0026-895X/02/6106-1385–1392$7.00
MOLECULAR PHARMACOLOGY

Mol Pharmacol 61:1385–1392, 2002

Vol. 61, No. 6
1628/987782
Printed in U.S.A.

Glucocorticoid Responsiveness of the Rat Phenylethanolamine *N*-Methyltransferase Gene

T. C. TAI, R. CLAYCOMB, S. HER, A. K. BLOOM, and DONA L. WONG

Department of Psychiatry, Harvard Medical School, Boston, Massachusetts; and Laboratory of Molecular and Developmental Neurobiology, McLean Hospital, Belmont, Massachusetts

Received January 23, 2002; accepted March 15, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Two newly identified, overlapping (1 bp) glucocorticoid response elements (GREs) at −759 and −773 bp in the promoter of the rat phenylethanolamine *N*-methyltransferase (PNMT; EC 2.1.1.28) gene are primarily responsible for its glucocorticoid sensitivity, rather than the originally identified −533-bp GRE. A dose-dependent increase in *PNMT* promoter activity was observed in RS1 cells transfected with a wild-type *PNMT* promoter-luciferase reporter gene construct and treated with dexamethasone (maximum activation at 0.1 μM). The type II glucocorticoid receptor antagonist RU38486 (10 μM) fully inhibited dexamethasone (1 μM) activation of the *PNMT* promoter, consistent with classical glucocorticoid receptors mediating corticosteroid-stimulated transcriptional activity. Relative IC_{50} values from gel mobility shift competition assays showed that the −759-bp GRE has a 2-fold greater affinity for the glucocorticoid receptor than the −773-bp GRE. Site-directed mutation of the −533-, −759-, and −773-bp GREs alone or in tandem demonstrated that the −759-bp GRE was also functionally more important, but both the −759- and −773-bp GREs are required for maximum glucocorticoid responses. Moreover, the −533-bp GRE, rather than increasing glucocorticoid sensitivity of the promoter, may limit corticosteroid responsiveness mediated via the −759- and −773-bp GREs. Finally, the glucocorticoid receptor bound to the −759- and −773-bp GREs interacts cooperatively with Egr-1 and/or AP-2 to stimulate *PNMT* promoter activity in RS1 cells treated with dexamethasone. In contrast, glucocorticoid receptors bound to the −533-bp GRE only seem to participate in synergistic activation of the *PNMT* promoter through interaction with activator protein 2.

Glucocorticoids are critical regulators of phenylethanolamine *N*-methyltransferase (PNMT; EC 2.1.1.28), the final enzyme in epinephrine biosynthesis, exerting both transcriptional and post-transcriptional influences. In vivo studies in rats have shown that depletion of corticosteroids by hypophysectomy decreases PNMT mRNA and enzyme expression (Evinger et al., 1992; Wong et al., 1992a, 1995, 1996; Evinger, 1998). These changes can be reversed by administration of adrenocorticotropin, which stimulates endogenous glucocorticoid production, or direct corticosteroid replacement by administration of the synthetic glucocorticoid dexamethasone. Changes in PNMT enzyme are a consequence of alterations in both gene transcription and proteolytic degradation (Berenbeim et al., 1979; Wong et al., 1985). In terms of the latter, corticosteroids sustain methionine adenosyltransferase and *S*-adenosylhomocysteine hydrolase, the metabolic enzymes responsible for maintaining the cosubstrate and methyl donor, *S*-adenosylmethionine. Sufficient AdoMet is thereby provided for PNMT enzymatic activity; in addition, however, the binding of AdoMet to PNMT protects it against proteolysis.

When intact rats are administered either dexamethasone or the glucocorticoid agonist RU28362, PNMT mRNA levels rise markedly (Wong et al., 1992b) because of increased gene transcription. Although it remains unclear whether glucocorticoids are essential for PNMT transcriptional activity, glucocorticoid receptor-deficient mice do not express adrenal medullary PNMT although chromaffin cells are otherwise ostensibly normal (Schmid et al., 1995; Finotto et al., 1999).

Glucocorticoid-induced transcriptional changes are mediated through glucocorticoid response elements (GREs) in the proximal 5′ flanking sequences of the *PNMT* gene promoter. At least one putative GRE has been identified for every species-specific *PNMT* gene, including human (Baetge et al., 1988; Kaneda et al., 1988), cow (Baetge et al., 1986; Batter et al., 1988), rat (Ross et al., 1990), and mouse (Morita et al., 1992). In the case of the rat *PNMT* gene, a GRE was identified at −533 bp when the gene was first cloned (Ross et al., 1990). Although this GRE seemed to be functional, its responsiveness to glucocorticoid activation seems both variable and weak. At best, glucocorticoid treatment (1 μM dexameth-

This work was supported by grant DK51025 from the National Institute of Diabetes and Digestive and Kidney Diseases, by the Spunk Fund, Inc., by the Sobel and Keller Research Support Fund, and by McLean Hospital.

ABBREVIATIONS: PNMT, phenylethanolamine *N*-methyltransferase; RU28362, 17-α-alkanyl-11β,17-dihydroxy-androsterone; GRE, glucocorticoid response element; GR, glucocorticoid receptor; AP-2, activator protein 2; RU38486, mifepristone.

asone) elicits no greater than a 2-fold induction of the *PNMT* promoter as demonstrated through transient transfection assays with *PNMT* promoter-luciferase reporter gene constructs (Ebert et al., 1998) or changes in PNMT mRNA measured by ribonuclease protection assays (Morita et al., 1996). However, glucocorticoid-activated glucocorticoid receptors (GR), bound to the −533-bp GRE, seem to interact cooperatively with other transcriptional activators bound to their cognate recognition sites [e.g., the immediate early gene transcription factor Egr-1 (Ebert et al., 1994) and the developmental transcription factor AP-2 (Ebert et al., 1998)] to synergistically stimulate the *PNMT* promoter.

This study is the first to definitively identify the primary GREs mediating the glucocorticoid responsiveness of the rat *PNMT* gene. The sites at −759 and −773 bp have been characterized extensively and their functionality has been established. Glucocorticoid receptors bound to the GREs are further shown to participate in cooperative interactions with two other PNMT transcriptional activators, Egr-1 and AP-2, which is probably important for their biological activity. Finally, it is demonstrated that glucocorticoid receptor activation of these GREs and/or their synergistic interactions with Egr-1 and AP-2 also stimulates the endogenous *PNMT* gene in a manner consistent with their stimulation of the *PNMT* promoter, whereas the original GRE (−533 bp) only shows apparent synergism with AP-2.

Materials and Methods

Plasmids and Oligonucleotides. The wild-type construct pGL3RP893 was generated by subcloning the proximal 863 bp of proximal rat *PNMT* promoter sequences into the plasmid pGL3Basic (Pharmacia-Upjohn, Kalamazoo, MI). Verification of the promoter fragment by DNA sequence analysis (Her et al., 1999) showed that the insert was 893 bp in length, rather than 863 bp as identified originally (Ross et al., 1990). The difference arises in GC-rich regions where G and C residue determination may be difficult and is consistent with a recent report on this proximal extent of *PNMT* promoter (Evinger, 1998). Hence, the full-length construct was redesignated pGL3RP893.

