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Phenylethanolamine *N*-Methyltransferase Gene Expression

SP1 AND MAZ POTENTIAL FOR TISSUE-SPECIFIC EXPRESSION*

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Phenylethanolamine *N*-methyltransferase (PNMT) promoter-luciferase reporter gene constructs (pGL3RP863, pGL3RP444, and pGL3RP392) transfected into COS1, RS1, PC12, NIH/3T3, or Neuro2A cells showed the highest basal luciferase activity in the Neuro2A cells. DNase I footprinting with Neuro2A cell nuclear extract identified protected PNMT promoter regions spanning the –168/–165 and –48/–45 base pair Sp1/Egr-1 binding sites. Gel mobility shift assays and transient transfection assays using site-directed mutant PNMT promoter-luciferase reporter gene constructs indicated that the elevated basal luciferase activity in the Neuro2A cells was mediated by Sp-1. Furthermore, activation of the PNMT promoter by Sp1 depends on both its binding affinity for its cognate target sequences and its intracellular concentrations. When Sp1 levels were increased through an expression plasmid, luciferase reporter gene expression rose well beyond basal wild-type levels, even with either Sp1 binding element mutated. Finally, another transcription factor expressed in the Neuro2A cells competes with Sp1 by interacting with DNA sequences 3′ to the –48 base pair Sp1 site to prevent Sp1 binding and induction of the PNMT promoter. The DNA consensus sequence, Southwestern analysis, and gel mobility shift assays with antibodies identify MAZ as the competitive factor. These findings suggest that Sp1 may potentially contribute to the tissue-specific expression of the PNMT gene, with the competition between Sp1 and MAZ conferring additional tissue-specific control.

The neurotransmitter/neurohormone epinephrine is a physiologically significant neuroregulator that assumes an important role in the stress response and is a major component in cardiac disease, immune dysfunction, and neuropsychiatric illness. Consequently, phenylethanolamine *N*-methyltransferase (PNMT)[1] (EC 2.1.1.28), its biosynthetic enzyme, and the mechanisms by which PNMT is genetically regulated have been of interest in attempts to better understand the etiologies of these disorders.

Examination of the PNMT gene from a variety of species (human (1, 2), cow (3, 4), mouse (5), and rat (6)) suggests that there may be common transcriptional regulatory proteins that control PNMT gene expression based on the presence of canonical DNA binding sequences for several known factors. We have previously demonstrated for the rat PNMT gene that one stimulatory transcriptional regulator is the factor Sp1 for which two binding elements exist within the proximal –863 bp of 5′ upstream PNMT promoter/regulatory sequence at –48 and –168 bp relative to the site of transcription initiation, +1 (7, 8). Binding elements for the immediate early gene transcription factor Egr-1 overlap 6 bp of the 3′ end of each of these Sp1 sites. Although both sites can function as Sp1 or Egr-1 activation sites, it appears that *in vivo*, the distal overlapping Sp1/Egr-1 binding element (–168 bp) may preferentially serve as a site for Egr-1 induction, whereas the proximal binding element (–48 bp) likely functions as an Sp1 site. This motif of overlapping or closely adjacent Sp1 and Egr-1 binding elements seems to be common among genes that possess these regulatory sites (9–12), and Sp1 or Egr-1 either activates or inhibits gene expression through interaction with their cognate DNA sequences.

The present studies further examine the role of Sp-1 in the regulation of PNMT gene expression. We demonstrate that the high basal activity of the PNMT promoter in the neuroblastoma-derived Neuro2A cell line is due to Sp1 acting through its –48 and –168 bp binding elements. In the case of the –48 bp Sp1 binding element, another transcription factor can bind to a recognition site 3′ to this Sp1 site, thereby preventing Sp1 binding and activation of the PNMT promoter through this proximal element. Consensus sequence match, Southwestern analysis, and gel mobility shifts assays with antibodies indicate that this factor is the transcription protein MAZ (13). Together, these findings suggest that Sp1 may be an important regulator of tissue-specific PNMT gene expression and that the exclusionary competition between Sp1 and MAZ may provide additional tissue-specific control.

EXPERIMENTAL PROCEDURES

Oligonucleotides and Plasmid Constructs—The 21-mer WTA (5′-CCTCCCCGCCCCCGCGCGTCC-3′) (rat PNMT promoter sequences from –180 to –160 bp), WTB (5′-GTCTGGGCGGGGGGGAGGGGA-3′) (PNMT promoter sequences from –59 to –39 bp), and mut45, 5′-GTCTGGGCGGGGaaaAGGGGA-3′ (point mutation in –45 bp Egr-1 consensus sequence) oligonucleotides have been previously described (7). Two additional 21-bp oligonucleotides were synthesized to use as mutant probes for the distal and proximal Sp1 sites at –168 and –48 bp, respectively: 1) mut168 (5′-CCTCaaCGCCCCCGCGCGTCC-3′; mutated –168 bp Sp1 binding element), and 2) mut48 (5′-GTCTaaGCGGGGGGGAGGGGA-3′; mutated –48 bp Sp1 binding element). The duplex WTA, WTB, mut45, mut168, and mut48 oligonucleotides were subcloned into the *Sma*I site of the vector pBluescript II SK(–) (Stratagene, La Jolla, CA), yielding pBSIISK(–)RPWTA, pBSIISK(–)-RPWTB, pBSIISK(–)RPmut45, pBSIISK(–)RPmut168, and pBSIISK(–)-RP48 respectively, to generate double-stranded DNA for gel mobility shift assays.

For all plasmid constructs, standard recombinant DNA technologies

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[1] The abbreviations used are: PNMT, phenylethanolamine *N*-methyltransferase; bp, base pair(s); PCR, polymerase chain reaction.

were used. The wild-type PNMT promoter-luciferase reporter gene construct (pGL3RP863) was obtained by subcloning the *Xho*I-*Hin*dIII restriction fragment from pRP863LUC (7), containing the rat PNMT promoter sequences from −863 to +18 bp, into the corresponding restriction sites in the multiple cloning region of the vector pGL3Basic (Promega, Madison, WI). Two deletion constructs, pGL3RP444 and pGL3RP392, containing 444 and 392 bp of proximal PNMT promoter sequence, respectively, were then generated by digesting pGL3RP863 with *Kpn*I or *Nhe*I, followed by self-ligation. The unaltered plasmid, pGL3Basic, was used as a promoterless control, whereas the plasmid pRSV-LacZ, containing the β-galactosidase gene, was used as a normalization control to correct for variable transfection efficiency as described previously (7) and below.

The Sp-1 expression construct, pCGNSp-1, and the vector construct, pCGN, were kindly provided by Dr. Thomas Shenk (Howard Hughes Medical Institute, Princeton University).

Cell Culture and Transient Transfection Assays—The rat pheochromocytoma PC12 and RS1 cells have been previously described (7). The neuroblastoma-derived Neuro2A, embryonic mouse fibroblast NIH/3T3, and monkey kidney COS1 cells were obtained from the American Type Culture Collection (Rockville, MD). All cells were maintained in Dulbecco's modified Eagle's medium containing gentamycin sulfate (50 μg/ml, United States Biochemical Corp., Cleveland, OH) at 37 °C in an atmosphere of 7% CO_2-93% air (7). In the case of the RS1 cells, 200 units/ml of hygromycin B (Calbiochem, La Jolla, CA) was also included. In addition, 5% bovine calf serum and 5% equine serum (Hyclone, Logan, UT) were added to the medium for the PC12, RS1, and NIH/3T3 cells, whereas 10% fetal bovine serum (Gemini Bio-Products Inc., Calabasas, CA) was used for the Neuro2A and COS1 cells.

