and its enhancer, which is cAMP and phorbol ester inducible, responds to 2 ubiquitous second-messenger systems. Nevertheless, there are several features of pENKAT12 expression in chromaffin cells that can be distinguished from its transcription in continuous cell lines, including PC12. For example, the basal levels of pENKAT12 expression are impressively higher (on the order of 10-fold) in chromaffin cells than in tumor lines, even when cultures are treated under identical conditions. In contrast to the tumor cell data, CAT inductions in chromaffin

Figure 5. Comparison of the drug induction profiles of pENKAT12 when expressed in PC12 or bovine chromaffin cells. Transfected cultures were treated for 24 hr with either 25 μ M forskolin (F) or 100 nM TPA (T) in the presence or absence of 0.5 mM IMX (I). When expressed in PC12 cells, IMX alone has no effect on transcription, while in chromaffin cells there is a nearly 4-fold induction of pENKAT12 expression. In PC12 cells, neither forskolin nor TPA affect pENKAT12 transcription in the absence of the phosphodiesterase inhibitor, IMX. In contrast, when pENKAT12 is expressed in bovine chromaffin cells, substantial inductions (up to 7-fold) are produced by forskolin or TPA in the absence of IMX. In the presence of IMX, the inductions of pENKAT12 transcription produced by forskolin and TPA are modestly augmented over those observed without IMX in the chromaffin system. Forskolin and TPA inductions of CAT activity in chromaffin cells in the presence of IMX are virtually identical to those observed in the PC12 transfection system.

cells by TPA and forskolin can be observed in the absence of the phosphodiesterase inhibitor, IMX. Moreover, although IMX alone has no appreciable effect on pENKAT12 expression in continuous cell lines, it produces a 4-fold increase in CAT activity in the chromaffin expression system. Though the reason for this observed difference is not yet clear, it may be due to cell-type specific differences in basal levels of cAMP or activities of phosphodiesterases. On the other hand, IMX may be exerting its influence by another route, such as the known interaction of methylxanthines with adenosine receptors, the state of which may differ between tumor and primary cells.

Expression of PNMT constructs and functional definition of a GRE

The experiments reported here provide several insights into the transcriptional control of the PNMT gene. All 4 PNMT promoter plasmids produce low levels of basal activity, which were detected only in primary chromaffin cells, presumably those which endogenously express PNMT. That none of the continuous cell lines tested produced appreciable expression of the PNMT/CAT constructs suggests that there are either requisite positive trans-acting factors missing in the tumor cell nuclei or negative trans-acting factors are present which preclude the transcription of PNMT in those cell lines. However, an exhaustive search to find transfection conditions permitting expression of these plasmids in cell lines remains to be completed.

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Figure 4. Histochemical staining of primary bovine chromaffin cell cultures transfected with pENK β GAL-1. *A* and *B*, Duplicate cultures to those in Figure 1 were transfected with a plasmid carrying the proenkephalin promoter fused to a lac Z reporter. After 16–24 hr, cultures were fixed and cells containing the plasmid were identified by histochemical reaction for β -galactosidase activity (see Materials and Methods). The transfected plasmid was expressed predominantly in cells with chromaffin morphology. The efficiency of transfection was estimated to be approximately 5%. Scale bars: low magnification (*A*), 200 μ m; high magnification (*B*), 50 μ m.

Figure 6. Localization of a region of the rat PNMT promoter which is responsive to glucocorticoid treatment. **A**, Maps of the promoters of the 4 PNMT expression plasmids pPNMT3000, 900, 300, and 100. The location of the GRE consensus sequence is indicated. **B**, Glucocorticoid inductions of these plasmids, expressed in bovine chromaffin primary cell cultures. In this series of experiments, the pPNMT3000 and pPNMT900 plasmids produced an average 5.3- and 4.45-fold (as high as a 6-fold) induction of CAT activity in response to 20 μ M dexamethasone (*Dex*) treatment, respectively. All CAT measurements were normalized to β -galactosidase activities of cotransfected pRSV β GAL (see Materials and Methods). The shorter promoter constructs, pPNMT300 and 100, which do not contain the GRE consensus sequence at -513, were not induced by the glucocorticoid. The sequence(s) producing the observed glucocorticoid response is therefore confined to a region spanning -273 to -863 bp of the PNMT gene. **C**, Northern analysis of the endogenous PNMT gene. Total RNA from cultures sham transfected and treated in parallel with those in **B** were blotted and probed first with a bovine cDNA for PNMT and then a probe for 18S RNA to normalize amounts of RNA added to the lanes. PNMT message levels in glucocorticoid depleted (*GC*⁻) cells dropped 50% relative to the serum-fed (*S*) cultures. Following dex treatment, PNMT message levels in depleted cells were induced 40-fold by 20 μ M dex. The calcium phosphate precipitate alone (+*Ca*) had no appreciable effect on PNMT mRNA levels compared with the untreated control (*C*).