Nested deletion constructs pGL3RP849, pGL3RP798, pGL3RP745, pGL3RP665, and pGL3RP557 were produced from the wild-type construct pGL3RP893 by 5′ exonuclease digestion, followed by religation. Constructs with site-directed mutations in the GREs were also developed. The wild-type construct pGL3RP790 and mutant construct pGL3RP790mut533 were generated by subcloning the *Xho*I-*Hin*dIII restriction fragments from pRP893LUC and pRP893mutGRE1LUC (Ebert et al., 1998) into the corresponding restriction sites of the pGL3Basic vector upstream of the luciferase reporter gene. The remaining mutant constructs, pGL3RP790mut759, pGL3RP790mut773, and pGL3RP790mut759/773, were produced from pGL3RP790 using the polymerase chain reaction as described below.

For gel mobility shift assays, protein-DNA complexes were formed using the wild-type 40-bp oligonucleotide GRE773/759. Competitor oligonucleotides included unlabeled GRE773/759, GRE773, GRE759, and palGRE, with nucleotide sequences as follows: 5′GTACCAAGAATGTGTTCTGCACTCTCTGTTCTTACACGAG3′ (−790 → −751, GRE773/759); 5′GTACCAAGAATGTGTTCTGCA3′ (−790 → −770, GRE773); 5′TTCTGCACTCTCTGTTCTTAC3′ (−776 → −756, GRE759); and 5′AGAGGATCTGTACAGGATGTTCTAGAT [palindromic (Scheidereit and Beato, 1984) or palGRE].

Egr-1 (Sukhatme et al., 1988) and AP-2 (Mitchell et al., 1987) expression and control constructs were kindly provided by Dr. Vikas Sukhatme (Harvard Medical School, Boston, MA) and Dr. Trevor Williams (Yale University, New Haven, CT) respectively.

Transient Transfection Assays. Transient transfection assays were executed as described previously (Her et al., 1999; Tai et al., 2001) in the rat pheochromocytoma-derived RS1 cells (Ebert et al., 1994). Briefly, cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% bovine calf serum, 5% equine serum (Hyclone, Logan, UT), 20 units/ml of hygromycin B (Calbiochem, La Jolla, CA), and 50 μg/ml gentamicin sulfate (U.S. Biochemicals Corp., Cleveland, OH) at 37°C in an atmosphere of 5% CO_2/95% air. All sera were charcoal-treated to remove endogenous glucocorticoids.

For transfection, cells were plated into 24-well culture dishes (2×10^5 cells/well) and held at 37°C and 5% CO_2/95% air overnight. Transfection was then performed using Superfect (QIAGEN, Inc., Valencia, CA) or polyethylenimine (Boussif et al., 1995) including 0.5 to 1.0 μg of wild-type or mutant *PNMT* promoter-luciferase reporter gene construct, 0 to 1.5 μg of expression or null construct, pCMVEgr-1 or pCMVETTL (Gupta et al., 1991) and/or pSPRSV-AP-2 or pSPRSV-NN (Williams and Tjian, 1991), and 0.3 μg of pRSV-LacZ normalization control construct. Total transfected DNA was adjusted to 3.0 μg by making up the difference with the pGL3Basic plasmid vector. After transfection, cells were exchanged to culture medium and maintained an additional 24 h. To examine the effects of dexamethasone and the antiglucocorticoid RU38486 (Roussel-UCLAF, Romaineville, France), transfected cells were exposed to dexamethasone (0–10 μM; Sigma Chemical, St. Louis, MO) for 6 h or pretreated with the antagonist for 1 h (0–10 μM), followed by 1 μM dexamethasone treatment for 6 h. The cells were then collected, lysed, and assayed for luciferase and β-galactosidase as described below.

Luciferase and β-Galactosidase Assays. After removal of the culture medium, the cells were washed twice with phosphate-buffered saline and then lysed in 100 μl of lysis buffer (Promega, Inc., Madison, WI). Cellular debris was removed by centrifugation at 800*g* and luciferase activity measured in 20 μl of cell lysate appropriately diluted to yield luciferase activity within the linear range as defined with purified luciferase using the Luciferase Assay System described previously (Ebert et al., 1994). Protein was determined by the method of Bradford (1976) and luciferase activity was adjusted for protein concentration. To correct for variation in transfection efficiency, β-galactosidase activity was also determined (Ebert et al., 1994) and luciferase activity expressed relative to β-galactosidase generated from the pRSV-LacZ control construct. As appropriate, the ratio of luciferase/β-galactosidase for the wild-type *PNMT* promoter-luciferase reporter gene construct was set to unity, and the ratio of luciferase/β-galactosidase for other constructs expressed relative to it. Alternatively, the ratio of luciferase/β-galactosidase expressed by *PNMT* promoter-luciferase constructs, wild-type, truncated or mutant constructs, in the absence of dexamethasone, was set to unity, and values in the presence of dexamethasone expressed relative to these respective untreated controls. At least six replicates were included in each sample group and experiments repeated two to three times.

Site-Directed Mutagenesis. Site-directed mutation of the −759- and −773-bp GREs was performed by polymerase chain reaction as described previously using the following 40-bp mutagenic primers, spanning upstream sequences from −790 to −751 bp in the *PNMT* promoter (Her et al., 1999): 5′GTACCAAGAATGTcaTCTGCACTCTCTGTTCTTACACGAG3′ (mut773); 5′GTACCAAGAATGTGTTCTGCACTCTCTcaTCTTACACGAG3′ (mut759); and 5′GTACCAAGAATGTcaTCTGCACTCTCTcaTCTTACACGAG3′ (mut773/759).

Gel Mobility Shift Competition Assays. As described previously (Her et al., 1999), protein-DNA complexes were generated using 1 ng of the wild-type oligonucleotide probe GRE773/759, end-labeled with [γ-^{32}P]ATP and T_4 polynucleotide kinase (3 nM, specific activity = 2.5×10^8 dpm/μg), and a truncated GR protein (Dr. Keith Yamamoto, University of California, San Francisco) (Freedman et al., 1988) in 20 μl of binding buffer consisting of 25 mM HEPES buffer, pH 7.9, 50 mM KCl, and 0.05 mM EDTA. Complexes were competed by including unlabeled oligonucleotides ranging in concentration from 1 to 1000 ng. The amount of residual complex was then quantified from its autoradiographic signal by scanning densitometry using Image software, version 1.52 (National Institutes of Health), on a Power MacIntosh 7500 computer and a Hewlett Packard 6100C scanner. IC_{50} values were determined by linear regression analysis of graphs of optical density units versus ln[competitor oligonucleotide concentration] and calculation of the x-intercept.

Statistical Analysis. Data are presented as the mean ± S.E.M. with $n = 6$ for each experimental group. Statistical significance between groups was determined by one-way analysis of variance followed by post hoc comparison using Student's t test. A p value ≤0.05 was considered statistically significant.

