Glucocorticoid Responsiveness of the Rat Phenylethanolamine N-Methyltransferase Gene

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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 IC₅₀ 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 (Berenheim 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-
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 the PNMT promoter sequences into the plasmid pGL3Basic (Pharmacia-Upsjohn, 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 Xhol-HindIII restriction fragments from pRP830LUC 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′-GTACCAAGAATGTGTTCTGCA3′ (−790 → −751, GRE773/759); 5′-GTACCAAGAATGTTGTGCTCA3′ (−790 → −770, GRE773); 5′-TTCGTGACACTCTGTCCTACAG3′ (−776 → −756, GRE759); and 5′-AGAGGATCTGACAGAGATGTCTTACAG3′ (palidromic (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% CO2/95% air. All sera were charcoal-treated to remove endogenous glucocorticoids.

For transfection, cells were plated into 24-well culture dishes (2 × 105 cells/well) and held at 37°C and 5% CO2/95% air overnight. Transfection was then performed using Superfect (QIAGEN, Inc., Valencia, CA) or polyethylenimine (Boussif et al., 1995) including 0.5 μg of wild-type or mutant PNMT promoter-luciferase reporter gene construct, 0 to 1.5 μg of expression or null construct, pCMV-Egr-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, Romainville, 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 800g 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 mutagenesis was performed using the following 40-bp mutagenic primers, spanning upstream sequences from −790 to −751 bp in the PNMT promoter (Her et al., 1999): 5′-GTACCAAGAATGTCTGCACTTCTGTTCTTACAGCAGAGTCAG (mut759); 5′-GTACCAAGAATGTCTGCACTTCTGTTCTTACAGCAGAGTCAG (mut759); and 5′-GTACCAAGAATGTCTGCACTTCTGTTCTTACAGCAGAGTCAG (mut773). Site-Directed 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, labeled with [γ-32P]ATP and T4 polynucleotide kinase (3 nM, specific activity = 2.5 × 106 dpm/μg), and a truncated GR protein (Dr. Keith Yamamoto, University of California, San Francisco) (Freeman 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 concent-
tion 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. IC₅₀ 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 these 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.

Matching of the DNA sequences in the glucocorticoid sensitive region to the consensus glucocorticoid response element, 5’ TAGAACANNNTCTTCT3’ (Scheidegger 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, TGTTCCT, 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 ³₂P-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 palindrome GRE sequences (Fig. 3A). All oligonucleotides interacted with the GR peptide as demonstrated by their displacement of the radiolabeled DNA (Fig. 3B). The abun-

![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.](Image)
dance of GR-GRE binding complex was quantified by scanning densitometry and relative IC$\text{_{50}}$ 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$\text{_{50}}$ values: 11.0 and 8.7, respectively). The affinities of the $\sim$759- and $\sim$773-bp GREs for the GR were $\sim$3.5 to 7.0-fold lower based on relative IC$\text{_{50}}$ values; the $\sim$759-bp GRE had $\sim$2-fold higher affinity (relative IC$\text{_{50}}$, 30.6) for the GR than the $\sim$773-bp GRE (relative IC$\text{_{50}}$, 60.8). The slopes of the regression curves for each competition assay did not significantly change, confirming that the differences in x-intercepts (IC$\text{_{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 $\sim$759- and $\sim$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 pGL3RP790mut773/759; and the triple mutant construct pGL3RP790mut533/773/759. 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 $\mu$M) (Fig. 4A). When the $\sim$533-bp GRE was mutated, rather than a decrease, a $\sim$1.5-fold increase in dexamethasone-stimulated PNMT promoter activation occurred at dexamethasone concentrations between 0.01 and 1 $\mu$M. By contrast, mutation of either the $\sim$759 or $\sim$773-bp GRE markedly attenuated dexamethasone-stimulated PNMT promoter activity. However, when the $\sim$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 $\mu$M dexamethasone). As observed with all GREs intact, 10 $\mu$M dexamethasone stimulated the PNMT promoter less than 1 $\mu$M dexamethasone.

![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 $\mu$M) or pretreated with RU38486 (0–10 $\mu$M) for 1 h, followed by 1 $\mu$M 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, $p \leq 0.001$.](https://molpharm.aspetjournals.org/issue/)

![Fig. 3. Affinity of glucocorticoid receptor for $\sim$773- and $\sim$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 $\sim$773-bp GRE (GRE773), the $\sim$759-bp GRE (GRE759), or the palindromic GRE (palGRE) (Scheidereit et al., 1983). Complex formation was quantified by computerized densitometry of the autoradiographic signals using NIH Image 1.52 (http://rsb.info.nih.gov/nih-image/). A, competitor DNA sequences. B and C, gel mobility shift assays and relative IC$\text{_{50}}$ values.](https://molpharm.aspetjournals.org/issue/)
−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 GREs were mutated, neither dexamethasone nor the combination of dexamethasone and RU38486 elicited any significant changes in PNMT promoter expression whatsoever.

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 declined precipitously. However, 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 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. 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, 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′AGAACANNTCTTCTT3′ 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 GRS 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 GRS. When the −533-bp GRE is mutated, leaving only these two GRS 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 GRSs 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 SCR1, GRIP1, CBP, P300, and PCAF (Wang et al., 1999). Current studies are now investigating the effects of selective silencing of the GRSs, 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 GRSs in the proximal sequences in the rat PNMT promoter. Through the newly identified −759- and −773-bp GRSs, 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 GRSs 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


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