Regulation of the Rat Phenylethanolamine N-Methyltransferase Gene by Transcription Factors Sp1 and MAZ

SONG HER, ROBERT CLAYCOMB, T. C. TAI, and DONA L. WONG

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

Received April 2, 2003; accepted August 11, 2003 This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The rat phenylethanolamine N-methyltransferase (PNMT) gene promoter contains 1-base pair (bp) overlapping consensus sequences for Sp1 and MAZ transcription factors at −48 and −38 bp, respectively. Gel mobility assays using PC-12-derived RS1 cell nuclear extracts or in vitro translated proteins showed that Sp1 and MAZ specifically bind to these elements, that MAZ displaces/prevents Sp1 binding, and that Sp1 and MAZ binding is mutually exclusive, with occupancy dependent on each factor’s concentration and affinity for its consensus element. In transfection assays, PNMT promoter activation by Sp1 and MAZ depends on promoter length, with −893 bp of sequence yielding greatest activation. Although MAZ has higher affinity for its binding element, it is a less effective activator. Changes in PNMT promoter activity for the constructs pGL3RP60 or pGL3RP893 using a fixed amount of MAZ expression construct and a variable amount of Sp1 expression construct or vice versa confirmed the latter. Mutation of the MAZ or Sp1 sites in pGL3RP60 attenuated but did not eliminate PNMT promoter activity, even though the proteins no longer bind to their consensus elements. Phosphatase treatment of RS1 cell nuclear extracts prevented MAZ- and Sp1-DNA binding complex formation. Although MAZ and Sp1 elevate endogenous PNMT mRNA in RS1 cells, MAZ preferentially increases intron-retaining whereas Sp1 preferentially increases intronless mRNA. Thus, expression of the PNMT gene seems to be modulated through competitive binding of phosphorylated Sp1 and MAZ to their consensus elements in the promoter. In addition, post-transcriptional regulation seems to be another important mechanism controlling PNMT expression.

During stress, epinephrine is secreted from the adrenal medulla into the bloodstream. After its release, levels of phenylethanolamine N-methyltransferase (PNMT; EC 2.1.1.28), the enzyme producing this catecholamine neurotransmitter from norepinephrine, increase (Ciaranello and Black, 1971; Kvetnansky et al., 1971; Viskupic et al., 1994; Wong et al., 2002), suggesting that regulation of PNMT expression may be an important component of the mammalian stress response.

To determine the genetic basis for stress-induced changes in epinephrine and PNMT gene expression, we have been identifying transcription factors that alter PNMT promoter activity. One such protein is Sp1, a ubiquitous mammalian transcription protein thought to be involved in the regulation of numerous genes (Briggs et al., 1986; Kriwacki et al., 1992). In the case of housekeeping genes that lack TATA and CAAT boxes, Sp1 seems to be the signal initiating transcriptional activation (Boisclair et al., 1993). For the rat PNMT gene, which possesses both TATA and CAAT boxes, it increases promoter activity by binding to consensus elements located at −168 and −48 bp upstream of the transcription initiation site (+1 bp) (Her et al., 1999).

In many genes, Sp1 sites overlap the binding elements of other transcription factors such as Egr-1 (Ackerman et al., 1991; Li et al., 1993; Ebert and Wong, 1995), MAZ (Parks and Shenk, 1996; Parks and Shenk, 1997), and YY1 (Dong and Pfister, 1999), and competition between Sp1 and these factors for binding site occupancy is thought to be an important mechanism controlling gene expression. Both Sp1 sites in the rat PNMT promoter were identified as overlapping 6 bp of functional binding elements for the immediate early gene transcription factor Egr-1 (Ebert and Wong, 1995). However, it now seems that the Egr-1 site of the distal paired Sp1/Egr-1 target sequences functions predominantly as an Egr-1 site, whereas the proximal paired Sp1/Egr-1 site functions as a binding site that favors interaction with the transcription factor MAZ (Her et al., 1999). Furthermore, competition between Sp1 and the c-myc associated zinc finger protein MAZ at this proximal site may be important for controlling PNMT gene expression.

This work was supported by a grant DK51025 from the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, the Marianne Gerschel Charitable Account, the Spunk Fund, Inc., the Denise Sobel and Norman Keller Research Support Fund, and McLean Hospital.

ABBREVIATIONS: PNMT, phenylethanolamine N-methyltransferase; bp, base pair(s); PCR, polymerase chain reaction; RT, reverse transcription; GAPDH, glyceraldehyde phosphate dehydrogenase; CIP, calf intestinal alkaline phosphatase; PP2A, protein phosphatase-2A.
Recently, two forms of PNMT mRNA were identified in PC-12 cells in vitro and mouse heart in vivo (Unsworth et al., 1999; Ziegler et al., 2002). The difference between these variants lies in post-transcriptional processing; the longer of the two forms is intron-retaining PNMT mRNA and the shorter form is intronless PNMT mRNA. PC-12 cells reportedly express very low levels of both mRNAs, and from the longer mRNA template a truncated, nonfunctional protein is generated, whereas the shorter form is intronless PNMT mRNA and the longer mRNA template a truncated, nonfunctional protein is produced. Translation of fully processed PNMT mRNA, of course, generates enzymatically active PNMT protein.

