Sequences Required for Induction of Neurotensin Receptor Gene Expression during Neuronal Differentiation of N1E-115 Neuroblastoma Cells*

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The promoter region of the mouse high affinity neurotensin receptor (Ntr-1) gene was characterized, and sequences required for expression in neuroblastoma cell lines that express high affinity NT-binding sites were characterized. Me2SO-induced neuronal differentiation of N1E-115 neuroblastoma cells increased both the expression of the endogenous Ntr-1 gene and reporter genes driven by NTR-1 promoter sequences by 3–4-fold. Deletion analysis revealed that an 83 base pair promoter region containing the transcriptional start site is required for Me2SO activation. Detailed mutational analysis of this region revealed that a CACCC box and the central region of a large GC-rich palindrome are the crucial cis-regulatory elements required for Me2SO induction. The CACCC box is bound by at least one factor that is induced upon Me2SO treatment of N1E-115 cells. The Me2SO effect was found to be both selective and cell type-restricted. Basal expression in the neuroblastoma cell lines required a distinct set of sequences, including an Sp1-like sequence, and a sequence resembling an NGFI-A-binding site; however, a more distal 5′ sequence was found to repress basal activity in N1E-115 cells. These results provide evidence that Ntr-1 gene regulation involves both positive and negative regulatory elements located in the 5′-flanking region and that Ntr-1 gene activation involves the coordinate activation or induction of several factors, including a CACCC box binding complex.

Neurotensin (NT)1 is a 13-amino acid peptide (1) that is expressed in a complex pattern in the limbic regions of the brain and in the gastrointestinal tract (2). NT and the related peptide neuromedin N are generated from a common precursor protein and are thought to have overlapping signaling functions (3, 4). There is considerable anatomical and functional evidence indicating that NT functions as a neuromodulator in the dopamine (DA) pathways in the central nervous system. The majority of midbrain DA neurons express the cloned high affinity NT receptor (NTR-1), and there is evidence that at least some DA neurons are directly contacted by NT-positive axons (5, 6). NT appears to excite midbrain DA neurons both directly (7, 8) and through inhibition of DA D2 autoreceptors (9) resulting in locomotor activation (10, 11). However, NT also attenuates amphetamine locomotor activation after intracerebroventricular administration or after direct application in the ventral striatum possibly through the inhibition of postsynaptic D2 signaling (12–14). These results suggest that the expression of NTR-1 in midbrain DA neurons is important for appropriate regulation of DA-mediated behaviors.

NTR-1 was cloned using an expression assay in frog oocytes, and sequence analysis revealed that it is a member of the G protein-coupled receptor superfamily (15). A lower affinity levo-cocaine-sensitive receptor (NTR-2) was subsequently cloned by low stringency hybridization using an NTR-1 cDNA probe (16, 17). Recent evidence suggests that NT does not stimulate signaling through NTR-2, suggesting that NT acts mainly through NTR-1 (18). NTR-1 is expressed at high levels in midbrain DA neurons, and dopamine regulates NTR expression in corticobasal structures in the rat brain (19–21). The ability of DA to modulate NTR expression suggests that changes in DA signaling result in plastic changes in NT signaling. This hypothesis is further supported by the observations that the indirect DA agonists cocaine and methamphetamine and D2 antagonists stimulate NT gene expression in the dorsal and ventral striatum (22, 23). Long term NTR-1 blockade and continuous infusion of NT also result in alterations in NTR-1 expression (24, 25). These results collectively indicate that DA, NT, and perhaps other signals can result in plastic changes in NTR-1 expression; however, the underlying mechanisms controlling NTR-1 expression are poorly understood.