For transient cotransfection assays, cells were plated in 24-well culture dishes at a density of 2×10^5 cells/well and maintained at 37 °C in an atmosphere of 7% CO_2-93% air overnight, and transfection was performed using SuperFect according to the manufacturer's recommendations (Qiagen, Inc., Chatsworth, CA). Each transfection assay included 1.0 μg of wild-type or mutant PNMT promoter-luciferase reporter gene construct, 0–1.5 μg of expression construct, and 0.3 μg of pRSV-LacZ. Following transfection, the cells were exchanged to culture medium and maintained for 36 h at 37 °C and 7% CO_2-93% air. Cells were processed and assayed for luciferase and β-galactosidase as described below

Luciferase and β-Galactosidase Assay—Following two phosphate-buffered saline (PBS) washes, cells were lysed in 120 μl of lysis buffer (Promega), and luciferase activity was measured as previously using the luciferase assay system (7). Purified luciferase was used to define the linear range of the assay, and luciferase activity was then determined in 20 μl of appropriately diluted cell lysate. Protein was measured by the Bradford method (14), and luciferase activity was expressed as pg of product/μg of protein. β-Galactosidase activity was determined as described previously (7), and luciferase activity was then corrected for transfection efficiency and expressed relative to control values as follows (7). Luciferase activity expressed from the plasmid vector pGL3Basic was first subtracted from the luciferase activity generated from each of the PNMT promoter-luciferase reporter gene constructs. Then, luciferase activity was expressed relative to β-galactosidase expressed from the pRSV-LacZ control. To correct for variable transfection within the same cell line, β-galactosidase activity from the pRSV-LacZ construct used for cotransfection with the full-length pGL3RP863 was set as unity and β-galactosidase activity in the cotransfections with the other constructs expressed relative to it. The ratios so generated were then used to correct each luciferase:β-galactosidase ratio so that β-galactosidase activity in each transfection was effectively identical. In a similar fashion, luciferase activity was normalized across cell lines. In this case, β-galactosidase activity from the pRSV-LacZ construct for the cotransfection using the full-length pGL3RP863 in the RS1 cell line was used as unity. Luciferase activity was then expressed relative to luciferase activity for the pGL3RP863 construct in RS1 cells set to unity. At least six replicates were included for each sample group, and experiments were repeated at least twice.

Site-directed Mutagenesis—Two single-stranded 21-mer mutagenic primers corresponding to the mut168 and mut48 sequences identified above were used for site-directed mutagenesis to inactivate the distal and proximal Sp1 sites, respectively. First, mutagenic megaprimers were generated for each mutant sequence by PCR using the 21-bp mutagenic primers paired with GLprimer2, a primer binding to the extreme 22 bp of 5′ coding region of the firefly luciferase gene contained within the pGL3Basic vector (Promega), pGL3RP863 as template, and $Vent_R$® DNA polymerase (New England BioLabs, Beverly, MA) to amplify the DNA (30 cycles, 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min). Then, a second PCR (10 cycles) was performed to extend each megaprimer using pGL3RP392 as template, followed by 20 cycles of PCR using RVprimer3 and the GLprimer2 once again to produce 392-bp oligonucleotides with either the −48 or −168 bp Sp1 mutated. PCR products were separated by agarose gel electrophoresis, and agarose was removed using a QIAXII Gel Extraction Kit (Qiagen Inc.). The PCR products were then subcloned into the pGL3Basic plasmid to generate pGL3RP392mut168 and pGL3RP392mut48. The double mutant construct pGL3RP392 mut168/48 was generated identically by using the mut48 primer and pGL3RP392mut168 as a template. Following selection and screening, the mutant constructs were verified by DNA sequencing (16).

Nuclear Extracts—Cells were propagated as described above using 100-mm culture dishes and nuclear extracts prepared according to the procedure of Andrews and Faller (15). Briefly, after the cells reached a density of 5×10^5–1×10^7 per dish, they were collected into 1.5 ml of ice-cold PBS, and washed once with cold PBS. The cells were then lysed in 400 μl of 10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride on ice for 10 min. Following collection of the nuclei by centrifugation for 10 s at $17{,}000 \times g$ and 4 °C in a microcentrifuge, nuclear extract was prepared by hypotonic lysis. The nuclei were resuspended in 20–100 μl of 20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride and maintained on ice for 20 min, followed by centrifugation for 2 min at $17{,}000 \times g$ and 4 °C. Extracts were separated from pelleted debris and stored at −70 °C until use for footprinting or mobility shift assays.

DNase I Footprinting Analysis—To generate the DNA probes for footprinting analysis, two oligomers, 5′-AAAGGGCGCCCTCCACATC-TC-3′ (nucleotides –204 to –184) and 5′-TCCTGTTGAGGCCGCCTAT-CT-3′ (nucleotides +18 to –3) were uniquely labeled at their 5′ terminus using [γ-^{32}P]ATP (NEN Life Science Products) and T4 polynucleotide kinase (Life Technologies, Inc), and PCR was performed as described for site-directed mutagenesis. The resulting PNMT promoter fragments (222 bp) were isolated by agarose gel electrophoresis and Qiagen gel extraction as described above. Approximately 7.5×10^4 dpm of each DNA probe was incubated with 70 μg of nuclear extract from Neuro2A or COS1 cells in 30 μl of binding buffer consisting of 25 mM HEPES buffer, pH 7.9, 50 mM KCl, 0.05 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol and containing 2 μg of poly(dI-dC)-poly(dI-dC) for 30 min on ice. After 10 min at room temperature, DNase I footprinting was performed. The optimal RQ1 DNase I (Promega) concentration (0.3 units/reaction) was determined empirically in preliminary experiments. DNase I cleavage patterns were identified by polyacrylamide gel electrophoresis (7% sequencing gel), mapping protected sequences by comparison of the autoradiographic banding patterns to sequencing ladders run in adjacent lanes on the gel (16).

Gel Mobility Shift Assays—Gel mobility shift assays were performed using 1 ng of the probes described above, end-labeled with [γ-^{32}P]ATP using T_4 polynucleotide kinase (3 nM, specific activity = 2.5×10^8 dpm/μg) and 3 μg of nuclear extract in 20 μl of binding buffer (see DNase I footprinting) containing 0.5 μg poly (dI-dC)-poly(dI-dC). To identify the transcription factors constituting the protein-DNA binding complexes, anti-Egr-1 (C-19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Sp1 (PEP2, Santa Cruz Biotechnology) or anti-MAZ (Dr. Kenneth Marcu, State University of New York, Stonybrook, NY) antibody was included in the binding reactions. Purified human Sp-1 protein was used as a control (Promega). Protein-DNA complex formation and antibody supershifts were analyzed on 5% polyacrylamide gels by autoradiography.

Southwestern Analysis—Twenty μg of Neuro2A cell nuclear protein was separated by SDS-polyacrylamide gel electrophoresis (10% gel) as described previously (17), along with human Sp1 as a control. Proteins were transferred to nitrocellulose and renatured by incubation in binding buffer consisting of 20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM dithiothreitol, 10% glycerol, 0.2% Nonidet P-40 at room temperature for 45 min. After prehybridization with binding buffer containing 0.5% nonfat dry milk, 1 μg/ml salmon sperm DNA, and 1×10^6 cpm/ml of [^{32}P]WTB probe at 4 °C overnight, the filter was rinsed and washed twice for 15 min with binding buffer. Protein-DNA complexes were then visualized by autoradiography, and the relative molecular weight of the complexing proteins determined using the prestained protein standards. Specifically, the ln M_r for each protein standard was plotted as a function of electrophoretic migration. Regression analysis was used to generate a linear equation from which the M_r of the unknown proteins could be determined by interpolation.