The present study was undertaken to further define the roles of the transcription factors MAZ and Sp1 in the regulation of the PNMT gene. We demonstrate that binding of MAZ to its consensus sequence (5′-GGGGAGGGGAG-3′), which lies in the 3′ direction from the proximal Sp1 target sequence (5′-CTGGGGCGGGG3′- and overlaps that sequence by 1 bp, prevents Sp1 binding to this site in the PNMT promoter. Although MAZ binds to its DNA recognition site with higher affinity than Sp1 to its binding site, thereby preventing Sp1 transactivation of the PNMT promoter, Sp1 is a stronger activator of the PNMT promoter. Both proteins also stimulate endogenous PNMT gene expression in vitro in the PC-12-derived RS1 cells and must be phosphorylated to bind and induce promoter-driven transcriptional activity. In the case of MAZ transactivation, the transcribed RNA is predominantly processed to an intron retaining mRNA. In contrast, Sp1 activation primarily increases intronless PNMT mRNA. These findings suggest that MAZ and Sp1 regulate PNMT gene expression through both mutually exclusive binding to their consensus elements and altered RNA processing, transcriptional regulatory mechanisms that may extend and be important for other genes as well.

**Materials and Methods**

Oligonucleotides and Plasmids. The 21-mer oligonucleotides and their complements [WTB, 5′-GTCTGGGCGGGGAGGAGGA′3′ (wild-type); mut38, 5′-GTCTGGGCGGGGAGGATG′3′ (point mutations in −38 bp MAZ consensus sequence, GGG→TTC; -47 to -45 bp); mut48, 5′-GTCTGGGCGGGGAGGAGGA′3′ (point mutations in −48 bp Sp1 consensus sequence, GGG→TTT; -46 to -45), and mut48/38, 5′-GTCTGGGCGGGGAGGATG′3′ (point mutations in both −48 and −38 bp Sp1 and MAZ consensus sequences, respectively)] were obtained from Gene Link (Madison, WI). Mutant PNMT-luciferase constructs were created (described above) end-labeled with [32P]ATP (PerkinElmer Life Sciences, Boston, MA) using T4 polynucleotide kinase (Invitrogen, Carlsbad, CA) and 3 μg of nuclear extract, human Sp1 protein (Promega), or the designated quantity of in vitro translated Sp1 and MAZ protein in 20 μl of binding buffer (25 mM HEPES buffer, pH 7.9, 50 mM KCl, 0.05 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol containing 0.5 μg of poly(dC-dC)-poly(dI-dI) for 30 min on ice. To confirm the transcription factors constituting the protein-DNA binding complexes, anti-Egr-1 antibody (C-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Sp1 antibody (PEP2; Santa Cruz Biotechnology) or anti-MAZ antibody (Dr. Kenneth Marcu, State University of New York, Stony Brook, NY) was included in the binding reactions. For gel mobility shift competition assays, a 300 M excess of the double-stranded competitor oligonucleotides identified above was added to the binding reaction. Protein-DNA complexes were separated on 5% polyacrylamide gels and visualized by autoradiography. For the gel mobility shift assays comparing the displacement of fixed MAZ by variable Sp1 or vice versa, radiolabeled WT probe was titrated to be in slight excess so that fixed transcription factor protein (0.5 footprinting units [fu]) was completely bound and variable transcription factor protein ranged from 0 to 2.00 fu.

**In Vitro Transcription and Translation.** The expression plasmids, pCMV-Sp1 (from Dr. Robert Tjian, University of California, Berkeley, CA) and pcDNA(MAZHH) (from Dr. Kenneth Marcu), with the T7 promoter upstream of each gene, were subjected to in vitro transcription-translation using the TNT T7 quick coupled transcription-translation system (Promega) to generate Sp1 and MAZ protein, respectively. The footprinting unit for each in vitro translated protein was determined by comparing protein-DNA complex formation to that of purified recombinant human Sp1 protein (Promega) in gel mobility shift assays.

**Cell Culture and Transient Transfection Assays.** RS1 cells (Ebert et al., 1994), a derivative of the PC-12 cell line, were maintained in Dulbecco’s modified Eagle’s medium containing 5% defined iron-supplemented bovine calf serum (Hyclone, Logan, UT), 5% donor equine serum (Hyclone), gentamycin sulfate (50 μg/ml; United States Biochemical Corp., Cleveland, OH), and hygromycin B (200 units/ml; Calbiochem, La Jolla, CA) at 37°C in an atmosphere of 5% CO2/95% air as described previously (Her et al., 1999).

Transient transfection assays were performed in 24-well plates using SuperFect (Her et al., 1999) or polyethylenimine (Boussif et al., 1995) as described previously. In general, 2 × 106 cells per well were plated using 1 to 3 μg of total double-stranded DNA consisting of the PNMT promoter-luciferase reporter gene construct, β-galactosidase construct, plasmid vector, and expression plasmid encoding MAZ and Sp1 as appropriate. Expression constructs included pGCNMAZ and pGCNSp1 (Dr. Thomas Shenk, Howard Hughes Medical Institute, Princeton University, Princeton, NJ). Cells were harvested 24 h after transfection, cell lysates were prepared, and total protein, luciferase, and β-galactosidase activities were determined (Her et al., 1999). Experiments were repeated at least three times with six replicates per sample for each experiment.