Tissue culture cell lines that express NTR-1 in a regulated or constitutive manner potentially provide model systems for understanding the mechanisms controlling Ntr-1 gene expression. The N1E-115 neuroblastoma cell line was isolated as part of a screen for catecholamine-producing neuronal cell lines from a mouse brain neuroblastoma (26), and Me2SO-induced neuronal differentiation of these cells (27) is accompanied by increased expression of high affinity NT-binding sites (28). The transition of these cells to a post-mitotic stationary phase also results in the induction of NTR expression (29). A neuroblastoma × glioma cell line has also been described that expresses high constitutive levels of high affinity NT-binding sites (30). The N1E-115 cells are a particularly attractive model to probe Ntr-1 gene regulation since the Ntr-1 gene is expressed in catecholaminergic neurons in vivo (31). They should also be useful for identifying signaling mechanisms that activate the Ntr-1 gene during neuronal differentiation (28).

To investigate the pathways controlling Ntr-1 gene expression in these and other cell types, we have cloned the mouse…

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1 The abbreviations used are: NT, neurotensin; RT-PCR, reverse transcriptase-polymerase chain reaction; PCR, polymerase chain reaction; kb, kilobase pair; bp, base pair; DA, dopamine; MEF, mouse embryonic fibroblast; PIPES, 1,4-piperazinediethanesulfonic acid; TRE, 12-O-tetradecanoylphorbol-13-acetate-response element; CRE, cAMP-response element.

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Ntr-1 gene and sequenced the promoter region. Detailed mutational analysis of the Ntr-1 promoter has revealed sequence elements that are crucial for Me\(_{2}\)SO induction and basal expression in N1E-115 cells that are conserved in the rat and human promoters. A CACCC sequence appears to be the most critical sequence element for Me\(_{2}\)SO responsiveness, and gel shift and DNase I footprinting experiments indicate that a Me\(_{2}\)SO-inducible complex binds to this site. Several sequence elements contribute to basal expression, including an Sp1-related site and a sequence that is similar to the initiator element first identified in the terminal deoxynucleotidyltransferase gene (32). We also present evidence that a transcriptional silencer controls the activity of this positive regulatory region. These results provide evidence that the Ntr-1 gene is transcriptionally activated during Me\(_{2}\)SO-induced neuronal differentiation of N1E-115 cells most likely through a mechanism involving the induction of a complex that binds to a sequence that includes a CACCC motif.

**EXPERIMENTAL PROCEDURES**

**Isolation of Mouse Ntr-1 Genomic Clones**—A mouse genomic library was constructed by ligating partially digested molecular weight DNA isolated from 2 embryonic stem cells (15–29-kb fragments) BamH1-digested λEMBL4 using standard methods (33). The library was screened using a 32P-labeled Smal fragment of the rat NTR-1 cDNA (15) that contains the 5‘ 1.3 kb of the cloned sequences and standard filter lift procedures (33). Positives were plaque-purified and a cDNA (15) that contains the 5‘ end of the cloned sequences was sequenced using the chain terminator method. A mouse genomic library (32). We also present evidence that a transcrip-

**Cell Culture**—N1E-115 cells were passaged in Dulbecco’s modified Eagle’s medium containing 4.5 g of glucose/liter and supplemented with 10% fetal bovine serum (Sigma) and 2 mM L-glutamine. NG108 cells were passaged in the medium described above except for 1.0 g of glucose/liter and supplemented with 10% fetal bovine serum and 2 mM L-glutamine.

**Transfections**—N1E-115 and MEF cells were subcultured from confluent dishes by diluting the cells 1:4 with fresh medium 3 days prior to transfection. All lines were subcultured the day before transfection at a density that resulted in 1 × 106 cells per 10-cm dish at the time of transfection. CaCl2-purified plasmid DNA was transfected by calcium phosphate precipitation using 10-cm dishes. Briefly, for each reporter plasmid, two 10-cm dishes were fed with 8 ml of fresh medium just prior to transfection, and 25 μg of plasmid DNA as a calcium phosphate precipitate was added to each dish. DNA precipitates were prepared by diluting 50 μg of plasmid DNA (luciferase reporter and pPGKgel standardization plasmids) into 700 μl of NTE (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA), 100 μl of 2 mM CaCl2, and 800 μl of transfection buffer (50 mM HEPES, pH 7.1, 180 mM NaCl, 2 mM NaPO4), and the mixtures were incubated at room temperature for 20–30 min. The calcium phosphate precipitate (.800 μl) was added dropwise to the cells, and after 16 h the medium was replaced, and the cells from one 10-cm dish were resuspended by trituration and plated into two 60-mm dishes. The cells were allowed to adhere to the dish for 1 h; 1.5% Me\(_{2}\)SO was added to one dish of each pair, and the cells were incubated for an additional 72 h. Cells were incubated for 72 h, and cell extracts were prepared after washing the cells with ice-cold phosphate-buffered saline by lysis in buffer containing 1% Triton X-100 as described previously (34). Luciferase and β-galactosidase activities were determined as described previously (35).