Statistic Analysis—All data are presented as the mean ± S.E. with

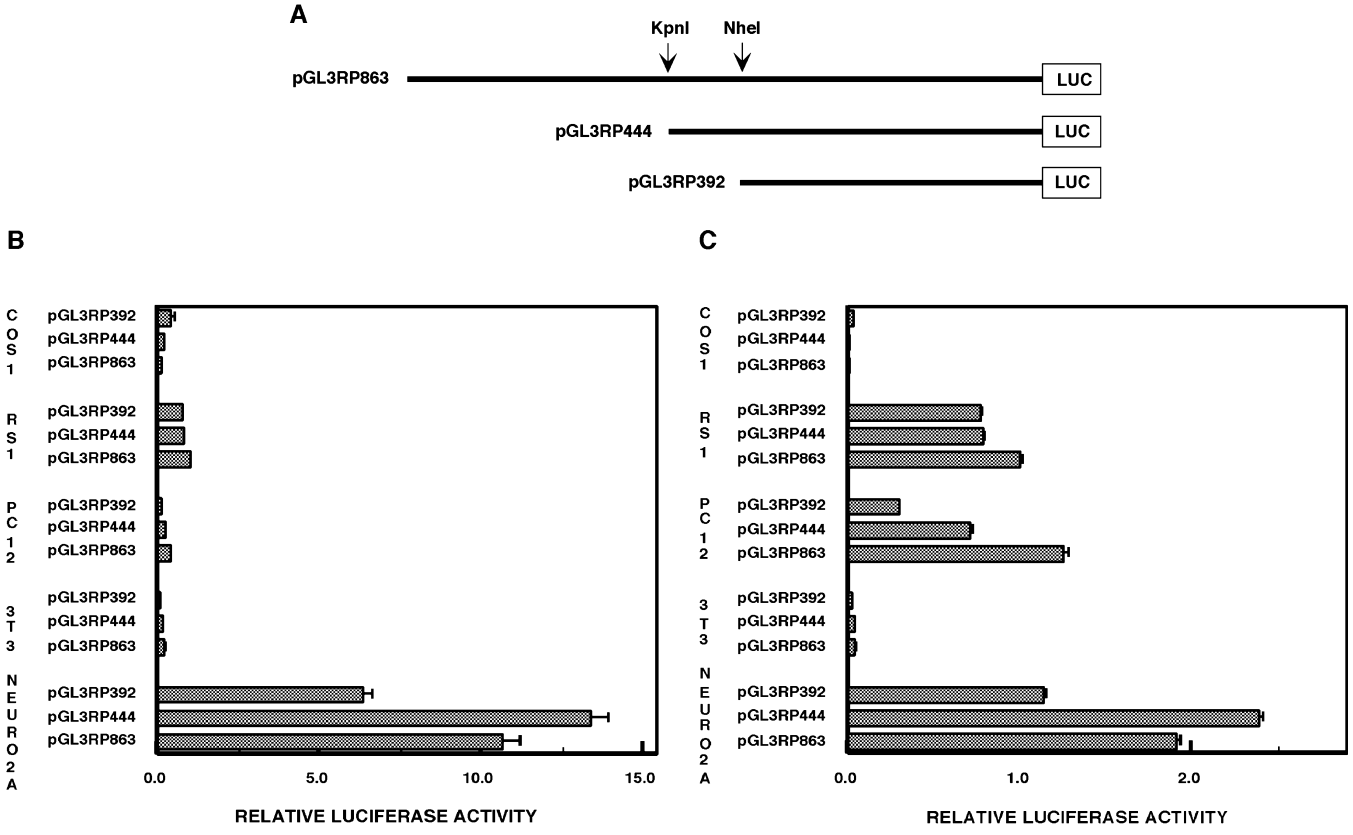

FIG. 1. **Cell-specific luciferase activity of PNMT promoter-luciferase reporter gene constructs.** The PNMT promoter-luciferase gene constructs pGL3RP863, pGL3RP444, and pGL3RP392, containing 863, 444, and 392 bp of rat 5′ upstream PNMT promoter/regulatory sequences were transfected into COS1, PC12, RS1, NIH/3T3 and Neuro2A cells and luciferase activity determined as described under "Experimental Procedures." *A,* schematic of the nested deletion constructs pGL3RP863, pGL3RP444, and pGL3RP392. *B,* luciferase activity corrected for variable transfection efficiency within each cell line. Luciferase activity was first corrected for variable transfection efficiency within each cell line using the normalization control plasmid, pRSV-LacZ. Luciferase activity was then expressed relative to luciferase expression for the full-length pGL3RP863 in RS1 cells (7). *C,* luciferase activity corrected for variable transfection efficiency across cell lines. In this case, luciferase activity was first normalized across all cell lines for variable transfection efficiency using the normalization control plasmid, pRSV-LacZ and setting β-galactosidase expression from this construct in the RS1 cells as unity. Then, luciferase activity was expressed relative to luciferase expression of the pGL3RP863 construct in the RS1 cells as previously.

an $n \geq 6$ for each experimental group. The statistical significance of the difference between two groups was determined using Student's t test. A p value of ≤ 0.05 was considered statistically significant.

RESULTS

Cell-specific Basal PNMT Promoter Activity—To examine the dependence of PNMT promoter activity on potential cell-specific transcription factors, three nested deletion PNMT promoter-luciferase reporter gene constructs, pGL3RP863, pGL3RP444, and pGL3RP392, containing 863, 444, and 392 bp of PNMT 5′ upstream promoter sequence, respectively, were cotransfected into PC12, RS1, Neuro2A, 3T3 or COS1 cells along with the β-galactosidase control construct pRSV-LacZ, and relative luciferase activity was determined (Fig. 1). Fig. 1*A* provides a schematic representation for each PNMT promoter-luciferase reporter gene construct, identifying the *Kpn*I and *Nhe*I restriction sites used to generate the deletion constructs. Because transfection efficiency may vary between transfections and with different constructs, luciferase activity was corrected for variable expression of both the control pRSV-LacZ construct and PNMT promoter-luciferase reporter gene construct as detailed under "Experimental Procedures." When transfection efficiency for each construct was normalized within a given cell line (Fig. 1*B*), the highest basal luciferase activity was observed in the Neuro2A cells with 10–100-fold lower expression ($p \leq 10^{-3}$) of luciferase from the nested deletion constructs transfected into the COS1, RS1, 3T3, and PC12 cells. Transfection efficiency may also vary between different cell lines. Two control constructs, pRSVLacZ and pRSVLUC (data not shown), showed that the COS1 and Neuro2A cells had the highest transfection efficiency, *i.e.* expressed higher β-galactosidase or luciferase activity, with lower transfection efficiency in the 3T3 cells, and the lowest transfection efficiency in PC12 and RS1 cells. When PNMT promoter driven luciferase reporter gene activity was corrected to account for these inter-cell line differences (Fig. 1*C*), the Neuro2A cells still showed the highest levels of luciferase expression, although luciferase levels expressed from the constructs in the PC12 and RS1 cells approached that in the Neuro2A cells. However, luciferase activity generated from the same PNMT promoter-luciferase reporter gene constructs in the 3T3 and COS1 cells remained markedly lower and barely detectable. Thus, PNMT promoter activity appears highest in cell lines derived from tissues likely to express PNMT.

In addition, in the Neuro2A cells, the construct pGL3RP444 showed the highest basal luciferase expression. By comparison, luciferase activity expressed from the pGL3RP863 and pGL3RP392 plasmid constructs in the Neuro2A cells was approximately 20 and 50% lower, respectively.

Together these results suggest that basal PNMT promoter activity varies depending on the host cell line as well as the extent of PNMT promoter sequences included in the reporter gene constructs. This specificity is likely due to the fact that the tissues from which these cell lines were derived express different transcriptional proteins, some of which may be im-