**Endogenous PNMT mRNA.** RS1 cells (6 × 106) were seeded into 100-mm culture dishes in Dulbecco’s modified Eagle’s medium, transfected as above or treated with 1 μM dexamethasone and grown to 85 to 90% confluence (24 h) at 37°C and 5% CO2/95% air. Cells were harvested and resuspended in 1 ml of Tri Reagent (Sigma Chemical, St. Louis, MO) and total RNA isolated per manufacturer’s protocol. All samples were treated with DNase I (1 unit of DNase I/2 μg of total RNA; Ambion, Austin, TX) for 30 min at 37°C before use.

For reverse transcription (RT)-PCR, 1 μg of total RNA was annealed with 100 μg of random hexamers (Invitrogen) at 65°C for 5 min and reverse transcribed with Superscript RNase H− reverse transcriptase (Invitrogen) according to manufacturer’s instructions. PCR was executed using 100 ng of RT product by adding reaction mix to a total volume of 25 μl so that final concentrations of the components were 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 200 nM each of dATP, dCTP, dGTP, and dTTP, 0.2 μM each of PNMT 5′ and 3′ complementary oligonucleotides.
and 3' primers (5'–CAGACTTCTTGGAGGTCAACCTG-3', 5'TTAT-
TATGGTCCACTTCGGGTG-3' respectively) or glyceraldehyde phos-
phate dehydrogenase (GAPDH; 5' and 3' primers (5'–ATGGTCGT-
GCTGAGTATGTCG-3', 5'–CATGTCAGATCCACAGGGATAC-3' re-
spectively), 0.1 μCi of [α-32P]dATP and 2 units of TaqDNA poly-
merase (Promega). After denaturation at 94°C for 2 min, primers
were annealed at 61°C for 1 min and primer extension was executed
at 72°C for 1 min. The denaturation, annealing, and extension cycle
was repeated 35 times for PNMT and 18 times for GAPDH cDNA.
DNA was isolated and PNMT and GAPDH amplification products
were combined and purified on 5% polyacrylamide gels for autora-
diography.  

Dephosphorylation of Nuclear Extract. Nuclear extracts were
prepared and adjusted to a concentration of 20 μg/μl as described
previously (Her et al., 1999). Dephosphorylation of the proteins (20
μg) was executed by exchanging nuclear extract to a buffer consist-
ing of 25 mM HEPES, pH 7.5, 34 mM KCl, and 50 mM MgCl2
containing protease inhibitors (complete, Mini, EDTA-free protease
inhibitor-cocktail; Roche, Mannheim, Germany) and treating with
1.0 unit of calf intestinal alkaline phosphatase and/or 0.1 units of
protein phosphatase-2A catalytic subunit (Promega) at 30°C for 30
to 60 min. The dephosphorylation reaction was terminated by addition
of a mixture of phosphatase inhibitors, NaF, sodium vanadate, po-
tassium pyrophosphate, and sodium phosphate to final concentra-
tions of 10 mM, 10 mM, 10 mM, and 5 mM, respectively. Control
nuclear extract was prepared by addition of the inhibitor mix to
nuclear extract in the absence of phosphatase treatment.  

Statistical Analysis. The statistical significance of the difference
between two groups was determined using Student’s t test. A p value
of ≤0.01 was considered statistically significant.

Results  
Sp1 and MAZ Binding to Consensus Elements in the
PNMT Promoter. Sp1 and MAZ, present in Neuro2A cell
nuclear extracts, were previously shown to bind to their
respective –48 bp and –38 bp overlapping (1 bp) consensus
sites in the rat PNMT promoter in a mutually exclusive
manner (Her et al., 1999). Gel mobility shift assays were
performed to confirm that both transcription proteins are
also present in nuclear extracts isolated from the rat adrenal
medulla-derived RS1 cells (Ebert et al., 1994) and similarly
interact with the Sp1/MAZ binding elements (Fig. 1). The
32P-labeled WTB probe (wild-type sequence) was used to
detect MAZ protein-DNA complex formation, whereas the
32P-labeled mut38 probe (mutated MAZ binding element)
was used to detect Sp1 protein-DNA complex formation (Fig.
1A) because the higher affinity of MAZ for its binding site
will preclude the binding of Sp1 to its consensus sequences in
the PNMT promoter (Her et al., 1999). As shown in Fig. 1B,
two major radiolabeled protein-DNA complexes were gener-
ated with the RS1 cell nuclear extracts and the WTB probe
(WSA and IgG lanes); the larger, slower complex was more
abundant. Inclusion of either anti-Egr-1 or anti-Sp1 antibody
did not affect either complex. However, anti-MAZ antibody,
although it apparently did not alter the electrophoretic mo-
bility of the complexes, seemed to disrupt or prevent protein-
DNA complex formation as shown by the marked reduction of
both radiolabeled bands. Thus, MAZ is expressed in the RS1
cell nuclei and binds to the wild-type PNMT promoter Sp1/
MAZ target sequences.