Results

Glucocorticoid Responsiveness of the *PNMT* Promoter. To examine the glucocorticoid responsiveness of the *PNMT* promoter, the proximal 893 bp of 5′ promoter/regulatory sequences were subcloned into the plasmid pGL3Basic (Pharmacia-Upjohn, Kalamazoo, MI) upstream of the firefly luciferase reporter gene. When this wild-type construct (pGL3RP893) was transiently transfected into the PC-12–derived RS1 cells (Ebert et al., 1994) and the cells treated with 1 μM dexamethasone, luciferase activity was induced ~6.0-fold, markedly higher than reported previously (Fig. 1).

To identify the glucocorticoid responsive DNA sequences within the promoter, nested deletion mutant *PNMT* promoter-luciferase reporter gene constructs were generated by 5′ exonuclease digestion. Transient transfection assays in the absence and presence of dexamethasone (1 μM) showed that the DNA sequences lying between −745 and −798 bp were responsible for the marked glucocorticoid induction of the promoter (Fig. 1). Two of three plasmid constructs (pGL3RP557 and pGL3RP665) harboring only the proximal −533-bp GRE (previously designated −513 bp) (Ross et al., 1990) did not show any apparent corticosteroid activation, whereas a third (pGL3RP745) showed a 2-fold stimulation of the *PNMT* promoter. In addition, sequences beyond −798 bp seemed to limit the glucocorticoid responsiveness of the *PNMT* promoter. Highest dexamethasone stimulation of *PNMT* promoter-driven luciferase activity occurred with the construct containing 798 bp of promoter sequence (pGL3RP798). Longer constructs expressed lesser amounts of luciferase; the full-length construct pGL3RP893 showed only a 6.0-fold induction by comparison to the 12.0-fold induction seen with the construct pGL3RP798.

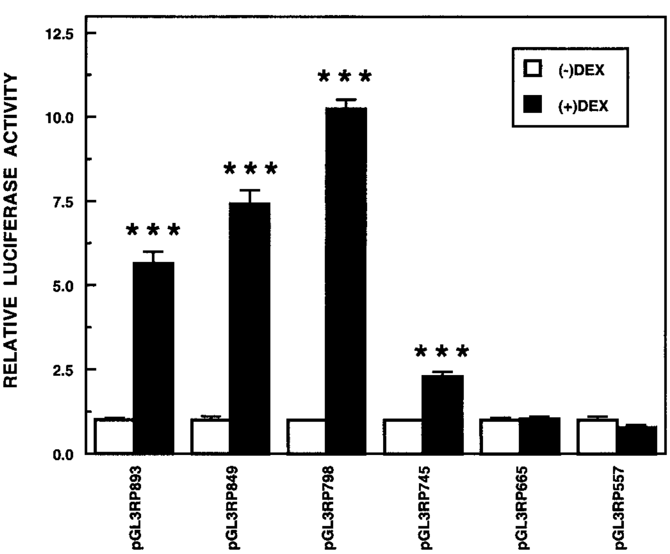

Fig. 1. Glucocorticoid activation of the *PNMT* promoter. The wild-type construct, pGL3RP893, and nested deletion *PNMT* promoter-luciferase reporter gene constructs were transfected into RS1 cells, the cells were treated with 1 μM dexamethasone, and luciferase activity was measured as described in *Materials and Methods*. ***, significantly different from respective control, $p < 0.001$.

Matching of the DNA sequences in the glucocorticoid sensitive region to the consensus glucocorticoid response element, 5′TAGAACANNNTCTTCT3′ (Scheidereit and Beato, 1984), using the Transfac database (version 3.2) identified two new GREs. Based on the highly conserved 3′ hexanucleotide sequence of the consensus GRE, TGTTCT, the 3′ termini of these GREs were fixed at −759 and −773 bp, with a 1-bp overlap of their 5′ and 3′ ends, respectively.

Effects of Dexamethasone and RU38486 on *PNMT* Promoter Activation. Because longer constructs seemed to contain sequences inhibiting full glucocorticoid responsiveness of the *PNMT* promoter, the construct pGL3RP790, which shows comparable dexamethasone sensitivity to pGL3RP798, was used in subsequent studies (data not shown). A dose-response curve was first executed for dexamethasone (Fig. 2A). When RS1 cells transfected with pGL3RP790 were treated with 0 to 10 μM dexamethasone for 6 h, a dose-dependent rise in *PNMT* promoter-driven luciferase reporter gene expression was observed. Maximum stimulation of the promoter occurred at 0.100 μM. No significant change in activation was observed when the dexamethasone concentration was increased to 1 μM dexamethasone. However, luciferase activity decreased to levels equivalent to 0.01 μM dexamethasone when corticosteroid levels were increased to 10 μM.

Because dexamethasone is both a type I and type II GR receptor agonist, with greater preference for type I receptors, the effects of RU38486, a specific type II GR antagonist, was investigated. RS1 cells transfected with pGL3RP790 were pretreated with RU38486 (0–10 μM) for 1 h, followed by treatment with 1 μM dexamethasone for 6 h and *PNMT* promoter-driven luciferase expression determined. As shown in Fig. 2B, RU38486 inhibited the dexamethasone-mediated rise in luciferase. A significant reduction in dexamethasone-stimulated *PNMT* promoter activity was apparent at 0.001 μM, with complete inhibition at 10 μM RU38486.

Glucocorticoid Receptor Binding to the Upstream GREs and Activation of the *PNMT* Promoter. The previous results are consistent with type II GRs mediating *PNMT* promoter transcriptional activation. To demonstrate the specificity of the GR and its relative binding affinity for the newly identified GRE target sequences, gel mobility shift competition assays were executed (Fig. 3). Protein-DNA binding complex was formed between a 40-bp ^{32}P-labeled wild-type oligonucleotide spanning both the −759- and −773-bp GREs and 5 bp of 5′ and 3′ flanking sequence and a truncated GR protein (Dr. Keith Yamamoto, University California, San Francisco). The complex was competed with increasing amounts of unlabeled oligonucleotide (1–1000 ng), including the unlabeled 40-bp oligonucleotide and 21-bp oligonucleotides encoding the −759-bp, −773-bp, and palindromic GRE sequences (Fig. 3A). All oligonucleotides interacted with the GR peptide as demonstrated by their displacement of the radiolabeled DNA (Fig. 3B). The abundance of GR-GRE binding complex was quantified by scanning densitometry and relative IC_{50} values determined by regression analysis of signal intensity versus ln[competitor concentration] (Fig. 3, B and C). The palindromic GRE and the oligonucleotide harboring both upstream GREs had the highest affinity for the GR (relative IC_{50} values: 11.0 and 8.7, respectively). The affinities of the −759- and −773-bp GREs for the GR were ~3.5 to 7.0-fold lower based on relative IC_{50} values; the −759-bp GRE had ~2-fold higher affinity (relative IC_{50}, 30.6) for the GR than the −773-bp GRE (relative IC_{50}, 60.8). The slopes of the regression curves for each competition assay did not significantly change, confirming that the differences in x-intercepts (IC_{50}) were caused solely by affinity of the DNA sequences for the GR and not differences in receptor abundance as well.