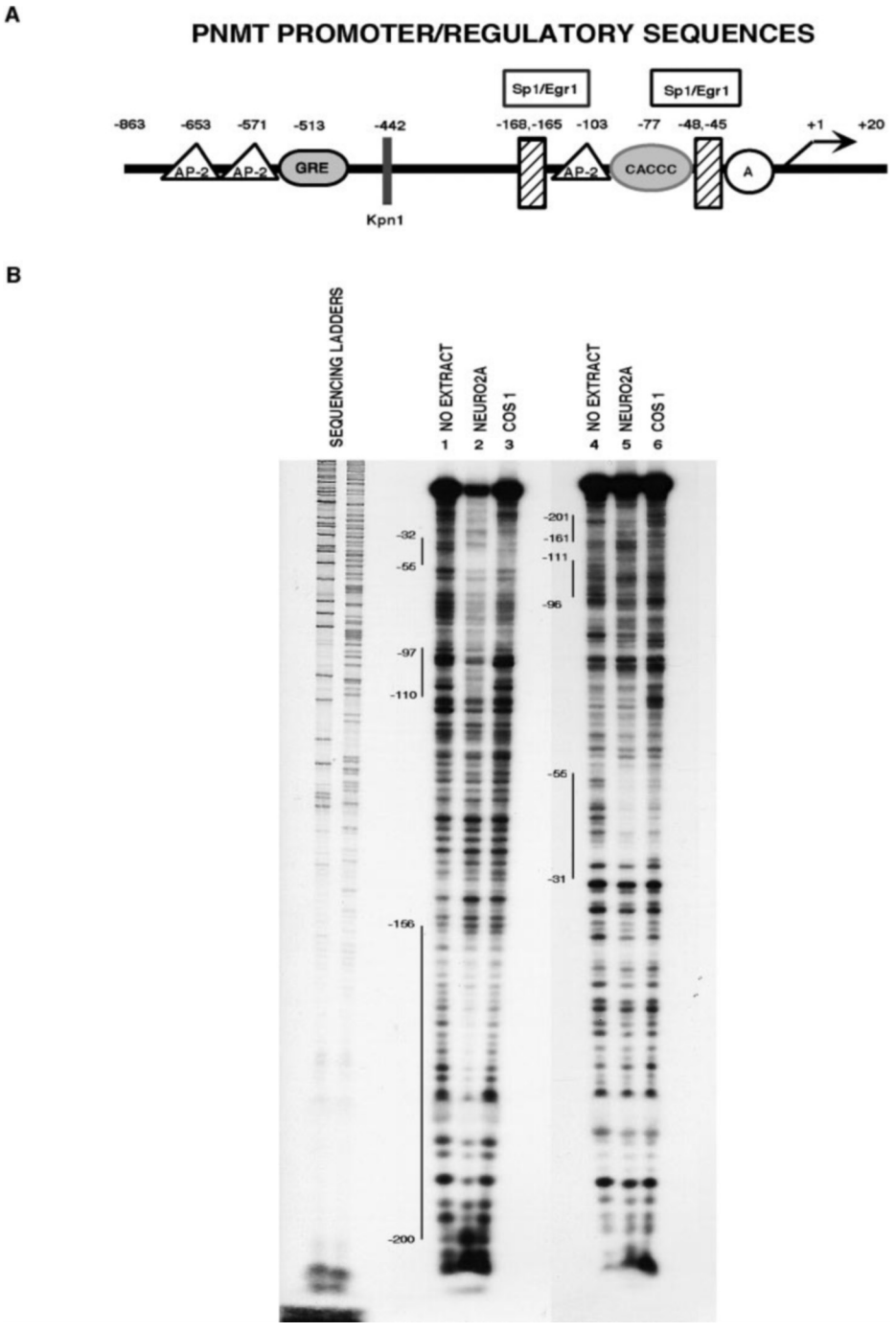

FIG. 2. **DNase I footprinting with Neuro2A and COS1 cell nuclear extracts.** A 222-bp DNA fragment spanning nucleotides +18 to –204 of the rat PNMT gene was generated by PCR using two 21-mer primers uniquely labeled with ^{32}P at their 5′ terminus (5′ → 3′, –204 to –184 bp and +18 to –3 bp). The fragments were incubated with nuclear extracts from Neuro2A and COS1 cells and then subjected to DNase I footprinting. *A,* schematic of the PNMT promoter depicting binding elements for known transcriptional regulators of the PNMT gene. *B,* autoradiogram from DNase I footprinting. *Lanes 1* and *4,* no extract; *lanes 2* and *5,* Neuro2A cell nuclear extract; *lanes 3* and *6,* COS1 cell nuclear extract. DNase I concentrations used for the footprinting were 0.001 unit/μl for *lanes 1* and *4* and 0.02 units/μl for *lanes 2, 3, 5,* and *6*. The protected nucleotides were mapped by comparison to sequencing products generated using the PCR primers above and the position of the footprints denoted by the vertical bars and nucleotide number to the *left* of each footprint.

portant for PNMT promoter activity and, hence, PNMT gene expression.

PNMT Promoter-specific Transcriptional Activators Expressed by Neuro2A Cells—As PNMT promoter activity was greatest in the Neuro2A cells and low in the COS1 cells, DNaseI footprinting was performed using uniquely labeled [^{32}P]DNA probes spanning nucleotides +18 → –204 bp and nuclear extracts from these cell lines to identify proximal DNA sequences to which transcription factors specific to the Neuro2A cells bind to stimulate the PNMT promoter. As shown in Fig. 2*B,* (*lane 2*), protected sequences using the Neuro2A cell nuclear extract included the nucleotide sequences from –32 to –55 bp, –97 to –110 bp, and –156 to –200 bp. On the complementary DNA strand (*lane 5*), corresponding footprints were observed spanning nucleotides –55 to –31 bp, –111 to –95 bp, and –195 to –161. In contrast, only sequences spanning nucleotides –32 to –55 bp (–55 to –31 bp on the complementary strand) showed a definite footprint with the COS1 cell extracts (*lanes 3* and *6*). The footprinted regions coincided with previously identified binding elements for several transcription factors shown to activate PNMT gene expression, including Sp1 (–168 bp)/Egr-1 (–165 bp), AP-2 (–103 bp), and Sp1 (–48 bp)/Egr-1 (–45 bp).

Sp1, a Transcriptional Activator of the PNMT Promoter in Neuro2A Cells—The putative AP-2 site at –103 bp shows low identity to the AP-2 consensus sequence and low affinity for AP-2 protein. In addition, gel mobility shift assays using an oligonucleotide spanning this region and nuclear extracts from the Neuro2A and COS1 cells showed no protein-DNA complex formation (data not shown). Therefore, attention was focused on the proximal and distal overlapping Sp1 and Egr-1 consensus sites. To determine whether Sp1 or Egr-1 was expressed in

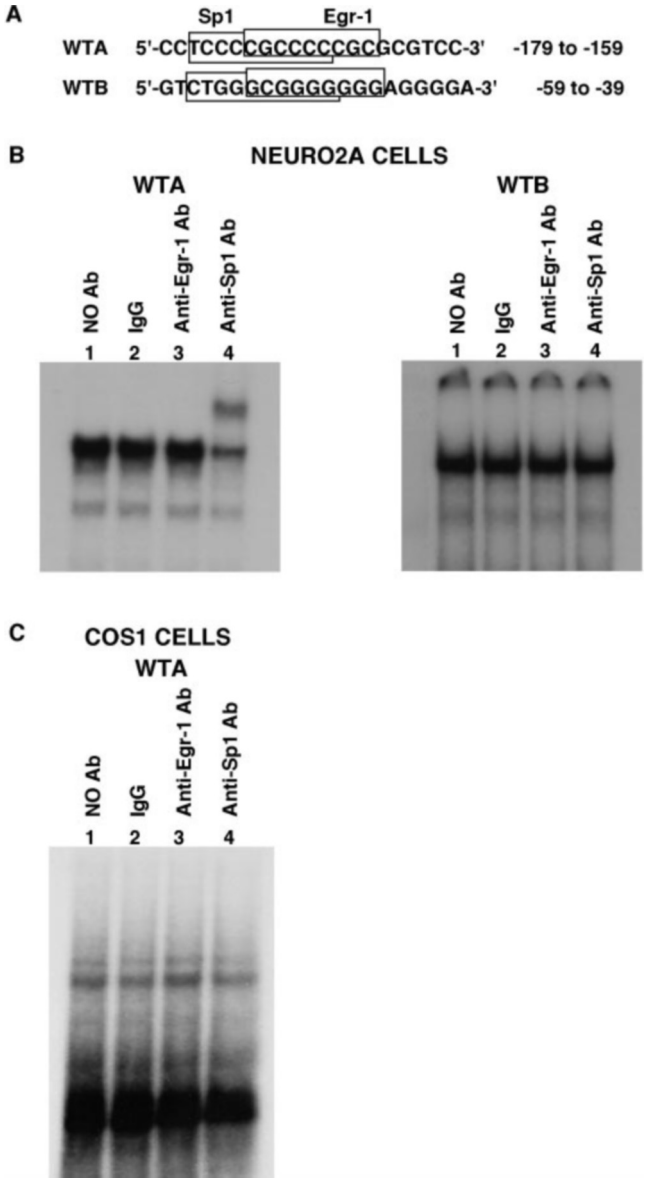

FIG. 3. **Neuro2A cell nuclear proteins binding to the Sp1 consensus elements.** Gel mobility shift assays were performed as described under "Experimental Procedures" using the 21-bp WTA (–168/–165 bp Sp1/Egr1 site) and WTB (–48/–45 bp Sp1/Egr1 site) oligonucleotide probes end-labeled with ^{32}P and nuclear extract from Neuro2A and COS1 cells. Antibodies were used to identify the proteins constituting the protein-DNA complexes. *A,* schematic of the WTA and WTB oligonucleotide probes. The nucleotide sequences for the WTA and WTB oligonucleotide probes are depicted. Sp1 and Egr-1 binding elements are identified. *B* and *C,* autoradiograms from gel mobility shift assays for Neuro2A (*B*) and COS1 (*C*) cell nuclear extracts using the [^{32}P]WTA and [^{32}P]WTB probes. *Lanes 1–4,* nuclear extract without antibody (*Ab*), IgG, anti-Egr-1 antibody (C-19, Santa Cruz Biotechnology), or anti-Sp1 antibody (PEP2, Santa Cruz Biotechnology), respectively.