**Luciferase Reporter Constructs**—The initial NTR luciferase reporter construct was created by ligating partially digested molecular weight DNA isolated from 2 embryonic stem cells (15–29-kb fragments) BamH1-digested λEMBL4 using standard methods (33). The library was screened using a 32P-labeled Smal fragment of the rat NTR-1 cDNA (15) that contains the 5‘ end of the cloned sequences and standard filter lift procedures (33). Positives were plaque-purified and a cDNA (15) that contains the 5‘ end of the cloned sequences was sequenced using the chain terminator method. A mouse genomic library (32). We also present evidence that a transcrip-

**RT-PCR**—Mouse brain poly(A)+ RNA was treated with DNase I to remove trace amounts of contaminating genomic DNA and RT-PCR reactions using avian myeloblastosis virus reverse transcriptase (Promega) and Taq polymerase (Roche Molecular Biochemicals) using the conditions specified by the manufacturer, except that RT reactions were performed at 50 °C and betaine was added to the PCR reactions. The reverse transcription reaction was initiated after heat inactivation of the DNase I (70 °C for 5 min) and addition of 5 μl of a gene-specific primer (PEX-411c 5’-TGGCGCAGAGAGGAGCGCACGCAG- GCT GCCGACG-3’) complementary to nucleotides −411 to −376 of the mouse Ntr-1 gene and 25 units of avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals). PCR reactions were performed using either mouse brain cDNA or embryonic stem cell genomic DNA templates mixed with 10 pmol of the PEX-411c primer and 10 pmol of one of three different 5‘ primers (−513, 5’-GTGGAAGCGCA-GAGGCGCACCCACGCTGCCGACG-3’; −548, 5’-TTTTGGATCCACTGTGGGCGCCCC-3’; −566, 5’-CTCAACACCCACCCCTGCTAAG-3’), the numbers correspond to the position of the 5‘ end of the oligonucleotide primer (19). PCR reactions were performed at 2.5 mM MgCl2 (Sigma) due to the high GC content of the promoter region, and all amplification cycles (94 °C, 15 s; 62 °C, 1 min; 72 °C, 1 min; the extension time was increased to 4 min on the last cycle) were performed after an initial 1-min denaturation step at 94 °C (41).

**DNase I Footprinting and Methylation Interference Assays**—32P-End-labeled probes spanning the promoter region were digested by digested with BamHI and SacI and cloned into the SacI 5‘ deletion construct described above digested with BamHI and SacI. This minimal promoter fragment was initially selected based on the positions of the transcriptional start sites that had been determined in rat and human (37, 38); however, the start point in the mouse actually lies 5‘ to the TATA box, in agreement with the transcription start sites created using the PCR overlap extension protocol (39). Brieﬂy, two overlapping PCR fragments were generated using either a 5‘ primer that ends at the promoter Smal site and introduces a BamHI site and a 3‘ primer containing the clustered point mutations flanked by 10 and 6 nucleotide stretches of complementary sequence on the 3‘ and 5‘ ends of the fragment (36) were digested with PstI and used to amplify the closest clone(s). PCR reactions were performed using the outer 5‘ and 3‘ primers described above. The resulting fragment was digested with BamHI and SacI, gel-isolated, and ligated into the SacI deletion clone in pXP-2 described above digested above digested with BamHI and SacI. The promoter regions of the mutant constructs were completely sequenced to verify that only the intended changes had been introduced.