To further examine the functionality of the −759- and −773-bp GREs, site-directed mutations were introduced into the various GREs as described under *Materials and Methods* to produce the single mutant constructs pGL3RP790mut533, pGL3RP790mut759, and pGL3RP790mut773; the double mutant construct pGL3R790mut773/759; and the triple mutant construct pGL3RP790mut533/759/773. The wild-type or mutant constructs were then transiently transfected into RS1 cells and *PNMT* promoter-driven luciferase activity was determined in the absence or presence of dexamethasone (1 μM) (Fig. 4A). When the −533-bp GRE was mutated, rather than a decrease, a ≥1.5-fold increase in dexamethasone-stimulated *PNMT* promoter activation occurred at dexamethasone concentrations between 0.01 and 1 μM. By contrast, mutation of either the −759 or −773-bp GRE markedly attenuated dexamethasone-stimulated *PNMT* promoter activity. However, when the −773-bp GRE was mutated, a dose-dependent increase in *PNMT* promoter-driven luciferase activity was still apparent, although maximum induction was only 3.1-fold (1 μM dexamethasone). As observed with all GREs intact, 10 μM dexamethasone stimulated the *PNMT* promoter less than 1 μM dexamethasone. When the

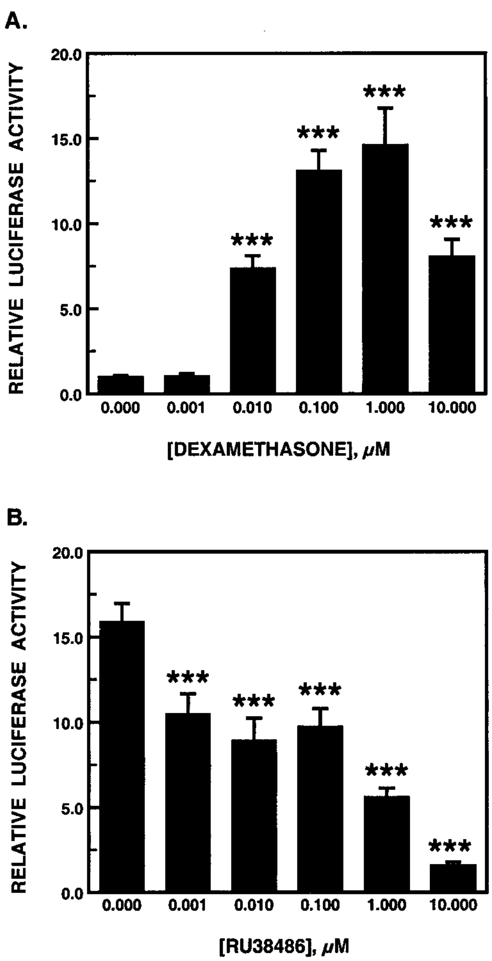

Fig. 2. Effects of dexamethasone and RU38486 on the *PNMT* promoter. The wild-type construct pGL3RP790 was transfected into RS1 cells as described under *Materials and Methods*. The transfected cells were treated with dexamethasone (0–10 μM) or pretreated with RU38486 (0–10 μM) for 1 h, followed by 1 μM dexamethasone. After 6 h, cells were collected and luciferase activity measured. A, dexamethasone responsiveness of the *PNMT* promoter. ***, significantly different from untreated wild-type control, $p \leq 0.001$. B, RU38486 effects on dexamethasone-responsiveness of the *PNMT* promoter. ***, significantly different from wild-type control, $p \leq 0.001$.

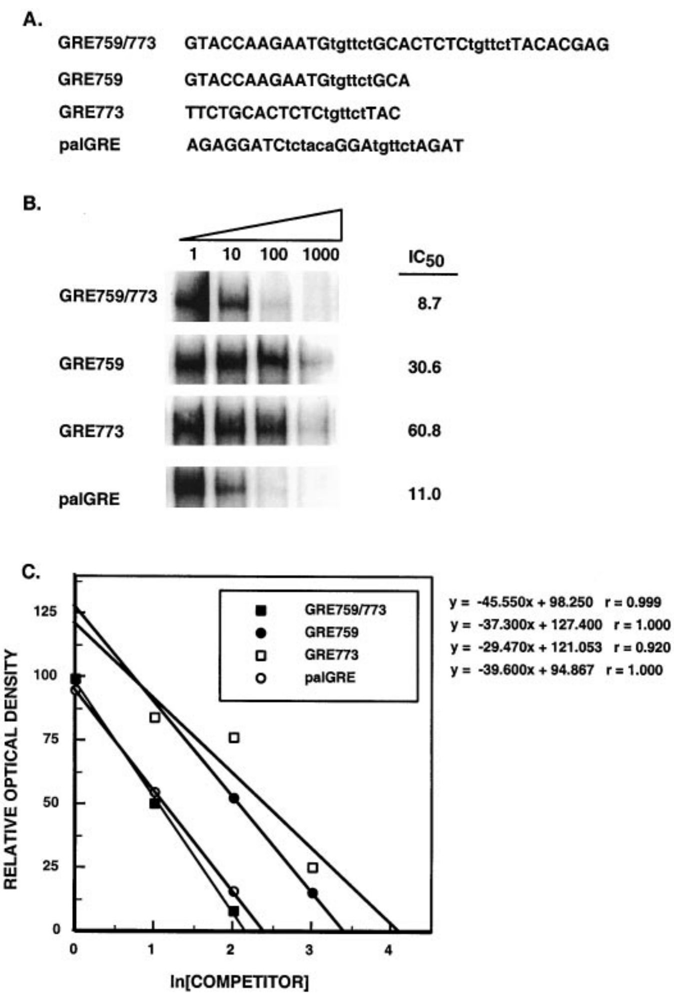

Fig. 3. Affinity of glucocorticoid receptor for −773- and −759-bp GREs. Gel mobility shift competition assays were performed as described under *Materials and Methods* using a truncated glucocorticoid receptor protein and the ^{32}P-labeled 40-bp wild-type oligonucleotide probe GRE773/759. The protein-DNA complex was competed with 1–1000 ng of homologous, unlabeled competitor DNA (GRE773/759) or 21-bp oligonucleotides encoding the −773-bp GRE (GRE773), the −759-bp GRE (GRE759), or the palindromic GRE (palGRE) (Scheidereit et al., 1983). Complex formation was quantified by computerized densitometry of the autoradiographic signals using NIH Image 1.52 (http://rsb.info.nih.gov/nih-image/). A, competitor DNA sequences. B and C, gel mobility shift assays and relative IC_{50} values.

−759-bp GRE was mutated, *PNMT* promoter-driven luciferase expression was not significantly different from untreated, control values.