the Neuro2A cells and responsible for the high basal levels of PNMT promoter activity in these cells, gel mobility shift assays were performed in the presence of specific antibodies. ^{32}P-labeled 21-bp oligonucleotide probes for both the –168/–165 and –48/–45 bp Sp1/Egr-1 sites (WTA, 5′-CCTCCCCGCCCCCGCGCGTCC-3′; WTB, 5′-GTCTGGGCGGGGGGGGAGGGGA-3′, respectively, Fig. 3*A*) were combined with Neuro2A or COS1 cell nuclear extract in the presence or absence of an anti-Egr-1 antibody (C-19) or an anti-Sp1 antibody (PEP2). As shown in Fig. 3*B,* the WTA probe, spanning the distal Sp1/Egr-1 site, produced several protein-DNA complexes with the nuclear extract from the Neuro2A cells (*lanes 1* and *2*). The major protein-DNA complex was supershifted by anti-Sp1 antibody (*lane 4*), whereas no supershifted complexes (Ref. 7, Santa Cruz Biotechnology) were observed with anti-Egr-1 antibody (*lane 3*). Several protein-DNA complexes were also observed using the duplex oligonucleotide probe WTB spanning the proximal Sp1/Egr-1 binding element and Neuro2A cell nuclear extract (Fig. 3*B*) or the WTA oligonucleotide probe and COS1 cell nuclear extract (Fig. 3*C*). However, neither the anti-Egr-1 nor anti-Sp1 antibody supershifted any of these complexes (Fig. 3, *B* and *C, lanes 3* and *4*). Finally, no protein-DNA complexes were observed when the WTB oligonucleotide probe was combined with nuclear extract from the COS1 cells (data not shown).

Thus, it would appear that the transcription factor Sp1 is one factor expressed by the Neuro2A cells that may be responsible for the higher basal activity of the PNMT promoter observed in these cells. However, binding of Sp1 was only evident at –168 bp Sp1 site and not at the –48 bp Sp1 site.

Inhibition of Sp1 Binding at Proximal Sp1 Site—The proximal –48 bp Sp1 binding element has been reported to be the higher affinity Sp1 site, and evidence suggests that it may be the biologically functional Sp1 site (8). It was therefore puzzling why the WTB probe spanning this Sp1 site did not form an Sp1-DNA complex with the Neuro2A cell nuclear extract. The possibility that another transcription factor expressed in the Neuro2A cells might be binding to the WTB oligonucleotide probe, thus preventing Sp1 binding, was examined. Gel mobility shift assays were performed with both the oligonucleotides WTA and WTB (Fig. 4*A*) and Neuro2A cell nuclear extract or purified Sp1 protein. In addition, an oligonucleotide, mut45 (Fig. 4*A*), with point mutations in the DNA sequences 3′ to the proximal –48 bp Sp1 consensus site (GCGGGGaaa, within the Egr-1 site), leaving the Sp1 element intact, was also examined in the gel mobility shift assays. As shown in Fig. 4*B,* the WTA oligonucleotide probe again formed several protein-DNA complexes with the Neuro2A cell nuclear extract (*lane 3*). The major complex showed the same electrophoretic mobility as the protein-DNA complex formed with pure Sp1 protein (*lane 2*) and, as described above, was supershifted by anti-Sp1 antibody. The WTB oligonucleotide again formed several protein-DNA complexes with the Neuro2A cell nuclear extract as well (*lane 6*). The major and slowest migrating complex was not identical to the Sp1 complexes formed with the Neuro2A cell nuclear extract (Fig. 4*B, lane 3*) or Sp1 protein (*lane 2*) because it had a faster electrophoretic mobility and was not supershifted by either anti-Sp1 or anti-Egr-1 antibody as described above. However, Sp1 protein could form a protein-DNA complex with the WTB probe that did migrate identically. Moreover, the mut45 probe also formed a protein-DNA complex with both Sp1 protein (*lane 8*) and the Neuro2A cell nuclear extract (*lane 9*) with identical electrophoretic mobilities to the Sp1-DNA complexes seen with the WTA oligonucleotide. A second, less intense protein-DNA binding complex with slower electrophoretic mobility was also observed with both pure Sp1 protein and Neuro2A cell nuclear extract. The Neuro2A cells therefore appear to contain a transcriptional protein, other than Egr-1, that binds to DNA sequences overlapping the –45 bp Egr-1 site and thereby precludes Sp1 from binding to its consensus element at –48 bp.

To confirm that the protein-DNA complex formed with Neuro2A cell nuclear extract and mut45, with identical electrophoretic mobility to the complex formed between Sp1 and mut45, does contain Sp1, gel mobility shift assays were executed with the WTB and mut45 oligonucleotide probes and Neuro2A cell nuclear extract in the absence or presence of anti-Sp1 antibody (Fig. 5). Multiple protein-DNA complexes

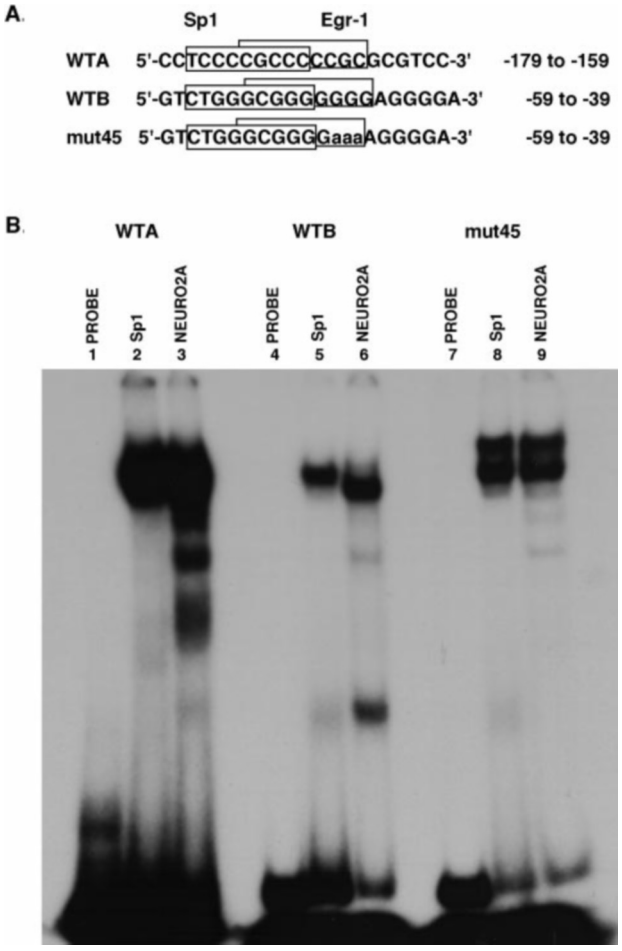

FIG. 4. **Displacement of Sp1 binding at −48 bp Sp1 consensus element.** Gel mobility shift assays were performed with purified human Sp1 protein (Santa Cruz Biotechnology) or nuclear extract from Neuro2A cells and the double-stranded DNA probes, WTA, WTB, and mut45, generated by digestion of pBSKII(−)WTA, pBSKII(−)WTB, and pBSKII(−)mut45 with *Eco*RI and *Bam*HI, followed by end labeling with [γ-^{32}P]ATP and T_4 polynucleotide kinase. Mut45 has point mutations in the −45 bp Egr-1 target sequences as described under "Experimental Procedures." *A,* schematic of WTA, WTB and mut45 oligonucleotide probes. The nucleotides sequence for each of the probes is depicted, and Sp1 and Egr-1 binding elements are identified as described above. *B,* autoradiogram of gel mobility shift assay. *Lanes 1, 4,* and *7,* no Sp1 or nuclear extract; *lanes 2, 5,* and *8,* Sp1 protein; *lanes 3, 6,* and *9,* Neuro2A cell nuclear extract.