**RNase Protection Assay**—Riboprobes were synthesized using either mouse Ntr-1 gene promoter fragments or a fragment of the rat NTR-1 cDNA subcloned into pGEM4 (Promega Biotech). The mouse Ntr-1 gene was digested with SpeI or SmaI and subcloned into either 293 or Sf9 insect cells (SacI92) derived from a 2.4-kb PstI fragment (colloidal gold 497-bp EcoRI/BamHI fragment (B/E 497, derived from a 1.2-kb BamHI subclone) into either SacI- or BamHI- and EcoRI-digested pGEM4. A 300-bp PstI fragment derived from pBSNTR2–2 (15) was subcloned into pGEM4 digested with PstI and treated with calf intestinal phosphatase to generate a probe for the quantitation of NTR-1 mRNA (PstI 300). To synthesize 32P-labeled riboprobes, plasmids were linearized with either EcoRI (B/E 497) or HindIII (SacI 293 and PstI 300) and transcribed with either T7 (B/E 497) or SP6 (SacI 293, PstI 300) RNA polymerase as described (40).

RNase protection assays were performed as described (40). Briefly, 10 μg of total RNA was mixed with 5 × 105 cpm of 32P-labeled riboprobe, dried, and dissolved in 30 μl of hybridization buffer (80% formamide, 40 mM PIPES, pH 6.7, 0.4 NaCl, 1 mM EDTA). Reactions were denatured by heating to 95 °C for 5 min and hybridized overnight (−16 h) at 45 °C. Following hybridization, 300 μl of RNase digestion buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 300 μM NaCl, 20 μg/ml nuclease P1, 2 μg RNase T1 was added, and the reactions were incubated at room temperature for 1 h. Reactions were terminated by the addition of 20 μl of 10 M HCl, and RNA was precipitated by 150 mM NaCl, 5 μl of NTR-1 promoter). PCR reactions were performed at 50 °C and betaine was added to the PCR reactions. The reverse transcription reaction was initiated after heat inactivation of the DNase I (70 °C for 5 min) and addition of 5 μl of a gene-specific primer (PEX-411c 5’-TGGCGCAGAGAGGAGCGCACGCAG- GCT GCCGACG-3’) complementary to nucleotides −411 to −376 of the mouse Ntr-1 gene and 25 units of avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals). PCR reactions were performed using either mouse brain cDNA or embryonic stem cell genomic DNA templates mixed with 10 pmol of the PEX-411c primer and 10 pmol of one of three different 5‘ primers (−513, 5’-GTGGAAGCGCAGGAGGCGCACCCACGCTGCCGACG-3’; −548, 5’-TTTTGGATCCACTGTGGGCGCCCC-3’; −566, 5’-CTCAACACCCACCCCTGCTAAG-3’), the numbers correspond to the position of the 5‘ end of the oligonucleotide primer (19). PCR reactions were performed at 2.5 mM MgCl2 (Sigma) due to the high GC content of the promoter region, and all amplification cycles (94 °C, 15 s; 62 °C, 1 min; 72 °C, 1 min; the extension time was increased to 4 min on the last cycle) were performed after an initial 1-min denaturation step at 94 °C (41).
tion of a 1.2-kb BamHI fragment subcloned into pGEM4 with either EcoRI or BamHI followed by Klenow fill-in with [32P]dATP. The plasmids were subsequently digested with either BamHI or EcoRI, respectively; the resulting fragments were separated on a 1% agarose gel, and the 496-bp labeled EcoRI/BamHI fragments labeled at either the EcoRI or BamHI sites were recovered by centrifugation through a Gen Elute column (Supelco) and ethanol precipitation. Nuclear extracts were prepared from N1E-115 cells that had either been treated with 1.5% Me2SO for 48 h or grown under control conditions as described previously (42) with minor modifications (43) using approximately 20 dishes of cells grown to confluency for each preparation. Protein concent-
centrations were determined using the Bradford method. DNase I foot-
print reactions were performed as described (35) by mixing 20,000 cpm
of DNase I (DNase I, pure, ribonuclease-free, Worthington). Reactions
were also performed without the addition of nuclear extract to identify
protected regions. The DNase I-treated reactions were phenol/chloro-
form-extracted, and recovered by ethanol precipitation. The DNA
autoradiography, and the region of the gel containing complexes 1 and
phoresis on a non-denaturing gel. The complexes were visualized by
DNA sequences Required for Ntr-1 Gene Activation