The effects of RU38486 on dexamethasone-stimulated *PNMT* promoter activation was also determined for all of the mutant constructs. As above, cells transfected with the constructs were pretreated with the antagonist (0–10 μM) for 1 h followed by 6 h of 1 μM dexamethasone treatment (Fig. 4B). The construct harboring a mutation in the −533-bp GRE (pGL3RP790mut533) showed *PNMT* promoter-driven luciferase expression similar to that of the wild-type construct (pGL3RP790). At concentrations of 0.1 and 1 μM, luciferase activity was, in fact, slightly higher than the wild-type control cells. The construct harboring a mutation in the −773-bp GRE (pGL3RP790mut773) showed a linear decrease in dexamethasone-stimulated, *PNMT* promoter-driven luciferase expression as would be expected based on its responses to dexamethasone described above. Similarly, the mutant construct with altered −759-bp GRE showed no significant dexamethasone and RU38486 responsiveness. Finally, when the −759- and −773-bp or the −533-, −759-, and −773-bp GREs were mutated, neither dexamethasone nor the combination of dexamethasone and RU38486 elicited any significant changes in *PNMT* promoter expression whatsoever.

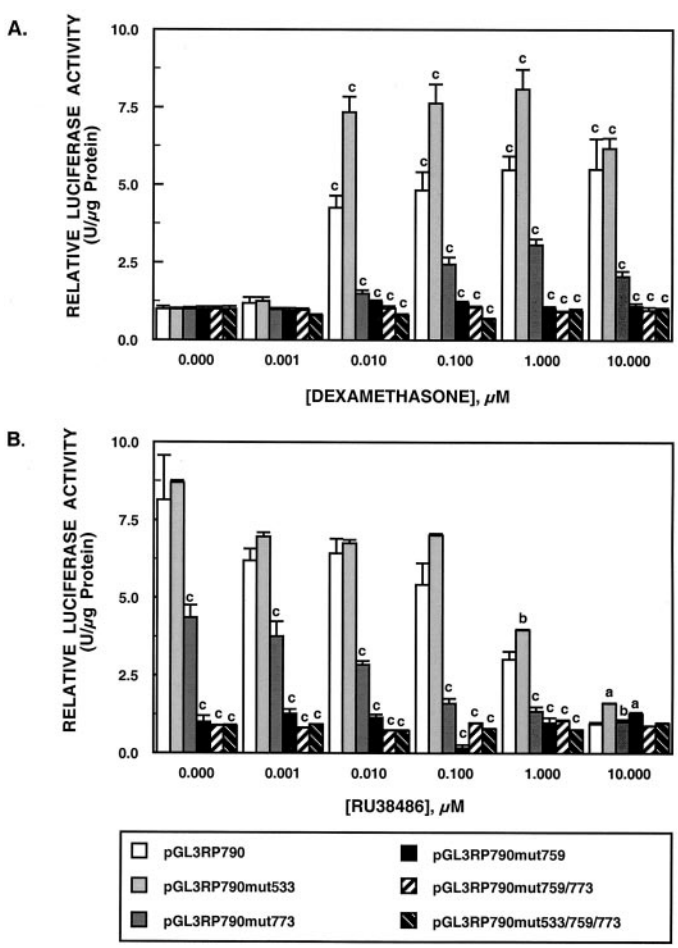

Fig. 4. Site-directed mutagenesis of GREs and *PNMT* promoter responsiveness to glucocorticoids. *PNMT* promoter-luciferase reporter gene constructs were generated with mutations in the −773, −759, −773 and −759, −533 and all three GREs (pGL3RP790mut773, pGL3RP790mut759, pGL3RP790mut773/759, pGL3RP790mut533, and pGL3RP790mut773/759/533, respectively). The wild-type construct pGL3RP790 or mutant constructs were transfected into RS1 cells and treated with dexamethasone (0–10 μM). Alternatively, transfected cells were pretreated with RU38486 (0–10 μM) for 1 h, followed by treatment with 1 μM dexamethasone for 6 h. Cell lysates were prepared and luciferase activity measured as described under *Materials and Methods*. A, glucocorticoid responsiveness of wild-type construct pGL3RP790 and mutant constructs pGL3RP790mut773, pGL3RP790mut759, and pGL3Rpmut773/759 in RS1 cells. c, significantly different from wild-type control, $p \leq 0.001$. B, effects of RU38486 on the glucocorticoid responsiveness of wild-type pGL3RP790 and mutant constructs, pGL3RP790mut533, pGL3RP790mut773/759, and pGL3RP790mut773/759/533 in RS1 cells. a, significantly different from dexamethasone-treated wild-type control, $p \leq 0.05$; b, significantly different from dexamethasone-treated wild-type control, $p \leq 0.01$; c, significantly different from dexamethasone-treated wild-type control, $p \leq 0.001$.

Cooperative Interactions between the GR and Other *PNMT* Transcriptional Activators. Many transcriptional regulators can independently and cooperatively stimulate gene expression. Although the −533-bp GRE in the rat *PNMT* promoter is apparently a weak independent glucocorticoid activation site, bound GR seemed to cooperatively activate the *PNMT* promoter through interactions with Egr-1 and/or AP-2 bound to their respective consensus elements at −165, −674, and −587 bp (Wong et al., 1998). As described earlier (Fig. 1), two deletion constructs, pGL3RP557 and pGL3RP665, which harbor the −533-bp GRE but not the −759 or −773-bp GREs, showed no significant glucocorticoid activation. However, dexamethasone did induce luciferase reporter gene expression 2.0-fold from a slightly longer construct, pGL3RP745, a construct containing functional AP-2 binding sites (Ebert et al., 1998).

To further investigate the role of the −533-bp GRE and the possible role of the −759- and −773-bp GREs in cooperative stimulation of the *PNMT* promoter, transient transfection assays were executed with the wild-type and mutant *PNMT* promoter-luciferase reporter gene constructs in the absence or presence of 1 μM dexamethasone and Egr-1 and/or AP-2 expression constructs. First, cooperativity between the GR and Egr-1 was investigated (Fig. 5A). Although dexamethasone stimulated only a 3.0-fold rise in *PNMT* promoter-driven luciferase activity in this case, mutation of the −533-bp GRE increased dexamethasone stimulation of the promoter 3.8-fold beyond that observed with the wild-type construct. In contrast, mutation of the −759-bp GRE, −773-bp GRE, both the −759- and −773-bp GREs, or the −533-, −759-, and −773-bp GREs almost completely abolished the glucocorticoid responsiveness of the *PNMT* promoter. Egr-1 alone stimulated a 2.6-fold increase in *PNMT* promoter-driven luciferase expression in the wild-type *PNMT* promoter-luciferase construct and in combination with dexamethasone, increased promoter activity slightly more than the additive inductions by the GR and Egr-1, consistent with GR and Egr-1 acting cooperatively to induce *PNMT* promoter-driven transcriptional activity. However, if the −533-bp GRE was mutated, Egr-1 or Egr-1 combined with dexamethasone stimulated the *PNMT* promoter 2- to 4-fold more than when the site was intact (4.5 and 9.8-fold, respectively). When the −759-bp GRE, the −773-bp GRE, the −759- and −773-bp GREs, or the −533-, −759-, and −773-bp GREs were mutated, stimulation of the *PNMT* promoter by Egr-1 and Egr-1 in combination with dexamethasone declined precipitously. However, Egr-1–mediated, *PNMT* promoter induction was still significantly greater than their respective controls in the case of the −759- and −533-/−759-/−773-bp mutant constructs (5.0- and 2.0-fold, respectively). It was previously demonstrated that AP-2 induction of the *PNMT* promoter required coactivation of the GR (Ebert et al., 1998). In the present study, AP-2 seems to independently stimulate *PNMT* promoter-driven luciferase activity in the case of the wild-type construct (3.7-fold), but synergistic activation by AP-2 and dexamethasone still occurred (20.7-fold). When the −533- or −773-bp GREs were mutated, similar responses were observed, although induction was markedly attenuated. However, if the −759-bp, −759- and −773-bp, or −533-, −759-, and −773-bp GREs were mutated, both the independent AP-2 and GR and AP-2 synergistic stimulation of the *PNMT* promoter was eliminated.