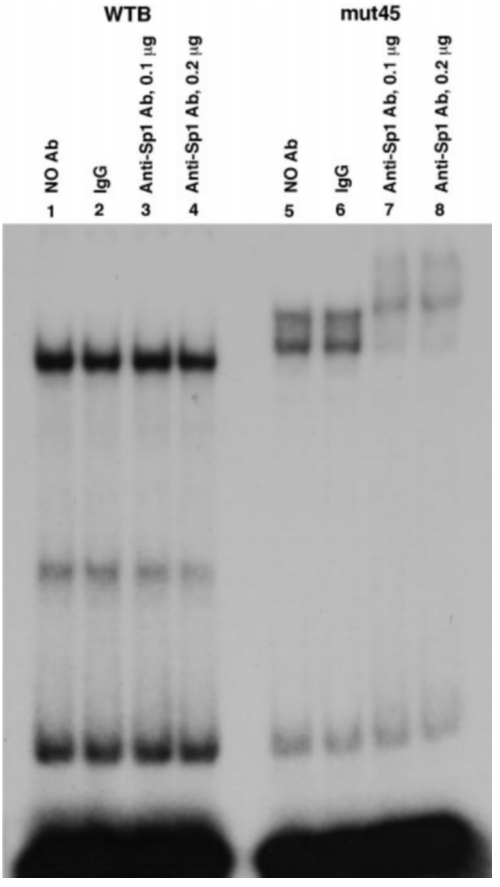

FIG. 5. **Confirmation of Sp1 binding to the −48 bp Sp1 consensus element.** Gel mobility shift assays were performed with the ^{32}P-labeled WTB or mut45 oligonucleotide probes and nuclear extract from Neuro2A cells in the absence or presence of anti-Sp1 antibody. Autoradiogram of gel mobility shift assay. *Lanes 1* and *5,* no nuclear extract; *lanes 2* and *6,* IgG; *lanes 3* and *7,* 0.1 μg of anti-Sp1 antibody; *lanes 4* and *8,* 0.2 μg of anti-Sp1 antibody.

were again apparent with the WTB oligonucleotide (*lanes 1* and *2*). However, none of the complexes were supershifted by the anti-Sp1 antibody (*lanes 3* and *4*). In contrast, the mut45 probe with a mutated Egr-1 site and intact Sp1 site showed the same two slower migrating complexes as described previously (*lanes 5* and *6*), both of which were supershifted by anti-Sp1 antibody (*lanes 7* and *8*). The fastest migrating, predominant complex was nearly completely supershifted, whereas the slower complex appeared only partially supershifted.

Thus, mutation of the 3′ −45 bp Egr-1 sequences prevents a DNA-binding protein in the Neuro2A cell nuclear extract from binding to the mut45 oligonucleotide, thereby permitting Sp1, present in the Neuro2A cell nuclear extract, to bind to the intact proximal −48 bp Sp1 site in the PNMT promoter.

Role of Sp1 in PNMT Promoter Activation—To further define the role of Sp1 in PNMT promoter activation, the effect of mutation of the −168 bp and/or the −48 bp Sp1 consensus sites on luciferase reporter gene expression was examined (Fig. 6). Changes in basal luciferase activity as well as the effects of Sp1 over expression were determined by cotransfecting the Neuro2A cells with the wild-type (pGL3RP392) or mutant PNMT promoter-luciferase reporter gene constructs (pGL3RP392mut168, pGL3RP392mut48, and pGL3RP392mut168/48, Fig. 6*A*) and the vector pCGN or the Sp1 expression construct pCGNSp1. In the case of basal luciferase activity, mutation of the −168 bp Sp1 binding element reduced luciferase activity to 75% of wild-type levels, whereas mutation of the −48 bp Sp1 binding element reduced luciferase to 50% of wild-type levels (Fig. 6*B*). If both Sp1 sites were altered, luciferase expression decreased to very low levels (28% of control). When intracellular levels of Sp1 were increased by cotransfection of the Sp1 expression plasmid pCGNSp1 along with the PNMT promoter-reporter gene constructs, luciferase expression could be further elevated, even in the case of the mutant constructs. Luciferase rose 2.5-fold above basal levels for the wild-type construct pGL3RP392 and each single mutant construct. However, each single mutant construct showed reduced luciferase activity by comparison to the wild-type construct (2.0- and 1.4-fold basal wild-type control for the −168 and −48 Sp1 mutant constructs, respectively), although luciferase activity expressed by the wild-type and the −168 bp mutant construct was not significantly different. In contrast, the PNMT promoter-luciferase reporter gene construct with the double Sp1 mutation showed no significant changes in luciferase in response to Sp1 over expression.

The effect of Sp1 over expression was also compared in the Neuro2A and COS1 cells to examine whether increasing Sp1 alone in the COS1 cells would increase PNMT promoter activ-

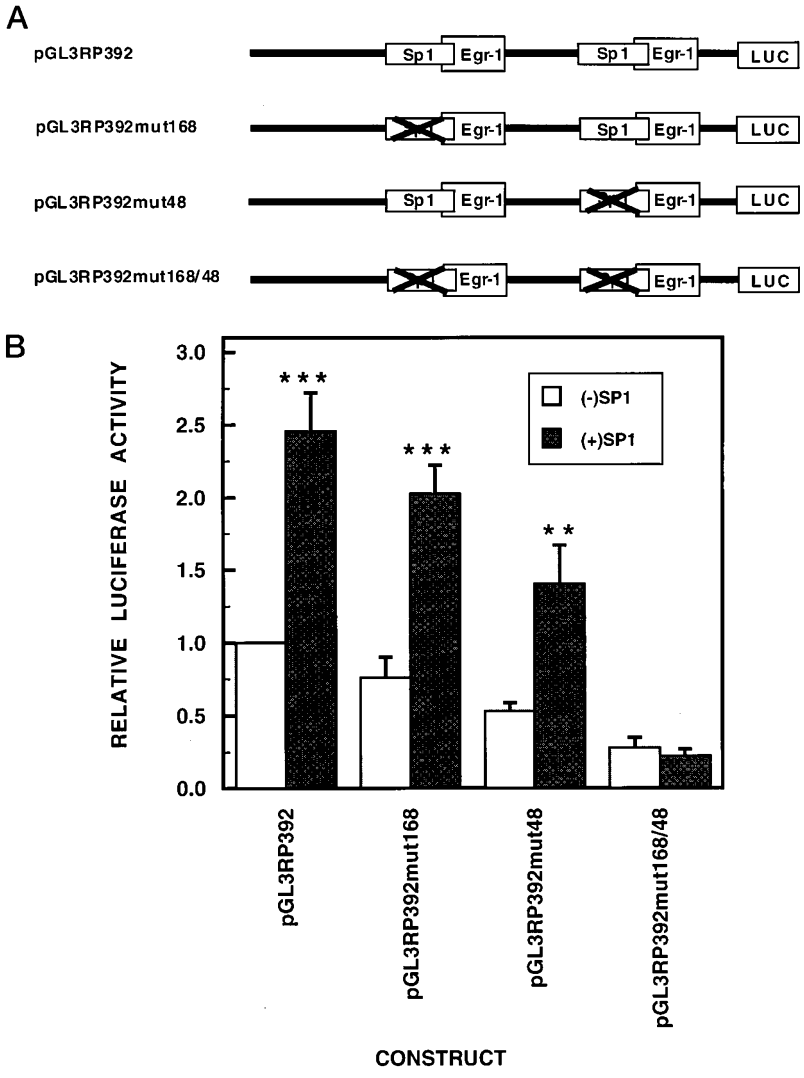

FIG. 6. **Basal and exogenous Sp1-stimulated luciferase activity from wild-type and mutant Sp1 PNMT promoter-luciferase reporter gene constructs.** The wild-type plasmid construct pGL3RP392 or mutant plasmid constructs pGL3RP392mut168, pGL3RP392mut48, or pGL3RP392mut 168/48 were cotransfected with the control plasmid pCGN and/or the Sp1 expression plasmid pCGNSp1 into Neuro2A cells, and luciferase activity was determined as described under "Experimental Procedures." *A,* schematic of pGL3RP392 (wild-type), pGL3RP392mut-168, pGL3RP392mut48, and pGL3Rpmut-168/48 constructs. Sp1 and Egr-1 binding elements are identified. *X* designates site directed mutation of binding element as described under "Experimental Procedures." *B,* relative luciferase activity expressed from wild-type and mutant PNMT promoter-luciferase reporter gene constructs in the absence (*white bars*) and presence (*shaded bars*) of exogenous Sp1. **, significantly different from the respective controls, $p \leq 0.01$; ***, significantly different from control, $p \leq 0.001$.

ity. Luciferase activity progressively rose as increasing amounts of the pCGNSp1 expression plasmid were cotransfected into the Neuro2A cells, whereas no significant induction of the wild-type expression construct pGL3RP392 was observed at all concentrations of Sp1 in the COS1 cells (Fig. 7).