A functional GRE in the PNMT promoter has been demonstrated here, spanning bases -513 to -528, relative to the CAP site. That this is the only GRE present within 3 kb of upstream sequence is supported by the fact that there is virtually no difference in the glucocorticoid response when 3 or 0.9 kb of upstream sequence is present in the construct and that removal of this region by site-directed mutagenesis abolishes the observed dex induction in the 0.9 kb construct. Furthermore, the addition of a 470 bp fragment encompassing this GRE con-

fers full glucocorticoid responsiveness to the minimal TK promoter.

It has been proposed that the human PNMT gene contains one putative GRE in its 5' flanking region and a second, weaker consensus sequence in the first intron (Baetge et al., 1988) and that the bovine gene contains 2 GREs in its 5' flank (Batter et al., 1988). Multiple GRE enhancers have been shown to function synergistically on other genes such as tyrosine amino transferase (Jantzen et al., 1987) or independently, as in the case of mouse

A.

B.

mammary tumor virus (Yamamoto, 1985; Buetti and Kuhnel, 1986). It is possible that there are additional GREs not contained on the promoter fragments we have examined and which may act in concert with the GRE defined here. However, as is the case for the tyrosine amino transferase gene, some of the consensus sequences for GREs, even though they bind glucocorticoid receptor in footprinting analyses, have subsequently been shown to make no appreciable contribution to glucocorticoid responsiveness (Jantzen et al., 1987). A detailed comparison of the 3 species of PNMT promoters, therefore, awaits functional analysis of the GRE motifs of the human and bovine genes, as well as a more complete examination of the 3' end of the rat gene. Nevertheless, based on the data presented here, it is clear that glucocorticoids can regulate the transcription of PNMT and that the consensus motif found in the rat gene at -513 bp is a functional, positively acting glucocorticoid response element.

Using chromaffin cells in primary culture, we have at our disposal a system in which to express fusion gene constructs using the PNMT promoter. Mutation analyses can now provide insights into the identity and definition of elements on this promoter. In addition to studies of PNMT function, the chromaffin system provides a biologically relevant paradigm in which to analyze the other catecholamine genes and their coexpressed neuropeptides. For, as demonstrated by the example of pENKAT12, the observed activity of a promoter introduced into an appropriate primary cell may differ from that seen in a tumor cell line. Transient expression in primary cells should also provide an invaluable aid in laying the groundwork for more lengthy and technically complex transgenic animal studies.

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Figure 7. Mutation analysis of the putative GRE in the rat PNMT promoter. **A**, Diagram of the fusion gene constructs used to identify the consensus sequence at -513 as a functional GRE element. The expression plasmid pBLCAT2 contains a minimal thymidine kinase (TK) promoter which lacks enhancer elements. The heterologous promoter plasmid, pPTK500, contains 472 bp of PNMT upstream sequence from -391 to -863 bp, containing the putative GRE, fused to the TK promoter. The mutant plasmid, pΔGRE, contains the PNMT 5' sequence to -863 bp of the wild-type promoter used in pPNMT900, except that the putative GRE motif at -513 bp has been replaced with a Pst I restriction site at that location by oligonucleotide-directed site-specific mutagenesis (see Materials and Methods). **B**, Expression of these plasmids in response to 20 μ M dex treatment. Bovine chromaffin cell primary cultures were transfected with the plasmids diagramed in **A**, and CAT activities, normalized to the cotransfected β -galactosidase activities, were determined in the presence or absence of glucocorticoid. The expression of enhancerless pBLCAT2 does not respond to dex treatment, while the upstream PNMT region containing the GRE consensus sequence (pPTK500) confers full glucocorticoid responsiveness to the minimal TK promoter. In parallel transfections the wild-type PNMT promoter construct (pPNMT900) produces an average 8.25-fold (and as high as a 10-fold) increase in CAT activity in response to dexamethasone, while expression of the site-specific mutant (pΔGRE) which lacks the GRE consensus at -513 bp is not induced by glucocorticoid treatment.

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