As reported earlier (Wong et al., 1998), greatest synergistic activation of the *PNMT* promoter was elicited in the presence of all three transcriptional activators, the GR, AP-2, and Egr-1 (~28.0-fold). Moreover, mutation of the −533-bp GRE increased *PNMT* promoter activity 4.0-fold beyond that observed with the wild-type construct (91.5-fold). In contrast,

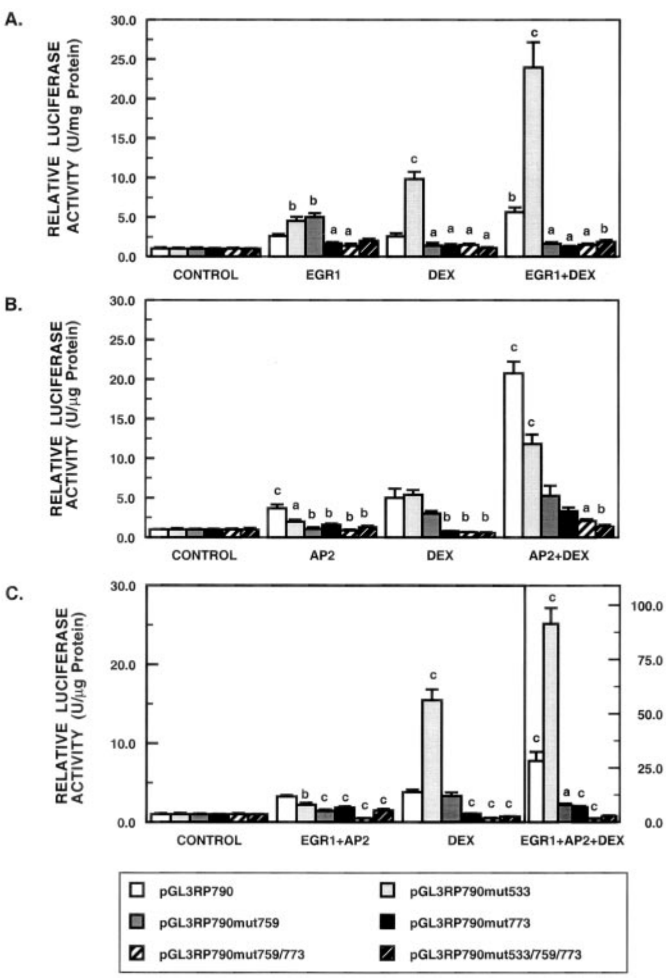

Fig. 5. Cooperative stimulation of the *PNMT* promoter by the glucocorticoid receptor and Egr-1 and/or AP2. RS1 cells were transfected with the wild-type construct pGL3RP790 or cotransfected with the wild-type construct and expression constructs for Egr-1, pCMVEgr-1, and AP-2, pSPRSV-AP-2, as described under *Materials and Methods*. The cells were sustained in the absence or presence of 1 μM dexamethasone for 6 h, after which luciferase activity was measured. a, significantly different from dexamethasone-treated wild-type control, $p \leq 0.05$; b, significantly different from dexamethasone-treated wild-type control, $p \leq 0.01$; c, significantly different from dexamethasone-treated wild-type control, $p \leq 0.001$.

mutation of either the −759 or −773-bp GREs markedly attenuated synergistic stimulation of the promoter (25% of wild-type) although a significant 6.0- to 7.9-fold activation remained. Finally, when both the −759- and −773-bp GREs as well as all three GREs were mutated, cooperative interactions were completely lost.

Discussion

Glucocorticoids are important regulators of *PNMT* gene expression, influencing adrenergic differentiation (Bohn et al., 1981; Teitelman et al., 1982; Bohn, 1983; Michelson and Anderson, 1992; Wong et al., 1992a; Schmid et al., 1995; Ebert et al., 1997) and the induction of PNMT in response to acute and chronic stress (Sabban et al., 1995; Sabban et al., 1998; Serova et al., 1998). Consistent with this role, a functional GRE was identified at −513 bp in the upstream sequences of the rat *PNMT* gene when it was first cloned (Ross et al., 1990). At best, however, only weak glucocorticoid responses are elicited through this GRE based on changes in *PNMT* promoter-reporter gene expression and PNMT mRNA expression in vitro (Wong et al., 1996; Ebert et al., 1997). Two additional glucocorticoid response elements have now been identified distal to the original GRE. These GREs, located at −759 and −773 bp upstream of the site of transcription initiation, overlap by 1 bp according to the consensus sequence defined by Scheidereit et al. (1983) and together contribute to a maximum 12.0-fold induction of the *PNMT* promoter in response to glucocorticoids.

The position of these new GREs has been designated by their 3′ termini based on the highly conserved 3′-hexanucleotide sequence TGTTCT in the 15-bp palindromic GRE, 5′AGAACANNNTCTTCT3′ identified above. These designations also correct for misalignment of the proximal *PNMT* promoter sequences arising from ~30 bp of G and C residues in GC rich regions where sequencing is difficult. Realignment of the promoter repositions the −513-bp GRE at −533 bp as well.

The relative contribution of each new GRE to the corticosteroid responsiveness of the *PNMT* promoter was investigated by site-directed mutagenesis, gel mobility shift competition assays, and examination of the effects of dexamethasone and the antiglucocorticoid RU38486. Both GREs must be intact to elicit full glucocorticoid sensitivity of the *PNMT* promoter, and the response seems greater than additive, indicating cooperativity between activated GRs bound to the GREs or bound GRs and other transcription factors. In addition, the −759-bp GRE has ~2-fold higher affinity for the GR, which was reflected by a greater attenuation of glucocorticoid-stimulated *PNMT* promoter activity when this GRE was mutated. Thus, this GRE is probably functionally more important. Finally, RU38486 effectively blocked glucocorticoid activation from either response element in a dose-dependent manner, consistent with the relative affinities of the GR for each GRE. The latter results also confirm that glucocorticoid activation of the *PNMT* gene promoter occurs through type II GRs because RU38486 is a classic GR antagonist.