Thus, the functionality of the Sp1 sites seems to depend on both the relative affinity of Sp1 for each of the Sp1 target sequences and the concentration of Sp1 protein available for occupancy of the sites. In addition, another transcription factor must be expressed by the Neuro2A cells that interacts with Sp1 to stimulate the PNMT promoter or a factor may preclude Sp1 activation of the PNMT promoter in the COS1 cells.

Sp1 Competition at the Proximal Sp1 Binding Element—As described above, another transcriptional protein present in the Neuro2A cell nuclear extract has a recognition site 3′ to the proximal −48 bp Sp1 site. It appears to form less protein-DNA complex with the WTB oligonucleotide (proximal Sp1/Egr-1 site) by comparison to the amount of Sp1-DNA complex formed with the WTA oligonucleotide (distal Sp1/Egr-1 site) when equivalent amounts of Neuro2A cell nuclear extract and probes (approximately equivalent specific activity) are used. The latter would suggest that the factor is less abundant, with apparent higher affinity for its recognition site than Sp1 for the proximal Sp1 site. In addition, when complexed to the WTB oligonucleotide probe, the factor has a faster electrophoretic mobility than Sp1 bound to the same probe, indicating that its relative molecular weight is less than that of Sp1. Examination of the DNA sequences 3′ to the −48 bp Sp1 site suggests that the factor may be MAZ (13) given the 11-bp match to the MAZ binding element (Fig. 8*A*). It also appears that the MAZ and Sp1 binding sites overlap by 1 bp. Southwestern analysis was performed to see if the binding protein was of the appropriate molecular mass for MAZ. As shown in Fig. 8*B,* two proteins form complexes with ^{32}P-labeled WTB. The larger, predominant protein has an apparent molecular mass of 105 kDa (*lane 1*), identical to the relative molecular mass of authentic human Sp1 protein (*lane 2*). The smaller, less abundant protein has a molecular mass of 60 kDa, which is the anticipated molecular mass for MAZ. Gel mobility shift assays with antibodies were used to confirm that MAZ was the protein in the Neuro2A cell nuclear extracts binding to the proximal Sp1/Egr-1 site (Fig. 8*C*). The ^{32}P-labeled WTB oligonucleotide probe was combined with Neuro2A cell nuclear extract in the presence of bovine serum albumin, IgG, anti-Egr-1, anti-Sp1, or anti-MAZ antibody. No supershifts were apparent with any of the antibodies, but the anti-MAZ antibody either disrupted or prevented DNA-complex formation, as evidenced by the marked reduction in the major radiolabeled protein-DNA complex. A low molecular mass complex was also apparent, and it was reduced in the presence of anti-MAZ antibody as well. Gel mobility shift assays with Sp1 protein are consistent, demonstrating that anti-Sp1 antibody supershifts the Sp1-WTB DNA complex, whereas anti-MAZ antibody has no effect whatsoever.

Thus, the binding of the transcription factor MAZ, a nuclear

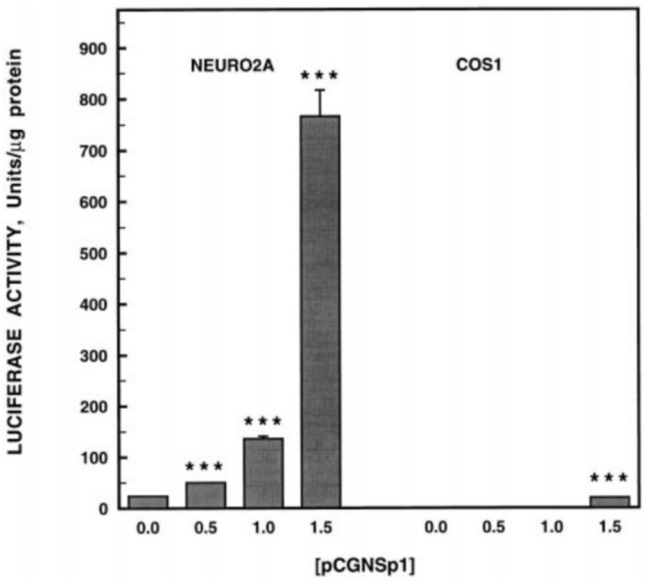

FIG. 7. **Sp1 responsiveness of PNMT promoter-luciferase reporter gene construct.** The wild-type (pGL3RP392) construct was cotransfected with increasing amounts of the Sp1 expression plasmid pCGNSp1 into Neuro2A and COS1 cells. The control plasmid pCGN was included to ensure that the total plasmid DNA transfected into the cells was equivalent. Luciferase activity was determined as described under "Experimental Procedures." ***, significantly different from control, $p \leq 0.001$.

protein present in the Neuro2A cells, to its consensus element (3′ to the −48 bp Sp1 site with a 1-bp overlap) in the rat PNMT promoter, prevents Sp1 binding and thereby inhibits Sp1 induction of the PNMT promoter through its proximal binding site. Although Sp1 seems to be the transcription factor responsible for the higher PNMT promoter activity observed in the Neuro2A cells, blockade of Sp1 activation at the −48 bp Sp1 site may limit Sp1 stimulation of the PNMT promoter and, hence, PNMT gene expression.

DISCUSSION

Although several transcriptional activators, including the glucocorticoid receptor, Egr-1, Sp1, and AP-2 (7, 8, 18, 19), and negative regulatory factors (20) have been shown to control the rat PNMT gene promoter and the endogenous PNMT gene, transcriptional regulatory proteins participating in the developmental-specific, tissue-specific, or stimulus-specific expression of PNMT remain to be determined. The present studies suggest that Sp1 and MAZ may be two transcriptional regulators participating in tissue-specific expression. Luciferase activity produced from PNMT promoter-luciferase reporter gene constructs containing 863, 444, and 392 bp of 5′ upstream PNMT promoter/regulatory sequence was high in cell lines derived from tissues likely to express PNMT, *i.e.* Neuro2A, PC12 and RS1 cells. In contrast, it was barely detectable in cell lines derived from tissues unlikely to express PNMT, COS1 and NIH/3T3 cells. DNase I footprinting further showed that the high basal luciferase levels in the Neuro2A cells might be due to nuclear proteins interacting with the Sp1/Egr-1 binding elements at –168/–165 and –48/–45 bp in the PNMT promoter. However, gel mobility shift assays demonstrated that Sp1, but not Egr-1, was present within the nuclei of Neuro2A cells. Consistent with these findings, mutation of the Sp1 sites in the PNMT promoter reduced basal luciferase activity expressed from PNMT promoter-reporter gene constructs. In addition, another Neuro2A cell nuclear protein appeared to bind to a consensus element 3′ to the proximal Sp1 site (one bp overlap), preventing Sp1 binding at this site and activation of the PNMT