In contrast, when RS1 cell nuclear extracts were combined
with the radiolabeled mut38 probe containing a mutated
MAZ binding element, only a single protein-DNA complex
was produced (Fig. 1C). This complex was much less abun-
dant than either of the protein-DNA complexes formed with
the WTB probe. In addition, anti-Sp1 antibody supershifted
the complex, whereas the other antibodies had no effect
whatsoever. Hence, Sp1 is also part of the complement of
nuclear proteins in the RS1 cells and, as observed previously,
MAZ seems to preferentially bind to the Sp1/MAZ site with
whatsoever. Hence, Sp1 is also part of the complement of
nuclear proteins in the RS1 cells and, as observed previously,
MAZ seems to preferentially bind to the Sp1/MAZ site with
the exclusion of Sp1 because of its apparent greater relative
affinity for its consensus element.

To further characterize the binding of Sp1 and MAZ to the
overlapping Sp1/MAZ elements in the PNMT promoter, gel
mobility shift competition assays were executed (Fig. 2).
MAZ-WTB and Sp1-WTB protein-DNA complexes were gen-
erated by combining RS1 cell nuclear extract or human Sp1

Fig. 1. Sp1 and MAZ protein-DNA binding complexes generated with RS1
cell nuclear extracts. Gel mobility shift assays were performed as described
under Materials and Methods using ei-
ther a 21-bp wild-type oligonucleotide
(WTB) probe containing the overlap-
ping Sp1/MAZ consensus elements or a
21-bp MAZ mutant oligonucleotide
probe (mut38) end-labeled with [32P]
and nuclear extract isolated from RS1
cells. Antibodies were included to iden-
tify the proteins constituting the pro-
tein-DNA complexes. A, schematic of
the WTB and mut48 oligonucleotides.
B and C, autoradiograms from gel mo-
bility shift assays. Bovine serum albu-
min (BSA), IgG, anti-Egr-1 antibody,
anti-Sp1 antibody, or anti-MAZ anti-
body was included in the binding reac-
tions as designated.

A. WTB

B. Probe

C. mutant

Antibody

BSA

Mut38

Sp1

IgG

Egr-1

MAZ

Sp1/DNA complex

MAZ/DNA complex

WTB

mut38

5' GTCTGGGCGGGGAGGGGA 3'

5' GTCTGGGCGGGG1 I T AGGGGA 3'

Probe

Antibody

MTB

IgG

Egr-1

Sp1

MAZ

mat38

Probe

Antibody

MAZ/DNA complex

Sp1/DNA complex

Downloaded from molpharm.aspetjournals.org at ASPET Journals on October 29, 2017
protein (Promega) with radiolabeled WTB probe. Competitor DNA included oligonucleotides with point mutations incorporated into unique regions of each consensus element to selectively interfere with MAZ or Sp1 binding (Fig. 2A). Specifically, two guanine nucleotides were converted to thymines in the Sp1 site (positions −54 and −55 bp, mut48) whereas three guanine nucleotides were converted to thymines in the MAZ site (positions −47, −46, and −45 bp, mut38). An oligonucleotide harboring both mutations was also generated by simultaneously modifying the MAZ and Sp1 sites in the same way. Consistent with previous findings, the mut48 competitor oligonucleotide, containing an intact MAZ binding element and mutated Sp1 site, and the WTB competitor oligonucleotide, in which both Sp1 and MAZ binding sites were intact, effectively displaced the wild-type radiolabeled probe from the MAZ protein-WTB complex (Fig. 2B). In contrast, the MAZ mutant oligonucleotide (mut38) and the Sp1/MAZ double mutant oligonucleotide (mut48/38) were ineffective competitors for MAZ. Similarly, the mut38 (MAZ site mutated and Sp1 site intact) and WTB probes effectively competed with radiolabeled WTB for human Sp1 protein, whereas the mut48 and mut48/38 oligonucleotides (Sp1 site mutated and both Sp1 and MAZ sites mutated, respectively).

\[
\begin{align*}
\text{A.} & \\
\text{WTB} & \quad 5' \text{GTCGCGCGGAGAGAGGAA} 3' \\
\text{mut48} & \quad 5' \text{GTCGCGCGGAGAGAGGAA} 3' \\
\text{mut38} & \quad 5' \text{GTCGCGCGGAGAGAGGAA} 3' \\
\text{mut38/48} & \quad 5' \text{GTCGCGCGGAGAGAGGAA} 3'
\end{align*}
\]

**Fig. 2.** Effects of selective mutagenesis on Sp1 and MAZ protein-DNA binding complex formation. Gel mobility shift assays were performed using the radiolabeled 21-bp wild-type oligonucleotide probe (WTB) in the presence of selective Sp1 and/or MAZ competitor oligonucleotides. A, schematic of the competitor oligonucleotides. Mut48 and mut38 contain mutated Sp1 and MAZ sites, respectively, whereas mut48/38 harbors both the Sp1 and MAZ mutations. The lowercase letters denote the base substitutions within each of the mutant binding sites. B and C, autoradiograms from gel mobility shift assays using RS1 cell nuclear extracts in the presence of a 300 M excess of each of competitor DNA relative to the WTB probe. Lane 1, no competitor; lane 2, WTB; lane 3, mut48 (Sp1 mutant); lane 4, mut38 (MAZ mutant); and lane 5, mut48/38 (Sp1 and MAZ mutant) oligonucleotides.

were ineffective competitors. Thus, competition with oligonucleotides with mutations in either the Sp1 and/or MAZ binding elements predictably alters the ability of the transcription proteins to bind to their respective PNMT promoter consensus sequences.