**RESULTS**

**Cloning and Characterization of the Mouse Ntr-1 Gene**—
Mouse NTR-1 clones were isolated by screening a genomic library constructed from D3 mouse embryonic stem cell DNA with a
[32P]-labeled 1.3-kb SacI 5′ fragment of NTR-1 cDNA (15), and two were further analyzed (c2 and 10-1) by restriction
mapping and Southern blotting. Exon one was localized to
an ~7-kb HindIII fragment that contained sequences extend-
ing ~2.0 kb 5′ to the initiator methionine codon. A portion of
the first exon and the 5′-flanking region were sequenced (Fig.
1A), and comparison with the corresponding regions of the rat
and human (38) NTR-1 genes revealed a region that is
highly conserved between all three species (Fig. 1B). This re-

**Cell Density and Me2SO Treatment Increase Ntr-1 Gene Ex-
pression in N1E-115 Cells**—N1E-115 cells plated at low density
are devoid of high affinity NT-binding sites; however, large increases in binding activity are observed when the cells are
grown to high density (29) or treated with Me2SO (28). To
examine whether these culture conditions result in increased
Ntr-1 gene expression, NTR-1 mRNA levels were quantitated
by nuclease protection assay using a [32P]-labeled antisense
riboprobe corresponding to nucleotides 296–632 of the rat
NTR-1 cDNA (PstI 300). N1E-115 cells were plated at low
density (5 × 10^5 cells/15-cm dish) and propagated without
A

FIG. 1. Sequence of the promoter region of the mouse Ntr-1 gene and comparison with other NTR-1 sequences and the dopamine D_2 receptor gene. A, a HindIII restriction fragment of the mouse Ntr-1 gene was subcloned, and the sequence of the 5' flanking region and a portion of exon one was determined on both strands using the chain terminator method. The positions of the transcriptional start site (\( +1 \)) and the 5' end of a mouse NTR-1 cDNA clone (*) are shown.

B

C

Sequences Required for Ntr-1 Gene Activation

\[ \text{Sequences Required for Ntr-1 Gene Activation} \]

\[ \text{Sequences Required for Ntr-1 Gene Activation} \]

\[ \text{Sequences Required for Ntr-1 Gene Activation} \]
subculture but with frequent medium changes in the first set of experiments as described (29). Cells were harvested from duplicate cultures at the indicated times; RNA was prepared, and NTR-1 mRNA levels were quantified using an RNase protection assay (Fig. 4A). Culturing the cells at low density resulted in an initial decline in Ntr-1 gene expression, followed by a gradual increase during growth to stationary phase. NTR-1 mRNA levels increased 20-fold compared with day 3 levels after 17 days of culture but declined precipitously thereafter as the cultures deteriorated (Fig. 4). To examine Ntr-1 gene expression following Me2SO treatment, cells were plated in duplicate in medium containing reduced serum and treated with 1.5% Me2SO for the indicated times (Fig. 4B). Me2SO treatment resulted in a 4-fold increase in Ntr-1 gene expression over the course of 72 h. These results indicate that increased Ntr-1 gene expression underlies the previously observed increases in NT-binding sites in these cells (28, 29).