In keeping with previous reports, the −533-bp GRE did not seem to markedly affect *PNMT* promoter activation through glucocorticoid exposure alone. Two *PNMT* promoter constructs (pGL3RP665 and pGL3RP557) containing this GRE, but not the −773- and −759-bp upstream GREs, failed to show an increase in *PNMT* promoter activity when treated with corticosteroids. However, a 2-fold induction of the luciferase reporter gene was observed with a slightly longer construct pGL3RP745. The latter also harbors two functional AP-2 binding elements at −674 and −587 bp (Ebert et al., 1998). In contrast to our earlier results, the present findings suggest that AP-2 alone can elicit a significant, but limited induction of the *PNMT* promoter. Most notable again is the marked cooperative induction of the promoter by GR bound to the −533-bp GRE and AP-2. AP-2 and dexamethasone synergism is demonstrated by the nearly 2.0-fold reduction (relative to wild-type) in *PNMT* promoter-driven luciferase activity when the −533-bp GRE is mutated. Curiously, when the −759- and −773-bp GREs are mutated and the −533-bp GRE left intact, synergism disappears. It may be that binding of GRs to these GREs alters *PNMT* promoter conformation in a fashion that favors the interaction between GRs bound to the −533-bp GRE and AP-2. Alternatively, GR bound to the −533, −759, and −773-bp GREs may interact with one another and/or AP-2. Whereas the −533-bp GRE participates in AP-2 and GR cooperative activation of the *PNMT* promoter, synergism between bound GR and the immediate early gene transcription factor Egr-1 does not occur, because there is no significant difference in their combined effects on *PNMT* promoter activity when this site is mutated. The latter results further suggested that previously reported cooperative induction by Egr-1 and dexamethasone might involve the newly identified tandem GREs. When the −533-bp GRE is mutated, leaving only these two GREs intact, Egr-1 and dexamethasone elicit a 5.0-fold higher stimulation of the *PNMT* promoter than observed with the wild-type construct where all three GRE sites are intact. Moreover, when these GREs are mutated independently, together or along with the −533-bp GRE, activation by Egr-1 and dexamethasone is effectively eliminated. Thus, the −759- and −773-bp GREs do seem to be the essential GREs for cooperative activation of the *PNMT* promoter by Egr-1 and the GR. In addition, the −759- and −773-bp GREs also participate in synergistic activation of the *PNMT* promoter with AP-2, although their contribution to GR and AP-2 activation of the promoter is less than that orchestrated through the −533-bp GRE. When the −533-bp GRE is mutated, AP-2 still elicited a residual ~12.0-fold stimulation of *PNMT* promoter-driven luciferase expression. Finally, these independent synergistic effects are reflected in the combined effects of AP-2, Egr-1, and the GR on the wild-type and mutant constructs.

Clearly, these cooperative interactions are complex and very dependent on promoter length, acetylated histones, and DNA folding and interaction as well as the availability of coactivator complexes containing factors such as SCRC1, GRIP1, CBP, P300, and PCAF (Wang et al., 1999). Current studies are now investigating the effects of selective silencing of the GREs, Egr-1 and AP-2 binding elements in *PNMT* promoter constructs and endogenous *PNMT* gene using viral vector driven antisense strategies.

In summary, the present study provides the first definitive identification and characterization of the functional GREs in the proximal sequences in the rat *PNMT* promoter. Through the newly identified −759- and −773-bp GREs, marked and selective glucocorticoid activation occurs, indicating that they are the primary targets through which glucocorticoid sensitivity is conferred. In addition, both the −533-bp GRE and these newly identified GREs seem to participate in cooperative or facilitatory activation of the *PNMT* promoter, the former with AP-2 and the latter with AP-2, Egr-1, and/or both.

References

Baetge EE, Behringer RR, Messing A, Brinster RL, and Palmiter RD (1988) Transgenic mice express the human phenylethanolamine *N*-methyltransferase gene in adrenal medulla and retina. *Proc Natl Acad Sci USA* **85:**3648–3652.

Baetge EE, Suh YH, and Joh TH (1986) Complete nucleotide and deduced amino acid sequence of bovine phenylethanolamine *N*-methyltransferase: Partial amino acid homology with rat tyrosine hydroxylase. *Proc Natl Acad Sci USA* **83:**5454–5458.

Batter DK, D'Mello SR, Turzai LM, Hughes HB III, Gioio AE and Kaplan BB (1988) The complete nucleotide sequence and structure of the gene encoding bovine phenylethanolamine *N*-methyltransferase. *J Neurosci Res* **19:**367–376.

Berenbeim DM, Wong DL, Masover SJ, and Ciaranello RD (1979) Regulation of synthesis and degradation of rat adrenal phenylethanolamine *N*-methyltransferase. III. Stabilization of PNMT against thermal and tryptic degradation by S-adenosylmethionine. *Mol Pharmacol* **16:**482–490.

Bohn MC (1983) Role of glucocorticoids in expression and development of phenylethanolamine *N*-methyltransferase (PNMT) in cells derived from the neural crest: a review. *Psychoneuroendocrinology* **8:**381–339.

Bohn MC, Goldstein M, and Black IB (1981) Role of glucocorticoids in the adrenergic phenotype in rat embryonic adrenal gland. *Dev Biol* **82:**1–10.

Boussif O, Lezoualc F, Zanta MA, Mergny MD, Scherman D, Demeneix B, and Behr J-P (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc Natl Acad Sci USA* **92:**7297–7301.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.

Ebert SN, Balt SL, Hunter JPB, Gashler A, Sukhatme V, and Wong DL (1994) Egr-1 activation of rat adrenal phenylethanolamine *N*-methyltransferase gene. *J Biol Chem* **269:**20885–20898.

Ebert SN, Ficklin MB, Her S, Siddall BJ, Bell RA, Morita K, Ganguly K, and Wong DL (1998) Glucocorticoid-dependent action of neural crest factor AP-2: Stimulation of phenylethanolamine *N*-methyltransferase gene expression. *J Neurochem* **70:** 2286–2295.

Ebert SN, Lindley SE, Bengoechea TG, Bain D, and Wong DL (1997) Adrenergic differentiation potential in PC12 cells: influence of sodium butyrate and dexamethasone. *Mol Brain Res* **47:**24–30.

Evinger MJ (1998) Determinants of phenylethanolamine-*N*-methyltransferase expression, in *Catecholamines: Bridging Basic Science with Clinical Medicine* (Goldstein DS, Eisenhofer G, and McCarty R eds) Vol. 42, pp. 73–76, Academic Press, San Diego.

Evinger MJ, Towle AC, Park DH, Lee P, and Joh TH (1992) Glucocorticoids stimulate transcription of the rat phenylethanolamine *N*-methyltransferase (PNMT) gene *in vivo* and *in vitro*. *Cell Mol Neurobiol* **12:**193–215.

Finotto S, Krieglstein K, Schober A, Deimling F, Lindner K, Bruhl B, Beier K, Metz J, Garcia-Arraras JE, Roig-Lopez JL, et al. (1999) Analysis of mice carrying targeted mutations of the glucocorticoid receptor gene argues against an essential role of glucocorticoid signalling for generating adrenal chromaffin cells. *Development* **126:**2935–2944.