**Competitive Binding of Sp1 and MAZ.** As described earlier, the proximal Sp1 binding element overlaps the MAZ binding element by 1 bp, with the Sp1 consensus sequences extending an additional 9 bp in the 5’ direction and the MAZ consensus sequences extending 10 bp in the 3’ direction (Fig. 3A). MAZ seems to bind selectively to its consensus element when both MAZ and Sp1 are present, although binding depends on the relative abundance and relative affinity of each factor for its site. However, when the MAZ site is mutated, Sp1 readily binds to its target sequences (Fig. 1).

To further investigate the competitive binding of MAZ and Sp1 to their overlapping binding elements, gel mobility shift assays were performed using in vitro-translated Sp1 and MAZ proteins. Radiolabeled wild-type WTB probe was combined with increasing concentrations of MAZ in the presence of a fixed concentration of Sp1 or vice versa under conditions in which the fixed protein was fully bound and probe in slight excess (Fig. 3B). Both MAZ and Sp1 formed a single, distinct protein-DNA complex with WTB. Because the concentration of MAZ was increased relative to Sp1, the amount of MAZ-WTB complex correspondingly rose, whereas the amount of Sp1-WTB complex diminished. Conversely, as the concentration of Sp1 was increased relative to MAZ, the amount of Sp1-WTB complex rose and the amount of MAZ-WTB complex declined. However, MAZ clearly has higher binding affinity for its target sequences because it fully displaces Sp1 binding at a lower concentration than is required for Sp1 displacement of MAZ. This difference in binding affinity is further underscored by the fact that, for the results shown, the concentration of Sp1 protein-DNA complex in the absence of MAZ is three times that of MAZ protein-DNA complex in the absence of Sp1 based on the relative intensities of each complex formed with the same amount of radiolabeled WTB.
probe and equivalent amounts of MAZ or Sp1 protein. Thus, about one third the amount of MAZ protein, by comparison with Sp1, seems to be required to displace Sp1 bound to the Sp1/MAZ sites. Finally, binding site occupancy of Sp1 and MAZ at their respective elements seems to be mutually exclusive, because only Sp1 or MAZ binding complexes are apparent, but no higher molecular weight protein-DNA complex, as would be expected if both Sp1 and MAZ were able to simultaneously bind to their target sequences.

Functional Effects of Sp1 and MAZ Binding to the PNMT Promoter. To assess the effects of MAZ and Sp1 on PNMT promoter activity, RS1 cells were cotransfected with PNMT promoter-luciferase reporter gene constructs of different lengths (pGL3RP60, pGL3RP392, pGL3RP893) and varying amounts of MAZ or Sp1 expression construct (pGCNMAZ and pGCNSp1, 0–0.7 μg). For all PNMT-reporter gene constructs, PNMT promoter-driven luciferase expression increased relative to the amount of MAZ or Sp1 expression plasmid included during transfection, with maximum promoter induction occurring at the highest concentration (Fig. 4). Induction was well beyond the low basal levels of PNMT promoter-driven luciferase expression caused by endogenous MAZ and Sp1 in the RS1 cells, and no significant difference was observed in basal luciferase activity for the different constructs (data not shown). If the amount of MAZ or Sp1 expression plasmid was increased beyond 0.7 μg, the magnitude of PNMT promoter-driven luciferase induction did not increase further. Rather, at higher expression plasmid concentrations (≥1.0 μg), luciferase activity declined, probably because of plasmid DNA exceeding optimum concentrations for transfection and reducing transfection efficiency (data not shown). Lastly, the magnitude of promoter induction seems to be related to promoter length, as is most evident with the longest construct containing 893 bp of upstream DNA sequence (pGL3RP893). For this construct, MAZ induction ranged from 2.6 to 35.3, whereas Sp1 induction ranged from 4.4 to 121.8. Sp1 is apparently the stronger of the two activators, inducing a 1.7- to 3.5-fold greater rise in PNMT promoter activity than MAZ.