**Promoter Sequences Required for Me2SO Induction in N1E-115 Neuroblastoma Cells**—To determine what promoter sequences are required for the response to Me2SO, a series of promoter deletion constructs in which up to 1.4 kb of 5' flanking sequences were fused to a luciferase reporter gene were...
transfected into N1E-115 cells, and the cells were either grown under control conditions or treated with 1.5% MeSO for 72 h. To control for variations in transfection efficiency, a PGK-βgal plasmid was co-transfected, and β-galactosidase activity was used to standardize luciferase activity. The initial series of constructs revealed two interesting features of the regulatory region. First, sequences upstream of −640 (we have used the numbering system previously used for the rat and human NTR-1 genes (37, 38) where +1 is the A of the initiator methionine codon) appear to suppress basal promoter activity (Fig. 5A). Second, promoter elements required for MeSO induction are located between a SmaI site at −640 and a SscI site at −448 (Fig. 5A). The −640 deletion construct is induced 3–5-fold upon MeSO treatment; however, deletion to the SscI site at −448 completely abolishes MeSO induction, and this construct is actually repressed after MeSO treatment (Fig. 5A). The effect of MeSO was selective, since the expression of a reporter gene controlled by the Rous sarcoma virus-long terminal repeat was not affected by MeSO treatment (data not shown). These experiments define an MeSO-responsive region between −640 and −448 and indicate that sequences upstream of −640 suppress basal promoter activity.

To determine more accurately the boundaries of the regulatory region, additional 5′ and 3′ deletion mutants were constructed and tested in N1E-115 cells (Fig. 5B). The response to MeSO was maintained through deletion to −589; however, deletion to −569 nearly abrogated the response. In contrast, basal activity decreased in a graded fashion as sequences were deleted from the 5′ end, except for a small increase when sequences between −589 and −569 were deleted, perhaps indicating that a weak repressor element is located in this region. Deletion in the 3′ direction from the SscI site at −448 to −526 had a severe impact on both basal activity and MeSO induction. Further 3′ deletions had no effect until removal of an Sp1-like sequence (compare constructs 3′-590 and 3′-603 that have deletion end points on either side of the Sp1-like site), which essentially eliminated expression, indicating that this Sp1-like site is important for basal expression. These results indicate that the MeSO regulatory region lies in the 83-bp region located between −507 and that the region between −640 and −507 is required for full basal expression. This regulatory region is highly conserved in the rat and human NTR-1 promoters (Fig. 1B), indicating that it is functionally important in vivo.

The mPAL was examined in more detail by evaluating the effects of clustered mutations along the length of the palindrome (Fig. 6A, mPal mut-1 to -6). Several mutations reduced basal activity; however, mPal mut-6 had the largest effect, reducing activity by ~70%. Removal of this sequence in the 3′-526 deletion construct greatly reduced both basal activity and MeSO induction (Fig. 5, A and B). Mutations near the 5′ end of the mPal all had similar effects, reducing basal activity by about half (Fig. 6A, mPal mut-1, -3, and -5). The transcriptional start site lies within the region affected by mPal mut-3. Deletion of the entire mPAL (Fig. 6A, dmPal) reduced basal activity and MeSO induction (Fig. 5B, A and B). Similar to the deletion experiments, the dmPal construct is induced 3–5-fold upon MeSO treatment, and this construct is actually repressed after MeSO treatment (dmPal). These experiments define an MeSO-responsive region between −640 and −448 and indicate that sequences upstream of −640 suppress basal promoter activity.

The transcriptional start site of the Ntr-1 gene is located in a CpG island. The sequence of the promoter region was analyzed in 100 nucleotide blocks for G/C content and the frequency of CpG dinucleotides. The ratio of CpG/GpC was calculated, and values of 0.6 in regions that had 50% G + C content were defined as meeting the criteria for CpG islands as described (43). A 500-bp region extending from approximately 140 bp upstream of the transcriptional start site to 350 bp into exon one has the characteristics of a CpG island. A graph of the ratio of CpG/GpC is shown, and the positions of the CpG and CpG dinucleotides are depicted schematically below along with a schematic of the mouse NTR-1 promoter region. The transcriptional start site is denoted by an arrow, and the coding region is depicted as a black box.

**Fig. 3.** The transcriptional start site of the Ntr-1 gene is located in a CpG island. The sequence of the promoter region was analyzed in 100 nucleotide blocks for G/C content and the frequency of CpG and CpG dinucleotides. The ratio of CpG/GpC was calculated, and values of 0.6 in regions that had 50% G + C content were defined as meeting the criteria for CpG islands as described (43). A 500-bp region extending from approximately 140 bp upstream of the transcriptional start site to 350 bp into exon one has the characteristics of a CpG island. A graph of the ratio of CpG/GpC is shown, and the positions of the CpG and CpG dinucleotides are depicted schematically below along with a schematic of the mouse NTR-1 promoter region. The transcriptional start site is denoted by an arrow, and the coding region is depicted as a black box.