Freedman LP, Luisi BF, Korszun ZR, Basavappa R, Sigler PB, and Yamamoto KR (1988) The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature (Lond)* **334:**543–546.

Gupta MP, Gupta M, Zak R, and Sukhatme VP (1991) Egr-1, a serum-inducible zinc finger protein, regulates transcription of the rat cardiac alpha-myosin heavy chain gene. *J Biol Chem* **266:**12813–12816.

Her S, Bell RA, Bloom AK, Siddall BJ, and Wong DL (1999) Phenylethanolamine *N*-methyltransferase gene expression: Sp1 and MAZ potential for tissue specific expression. *J Biol Chem* **274:**8698–8707.

Kaneda N, Ichinose H, Kobayashi K, Oka K, Kishi F, Nakazawa A, Kurosawa Y, Fujita K, and Nagatsu T (1988) Molecular cloning of cDNA and chromosomal assignment of the gene for human phenylethanolamine *N*-methyltransferase, the enzyme for epinephrine biosynthesis. *J Biol Chem* **263:**7672–7677.

Michelson AM and Anderson DJ (1992) Changes in competence determine the timing of two sequential glucocorticoid effects on sympathoadrenal progenitors. *Neuron* **8:**589–604.

Mitchell PJ, Wang C, and Tjian R (1987) Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. *Cell* **50:**847–861.

Morita K, Bell RA, Siddall BJ, and Wong DL (1996) Neural stimulation of Egr-1 messenger RNA expression in rat adrenal gland: possible relation to phenylethanolamine *N*-methyltransferase gene regulation. *J Pharmacol Exp Ther* **279:**379–385.

Morita S, Kobayashi K, Hidaka H, and Nagatsu T (1992) Organization and complete nucleotide sequence of the gene encoding mouse phenylethanolamine *N*-methyltransferase. *Mol Brain Res* **13:**313–319.

Ross ME, Evinger MJ, Hyman SE, Carroll JM, Mucke L, Comb M, Reis DJ, Joh TH, and Goodman HM (1990) Identification of a functional glucocorticoid response element in the phenylethanolamine *N*-methyltransferase promoter using fusion genes introduced into chromaffin cells in primary culture. *J Neurosci* **10:**520–530.

Sabban EL, Hiremagalur B, Nankova B, and Kvetnansky R (1995) Molecular biology of stress-elicited induction of catecholamine biosynthetic enzymes. *Ann NY Acad Sci* **771:**327–338.

Sabban EL, Nankova BB, Serova LI, Hiremagalur B, Rusnak M, Saez E, Spiegelman

B, and Kvetnansky R (1998) Regulation of gene expression of catecholamine biosynthetic enzymes by stress, in *Catecholalmines: Bridging Basic Science with Clinical Medicine* (Goldstein DS, Eisenhofer G and McCarty R eds), pp. 564–567, Academic Press, San Diego.

Scheidereit C and Beato M (1984) Contacts between hormone receptor and DNA double helix within a glucocorticoid regulatory element of mouse mammary tumor virus. *Proc Natl Acad Sci USA* **81:**3029–3033.

Scheidereit C, Geisse S, Westphal HM, and Beato M (1983) The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of mouse mammary tumor virus. *Nature (Lond)* **304:**749–752.

Schmid W, Cole TJ, Blendy JA, and Schutz G (1995) Molecular genetic analysis of glucocorticoid signalling in development. *J Steroid Biochem Mol Biol* **53:**33–35.

Serova L, Sabban EL, Zangen A, Overstreet DH, and Yadid G (1998) Altered gene expression for catecholamine biosynthetic enzymes and stress response in rat genetic model of depression. *Mol Brain Res* **63:**133–138.

Sukhatme VP, Cao X, Chang LC, Tsai-Morris CH, Stamenkovich D, Ferreira PCP, Cohen DR, Edwards SA, Shows TB, Curran T, et al. (1988) A zinc finger-encoding gene coregulated with *c-fos* during growth and differentiation and after cellular depolarization. *Cell* **53:**37–43.

Tai TC, Morita K, and Wong DL (2001) Role of Egr-1 in cAMP-dependent protein kinase regulation of the phenylethanolamine *N*-methyltransferase gene. *J Neurochem* **76:**1851–1859.

Teitelman G, Joh TH, Park D, Brodsky M, New M, and Reis DJ (1982) Expression of the adrenergic phenotype in cultured fetal adrenal medullary cells: role of intrinsic and extrinsic factors. *Dev Biol* **89:**450–459.

Wang J-C, Stromstedt P-E, Sugiyama T, and Granner DK (1999) The phosphoenolpyruvate carboxykinase gene glucocorticoid response unit: identification of the functional domains of accessory factors HNF3β and HNF4 and the necessity of proper alignment of their cognate binding sites. *Mol Endocrinol* **13:**604–618.

Williams T and Tjian R (1991) Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. *Genes Dev* **5:**670–682.

Wong DL, Ebert SN, and Morita K (1996) Glucocorticoid control of phenylethanolamine *N*-methyltransferase gene expression: implications for stress and disorders of the stress axis, in *Stress: Molecular Genetic and Neurobiological Advances*. Proceedings of the Sixth International Symposium on Catecholamines and Other Neurotransmitters in Stress; 1995 Jun 19–24; Smolenice Castle, Slovakia (McCarty R, Aguilera G, Sabban E and Kvetnansky R eds) Vol. 2, pp. 677–693, Gordon and Breach Science Publishers, New York.

Wong DL, Hayashi RJ, and Ciaranello RD (1985) Regulation of biogenic amine methyltransferases by glucocorticoids via S-adenosylmethionine and its metabolizing enzymes, methionine adenosyltransferase and S-adenosylhomocysteine hydrolase. *Brain Res* **330:**209–216.

Wong DL, Lesage A, Siddall B, and Funder JW (1992a) Glucocorticoid regulation of phenylethanolamine *N*-methyltransferase in vivo. *FASEB J* **6:**3310–3315.

Wong DL, Lesage A, White S, and Siddall B (1992b) Adrenergic expression in the rat adrenal gland: multiple developmental regulatory mechanisms. *Dev Brain Res* **67:**229–236.

Wong DL, Siddall BJ, Ebert SN, Bell RA, and Her S (1998) Phenylethanolamine *N*-methyltransferase gene expression: synergistic activation by Egr-1, AP-2 and the glucocorticoid receptor. *Mol Brain Res* **61:**154–161.

Wong DL, Siddall B, and Wang W (1995) Hormonal control of rat adrenal phenylethanolamine *N*-methyltransferase: enzyme activity, the final critical pathway. *Neuropsychopharmacology* **13:**223–234.

Address correspondence to: Dona Lee Wong, Ph.D., Department of Psychiatry, Harvard Medical School, Laboratory of Molecular and Developmental Neurobiology, McLean Hospital, 115 Mill Street, MRC #116, Belmont, MA 02478. E-mail: dona_wong@hms.harvard.edu