Competitive Transactivation of the PNMT Promoter by MAZ and Sp1. To further evaluate the competitive effects between MAZ and Sp1 binding on PNMT promoter-driven transcriptional activity, transient cotransfection assays with the PNMT promoter-luciferase reporter gene constructs, pGL3RP60 or pGL3RP893, and MAZ and Sp1 expression constructs were performed in the RS1 cells. pGL3RP60 contains only the overlapping Sp1/MAZ binding elements at −48/−38 bp, whereas pGL3RP893 contains both the overlapping elements and an additional Sp1 site at −168 bp (Ebert and Wong, 1995). pGCNMAZ was transfected at a fixed concentration of 0.5 μg, whereas the concentration of pGCNSp1 was varied between 0 and 0.7 μg or vice versa. For both PNMT promoter-luciferase constructs, increasing the amount of pGCNSp1 relative to a fixed amount of pGCNMAZ stimulated a concentration-dependent rise in PNMT promoter-driven luciferase expression (Fig. 5). Maximum promoter induction occurred with 0.5 μg of pGCNSp1. A similar stimulation of PNMT promoter-driven luciferase expression occurred when the transfection concentration of pGCNMAZ was increased relative to a fixed amount of pGCNSp1 but less MAZ was required to maximally induce PNMT promoter activity (0.3 μg). Moreover, the pattern of response was identical when the concentration of fixed expression construct, whether pGCNMAZ or pGCNSp1, was reduced to 0.4 μg, with 0.5 and 0.3 μg of variable expression construct (Sp1 and MAZ, respectively) providing maximum PNMT promoter stimulation (data not shown).

As observed previously, PNMT promoter induction by Sp1 alone when paired with pGL3RP60 or pGL3RP893 was 2.0-fold higher than induction when the same amount of MAZ expression construct was cotransfected into the RS1 cells. However, the maximum magnitude of PNMT promoter stimulation, although not markedly different when Sp1 was titrated against fixed MAZ or vice versa, was 3.0-fold higher for the longer PNMT promoter-luciferase reporter gene construct pGL3RP893. Thus, higher concentrations of Sp1 plasmid are required to displace the effects of MAZ plasmid whereas lower concentrations of MAZ plasmid are required to displace the effects of Sp1 plasmid, consistent with MAZ having higher affinity for its binding element than Sp1 for its respective binding element.

PNMT Promoter Induction after MAZ and Sp1 Binding Domain Inactivation. To evaluate the effects of inactivation of the Sp1 and/or MAZ binding elements on PNMT promoter activity, site-directed mutations were incorporated into the −48 and/or −38 bp Sp1 and MAZ sites in the PNMT promoter-luciferase reporter gene construct pGL3RP60 as described earlier, and reporter gene expression was evaluated in transient transfection assays using RS1 cells and Sp1 and MAZ expression constructs (0.7 μg). The shorter 60-bp PNMT promoter (Her et al., 1999) harboring the MAZ and Sp1 binding elements was used to assess these effects to
focus on the downstream contributions of MAZ and Sp1 in the absence of potential interactions with other bound transcriptional regulators of the PNMT promoter, including Sp1 bound to the upstream Sp1 site (−168 bp). The concentration of expression construct was selected based on earlier results showing that this amount of either expression construct provided a robust activation of the PNMT promoter mediated through their target sequences in each construct. When the Sp1 site (−48 bp) was mutated, PNMT promoter-driven luciferase expression was attenuated 2-fold ($p \leq 0.001$) compared with the wild-type control construct (Fig. 6A). Mutation of the MAZ site (−38 bp) also attenuated Sp1 activation of the PNMT promoter even though the Sp1 site was intact. However, the reduction in promoter activation was less than occurred when the Sp1 element itself was mutated. When both Sp1 and MAZ sites were inactivated, Sp1 activation of the PNMT promoter was still not eliminated but the extent of activation was less than observed with mutation of either the Sp1 or MAZ elements. As seen previously, MAZ activation of PNMT promoter-driven luciferase activity was markedly lower than Sp1-mediated induction (Fig. 6B). Mutation of the MAZ element also attenuated MAZ activation (30%). However, mutation of the Sp1 site or both MAZ and Sp1 elements decreased MAZ activation of the PNMT promoter to the same extent.

These results suggest that Sp1 and MAZ may contribute to basal PNMT promoter expression in the RS1 cells, with Sp1 exerting stronger influences on promoter activity. However, despite gel mobility shift assays showing that the factors are unable to bind to their respective mutated consensus sites,
each still produces attenuated but significant activation of the PNMT promoter.

**Sp1 and MAZ Phosphorylation and PNMT Promoter Binding Activity.** It has previously been demonstrated that Sp1 must be phosphorylated to bind to consensus sites within a gene promoter to activate that promoter (Kadonaga and Tjian, 1987). To determine whether phosphorylation may in part contribute to the different affinities of Sp1 and MAZ for their respective binding sites, gel mobility shift assays were executed using RS1 cell nuclear extracts in which proteins had been dephosphorylated by treatment with calf intestinal alkaline phosphatase (CIP) and/or the protein phosphatase-2A catalytic subunit (PP2A). MAZ- and Sp1-DNA complexes were generated by combining untreated or phosphatase-treated RS1 cell nuclear extracts with the radiolabeled WTB and mut38 probes, respectively (Fig. 7). A single MAZ protein-DNA band was generated by combining radiolabeled double-stranded WTB probe with untreated nuclear extract, and two Sp1 protein-DNA bands were formed by combining radiolabeled duplex mut38 probe with untreated nuclear extract. Both CIP AND PP2A, alone or in combination, prevented MAZ-DNA complex formation; only PP2A prevented formation of the two Sp1 complexes. To date, antibodies selectively recognizing phosphorylated versus unphosphorylated MAZ and Sp1 protein are not available. However, when equal amounts of untreated or phosphatase-treated protein from the cell extracts were subjected to Western analysis with the anti-MAZ or anti-Sp1 antibody, equal amounts of phosphorylated versus dephosphorylated MAZ or Sp1 protein were detected (data not shown).