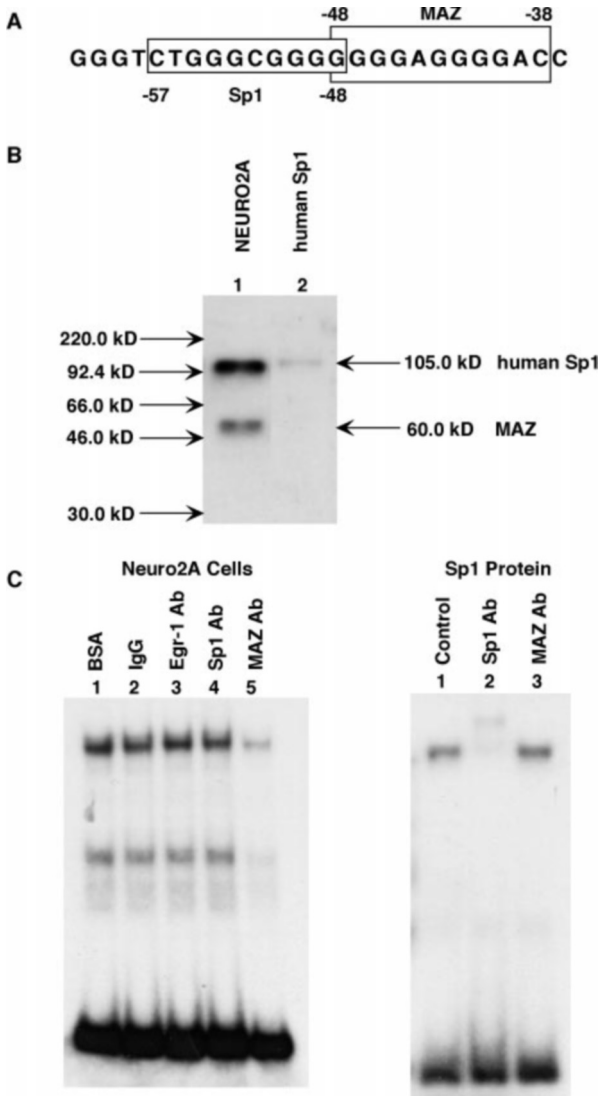

FIG. 8. **MAZ binding proximal to the Sp1 binding site.** Consensus sequence match using the Transfac version 3.2 data base indicated that the transcription factor MAZ might be the protein binding to the DNA sequences proximal to the Sp1 site. Southwestern analysis was performed to determine whether the molecular mass of the protein was that expected for MAZ, and gel mobility shift assays with antibodies were used to confirm the identity of MAZ protein. *A,* schematic of the Sp1 and MAZ sequences in the proximal PNMT promoter. The nucleotide sequence from –61 to –37 in the PNMT promoter is shown. The Sp1 binding element spans from –57 to –48, whereas the MAZ binding element spans sequences from –48 to –38. *B,* Southwestern analysis of Neuro2A cell nuclear extracts. The nuclear proteins contained in a 20-μg sample of Neuro2A cell nuclear extract were separated on 10% SDS-polyacrylamide gels as described under "Experimental Procedures." Following transfer to nitrocellulose, the proteins were visualized by forming protein-DNA complexes with ^{32}P-labeled WTB probe. Prestained molecular mass markers were included in order to determine the molecular mass of the proteins forming complexes with the WTB probe. Human Sp1 protein was run as a control. *Lane 1,* Neuro 2A cell nuclear extract; *lane 2,* purified human Sp1 (Santa Cruz Biotechnology). *C,* gel mobility shift assays with antibodies. Gel mobility shift assays were performed as described under "Experimental Procedures" using the 21-bp WTB probe end-labeled with ^{32}P and nuclear extract from Neuro2A cells or purified human Sp1 protein (Santa Cruz Biotechnology). Antibodies were used to identify the proteins constituting the protein-DNA complexes. Neuro 2A cell nuclear extract (*lanes 1–5*), bovine serum albumin (*BSA*), IgG, anti-Egr-1 antibody, anti-Sp1 antibody, and anti-MAZ antibody, respectively. Sp1 protein (*lanes 1–3*), Sp1 alone, anti-Sp1antibody, and anti-MAZ antibody, respectively.

promoter by Sp1. DNA consensus sequence identity, Southwestern analysis, and gel mobility shift assays with antibodies indicate that this factor is the 60-kDa protein MAZ, an activa-

tor of the c-myc (13, 21, 22), insulin (23), and $5HT_{1A}$ receptor (24) genes. Thus, the exclusion of Sp1 binding by MAZ may provide another mechanism contributing to tissue-specific PNMT gene regulation.

Previous studies showed that both Sp1 binding elements within the proximal 863 bp of 5′ PNMT promoter sequences likely participated in Sp1 activation of the PNMT gene. However, the −48 bp Sp1 site had higher affinity for Sp1 (8). We now demonstrate that Sp1 activation of the PNMT promoter depends on intracellular Sp1 concentrations as well. When intracellular Sp1 concentrations were increased by cotransfection of an Sp1 expression plasmid (pCGNSp1) along with the wild-type PNMT promoter-luciferase reporter gene construct into Neuro2A cells, luciferase activity rose 2.5-fold above basal values. Similar increases in luciferase expression were observed in PNMT promoter-luciferase reporter gene constructs with mutations in either the –168 or −48 bp Sp1 site. Only if both Sp1 binding elements were mutated was Sp1 unable to stimulate PNMT promoter induction. These results confirm that intact Sp1 binding elements are required for Sp1-mediated PNMT promoter activity and underscore the importance of considering both the relative abundance of transcriptional regulators and their affinities for their respective binding elements when investigating the actions of transcriptional regulatory proteins *in vitro*.

Gel mobility shift assays identified two Sp1 protein-DNA binding complexes arising from either the Neuro2A cell nuclear extract or Sp1 protein. Whereas the complex with the faster electrophoretic mobility predominated, both complexes were supershifted by anti-Sp1 antibody. Two forms of Sp1 protein have been identified with relative molecular masses of 95 and 105 kDa (25, 26). Both molecular mass forms of the protein are equivalently glycosylated, but the 105-kDa form is phosphorylated as well, and the latter accounts for the molecular mass difference (27). In Fig. 5, however, the slower migrating Sp1-DNA complex observed probably represents dimeric Sp1 bound to the −48 bp binding element, because the 5% polyacrylamide gels used for the gel mobility shift assays would not have resolved two Sp1-DNA complexes so similar in molecular mass. Consistent with this interpretation, Sp1, as other zinc finger proteins, reportedly forms functionally active dimers (28).

Finally, Sp1 binding at the proximal −48 bp Sp1 consensus element seems to be prevented by MAZ, another nuclear protein expressed in the Neuro2A cells, at an adjacent 3′ binding element. Both the Sp1 and MAZ binding elements consist predominantly of guanine and cytosine nucleotides, and many transcription proteins bind to GC-rich regions of DNA (29). In the case of the PNMT gene, we have previously shown that the immediate early gene transcription factor Egr-1 binds to sequences 3′ to the −48 bp Sp1 binding element; these sequences overlap the MAZ recognition site. However, we had further suggested that this proximal −48/−45 bp Sp1/Egr1 site likely functioned as an Sp1 activation element (7, 8). The DNA consensus element for MAZ has been defined as 5′-G(G/C)GG(C/A)GGGG(C/A)(G/T)-3′, whereas that for Sp1 is represented by 5′-(G/T)GGGCGG(G/A)(G/A)(C/T)-3′. In the rat PNMT gene, the promoter sequences spanning the nucleotides to which Sp1 and MAZ appear to bind include 5′-CTGGCGGGGGGGAGGGGACC-3′ (24). Based on the Sp1 and MAZ consensus sequences defined above, a core motif, 5′-GGGG(C/A)GGGG-3′, is common to both binding elements, indicating that either site may interact with Sp1 or MAZ. The 5′ flanking region of the $5HT_{1A}$ receptor gene has been shown to possess four MAZ binding sites that share this motif as well, and in the case of that gene, Sp1 binds to three of the four MAZ sites (24). However, Sp1 does not appear to bind to the nucleotides designated as constituting the MAZ site (–38 to −48 bp) and MAZ does not appear to bind to the nucleotides designated as the Sp1 site (–548 to –56 bp) in the PNMT gene. Evidence in support of distinct but overlapping binding elements for MAZ and Sp1 is provided by the finding that a MAZ-DNA complex forms in lieu of an Sp1-DNA complex if the MAZ binding sequences are intact, but only an Sp1 complex forms when the 3′ MAZ consensus sequence is mutated.

The exclusionary competition between MAZ and Sp1 once again underscores the importance of considering both binding affinities and relative abundance of transcription factors in gene activation. From Southwestern analysis, we see that in the Neuro2A nuclear extracts, MAZ is present at approximately 4-fold lower concentrations than Sp1. MAZ must therefore have a higher affinity for its binding site than Sp1 does for the −48 bp binding site. However, when Sp1 levels are raised, it is possible to drive PNMT promoter activity through Sp1 induction. Thus, increasing Sp1 concentrations disproportionately to MAZ seems to offset the lower affinity of Sp1 for its recognition site.

Both Sp1 and MAZ have been shown to facilitate transcription in TATA-less gene promoters by interacting with TATA-binding protein associated factors to facilitate TFIID binding to the promoter (24). We know, however, that the rat PNMT gene possesses an ATAAA box from which transcription initiation can occur (6). Recently, it has been demonstrated that Sp1 and MAZ can facilitate transcription of TATA box containing genes as well (30). Although less is known about the transcriptional regulatory properties of MAZ, Sp1 is known to be regulated developmentally and in response to specific stimuli (27, 31). Taken together, these various findings suggest a potential mechanism whereby MAZ and Sp1 may participate in the orchestration of tissue-specific expression of the PNMT gene. Present studies are further defining the independent and exclusionary control of the PNMT gene by MAZ and Sp1.

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