Thus, phosphorylation of both MAZ and Sp1 seems to be essential for binding to their respective recognition sites. Furthermore, MAZ and Sp1 protein expressed endogenously in the RS1 cells is phosphorylated and, in the case of Sp1, two phosphoproteins exist that contain different amounts of phosphate modifications, as reported previously (Kadonaga and Tjian, 1987).

**Endogenous PNMT mRNA.** To determine whether MAZ and Sp1 are important regulators of endogenous PNMT gene expression in addition to activating the PNMT promoter, RS1 cells were transfected with the MAZ and Sp1 expression constructs, total RNA was isolated, and PNMT mRNA was quantified by RT-PCR. As shown in Fig. 8, control RS1 cells predominantly express a long form of PNMT mRNA, corresponding to intron-retaining PNMT mRNA described by Unsworth et al. (1999). When intracellular levels of MAZ were increased, total PNMT mRNA rose markedly, but both intron-retaining and intronless PNMT mRNAs were produced; the amount of intron-retaining mRNA was ~2-fold greater. When intracellular levels of Sp1 were increased, total PNMT mRNA was elevated even more, but nearly all of the messenger RNA was the intronless form. By comparison, dexamethasone, which had previously been shown to markedly induce PNMT mRNA (Ebert et al., 1994), increased PNMT message to the greatest extent, so that primarily intronless mRNA was produced.

**Discussion**

The present study extends our previous findings demonstrating that the proximal Egr-1 consensus element in the rat phenylethanolamine N-methyltransferase promoter with its 3’ extent fixed at −38 bp upstream of the site of transcription initiation functions as a MAZ binding element (Her et al., 1999). MAZ (c-myc–associated zinc finger protein) is a transcription factor important for c-myc–mediated tumorigenesis. As suggested by its name, this protein regulates c-myc gene expression by providing both stimulatory (Komatsu et al., 1997) and inhibitory controls (Izzo et al., 1999). In most cases, however, the family of MAZ proteins (Logan et al., 1993; Sakatsume et al., 1996) activates gene promoters, including the SCL/TAL-1 promoter (Bockamp et al., 1995), the serotonin 1a receptor promoter (Parks and Shenk, 1996), the adenovirus major late promoter (Parks and Shenk, 1997), and the CD4 promoter (Duncan et al., 1995). The present studies show that MAZ also activates the rat PNMT gene promoter. However, in this promoter, the MAZ consensus element overlaps an Sp1 consensus element, and the 1-bp overlap is sufficient to mutually exclude the simultaneous binding of these two transcription factors. MAZ seems to bind...
preferentially because of higher affinity for its binding element than Sp1 for its recognition site. The consequence of this preferential binding may be restriction of PNMT promoter transactivation, because Sp1 more strongly induces the promoter. Finally, MAZ and Sp1 must be phosphorylated to bind and transactivate, and although both factors stimulate endogenous PNMT mRNA expression, Sp1 transactivation produces a greater amount of intronless mRNA template for translation of functional protein.

Consistent with these observations, introduction of point mutations into the MAZ binding element permitted Sp1-DNA complex formation but prevented MAZ-DNA complex formation when the MAZ mutant duplex oligonucleotide was combined with RS1 cell nuclear extracts (data not shown). Gel mobility shift competition assays confirmed the latter (Fig. 1). Similarly, site-directed mutation of the Sp1 consensus site permitted selective binding of MAZ but precluded Sp1 binding. However, site-directed mutation of the MAZ and/or Sp1 sites in the minimal promoter reporter gene construct pGL3RP60 attenuated but did not eliminate MAZ and/or Sp1-mediated PNMT promoter transcriptional activity, as would be expected when these proteins could no longer interact with their binding elements. In addition, inactivation of the MAZ consensus site attenuated Sp1 activation instead of increasing it, as would be expected if MAZ binding were prevented and Sp1 binding thereby facilitated. Similarly, MAZ activation of the PNMT promoter declined when the Sp1 site was mutated. Finally, mutation of both MAZ and Sp1 elements reduced, but did not eliminate either Sp1 or MAZ activation of the promoter. Together, these findings suggest that Sp1 and MAZ may participate in direct protein-protein interaction with the transcription initiation complex or other binding transcription factors, possibilities that are currently being investigated. Furthermore, for the single mutations, we cannot exclude the possibility that the point mutations may also affect DNA folding to facilitate interaction of MAZ or Sp1 (bound to their respective intact sites) with the transcription initiation complex.

Post-translational modification often affects the interaction of a transcription protein with its DNA binding element. In the case of Sp1, phosphorylation has been reported to increase its affinity for its consensus element, thereby leading to gene activation, and 105- and 95-kDa phosphoproteins have been identified (Kadonga and Tjian, 1987). The present results show that for the PNMT promoter, both MAZ and Sp1 must be phosphorylated to interact with their respective binding elements and stimulate promoter activity.

The selective binding of MAZ and Sp1 to their consensus sites within the PNMT promoter may have important biological consequences for PNMT gene expression. The RS1 cells (Ebert et al., 1994) used in the studies described here were derived from the PC-12 cell line, a progenitor cell line established from an adrenal medullary tumor (Greene and Tischler, 1976). Despite the fact that 85 to 90% of adrenal chromaffin cells exhibit the adrenergic phenotype, PC-12 cells seem to have lost their ability to express PNMT, presumably because of dedifferentiation during tumorigenesis. Glucocorticoids, acting through the glucocorticoid receptor, were previously thought to initiate PNMT expression and, hence, adrenergic differentiation (Bohn et al., 1981; Teitelman et al., 1982). However, other factors have been shown to be important, perhaps acting in conjunction with the glucocorticoid receptor (Ebert et al., 1994; Ebert and Wong, 1995; Her et al., 1999; Wong et al., 1998). MAZ and Sp1, given their mutually exclusive binding to regulatory sites in the proximal 60 bp of PNMT promoter, their relative abundance in the RS1 cells, and the role of MAZ in c-myc mediated oncogenesis, may contribute to the dedifferentiation and malignant transformation of adrenal medullary tissue to pheochromocytomas, the type of tumor from which the PC-12 cells were established. Although both MAZ and Sp1 activate the PNMT promoter to increase reporter gene and endogenous PNMT gene expression, we show that in the case of the endogenous PNMT gene, MAZ activation preferentially stimulates the production of an intron-retaining form of PNMT mRNA. From the latter, a truncated, enzymatically inactive PNMT enzyme is produced (Unsworth et al., 1999; Ziegler et al., 2002). In contrast, Sp1 preferentially stimulates the production of spliced, intronless PNMT mRNA from which active enzyme can be generated. Thus, although both MAZ and Sp1 stimulate the PNMT promoter to induce gene transcription, post-transcriptional controls restrict the production of functional protein through selective RNA processing.

It has previously been suggested that glucocorticoids might be the critical regulators of PNMT mRNA processing (Unsworth et al., 1999). The present findings are the first to demonstrate that differential PNMT RNA splicing is associated with other transcriptional activators of the PNMT gene as well and further that RNA processing may be an important regulatory mechanism for PNMT gene expression. Although intron retention has been described as a regulatory mechanism for viruses (Kienzle et al., 1999; Butsch and Boris-Lawrie, 2000; Poon et al., 2002), plants (Magaraggia et al., 1997), and fruit flies (Mattox et al., 1996; Gebauer et al., 1998), it was believed to occur infrequently in vertebrates. However, intron retention has now been reported for a variety of genes in higher organisms. Alternative splicing of a variant of bovine growth hormone pre-mRNA, for example, generates an mRNA possessing an intact upstream intron, which, like appropriately spliced mRNA, is translated to the cytosol (Dirksen et al., 1994). The murine periaxin gene also produces an intron retaining mRNA. Consequently, a premature stop codon is introduced, leading to translation of a truncated protein that is diffusely distributed throughout the cytoplasm of the Schwann cell, whereas the protein produced from intronless mRNA is restricted to the plasma membrane (Dytrych et al., 1998). In rats, several intron-retaining mRNA variants have been described as well that are translated into protein isoforms, such as that for the sodium channel 61A subunit (Kazen-Gillespie et al., 2000) and the histamine H3 receptor (Morisset et al., 2001). Intron-retaining species of mRNAs have been described for humans, too. In the case of human Rpp21, a protein in the ribonucleoprotein ribonuclease P complex, protein variants that arise from intron retention or alternative splicing of the primary transcript affect the assembly of RNase P and thereby its function (Jarrous et al., 2001). Intron retention in the processing of the RNA transcript for the membrane cofactor protein CD46 also results in a truncated protein with altered sequestration to the endoplasmic reticulum. Thus, intron retention seems to be an important post-transcriptional mechanism critical to the processing of a number of genes, including PNMT.

In summary, MAZ and Sp1 are both transcriptional regu-
lators of the PNMT gene that control its expression through differences in affinity for their respective binding elements within the PNMT gene promoter and differences in their intracellular abundance. They also exert important regulatory controls through their effects on PNMT transcript processing to either intron-retaining or intronless forms, thereby governing the production of functional enzyme via a post-transcriptional regulatory mechanism that may prove to be an important mode of regulation for controlling gene expression. Current investigations focus on identifying the role these two factors may play in the post-transcriptional regulation of PNMT.

References


Bohn MC, Goldstein M, and Black IB (1981) Role of glucocorticoids in the adrenergic modulation of PNMT.


Zhu S, Saltz S, Hunter JPB, Gashler A, Sukhatme V, and Wong DL (1994) Egr-1 sequence enhances alternative splicing of bovine growth hormone pre-mRNA.