**Fig. 4.** Cell density and MeSO induce Ntr-1 gene expression in N1E-115 cells. A, cell density experiments were performed in duplicate by plating N1E-115 cells at low density and incubation without further subculturing but with frequent medium changes as described under “Experimental Procedures.” Cells were harvested at the indicated times for RNA preparation. Total RNA isolated from each duplicate culture dish (10 μg) was hybridized with 32P-labeled Pst I 300 riboprobe and nuclease-treated, and the protected products were analyzed on sequencing gels. The relative levels of NTR-1 mRNA were quantitated using a PhosphorImager, and autoradiographs of the gels are shown below the graphs. The two lanes under each time point represent the protection products obtained with RNA preparations from each of the duplicate culture dishes. B, MeSO induction was analyzed by treating N1E-115 cells with 1.5% MeSO in duplicate for the indicated times and quantitation of NTR-1 mRNA as described in A.
expression by ~60%, consistent with this sequence being important for basal expression; however, the residual basal expression of this construct indicates that transcription can also initiate outside this region. A mutation near the center of the mPAL (mPal mut-2) had little effect on basal activity but severely curtailed Me2SO induction (Fig. 6A). Two other mutants that reduced basal expression had no effect on Me2SO induction (mPal mut-4 and -5), whereas the remaining mutations (mPal mut-1, -3, -6) reduced both basal and Me2SO-inducible expression. These results indicate that the mPAL is composed of multiple cis-regulatory sequences that contribute to basal and Me2SO-induced expression. The mPAL core (affected by mPal mut-2) appears to be most critical region involved in Me2SO induction, although sequences at the 5′ border of the mPAL are also important.

To examine the regulatory properties of the mPAL in more detail, one (mP12–4) or two (mP10–1) copies of an oligonucleotide spanning the mPAL were cloned upstream of the SacI deletion construct (5′–448) that displays very low basal expression and is actually inhibited by Me2SO treatment (Fig. 6A). Both constructs conferred some Me2SO responsiveness to the deleted promoter, but two copies were required to increase basal expression, although neither construct had full activity. To determine whether inclusion of sequences just upstream of the mPAL could restore full Me2SO responsiveness, a construct containing sequences between −589 and −507 cloned upstream of the SacI promoter deletion was tested (Fig. 6A, URmP-1). Inclusion of these more 5′ sequences restored Me2SO induction nearly completely, although the basal activity of this construct was increased only marginally (similar to the mP10-1 construct). These results provide additional evidence that sequences immediately upstream of the mPAL and the mPAL itself are required for full Me2SO responsiveness.

To define the additional sequences located in the upstream region that are required for full Me2SO induction, a series of mutations in this region were analyzed by transfection in NIE-115 cells (Fig. 6B, mut-1 to -4). Mutants 2–4 had little effect on Me2SO induction (Fig. 6B), and none of the mutants had a large effect on basal expression (data not shown). In contrast, mut-1, which specifically alters the conserved CACCC box, nearly abolished Me2SO inducibility (Fig. 6B). These results indicate that the conserved CACCC box that lies immediately upstream of the mPAL is required for Me2SO induction. This result was somewhat surprising in view of the fact that 5′ deletion to −569 results in a large drop in Me2SO responsiveness (Fig. 5, 5′–569), and this deletion removes the sequences mutated in mut-2 to -4. This apparent discrepancy could result from either functional redundancy within the region mutated in mut-2 to -4 (e.g., the sequence GTGGC is directly repeated), the presence of a cis-regulatory element within the deleted region that is not inactivated by any of the individual mutations, or the creation of junction sequences during cloning that have an adventitious effect on activity. Thus, although it remains possible that one or more sequences in the region affected by mut-2 to mut-4 contribute to Me2SO responsiveness, the CACCC box is clearly an important cis-regulatory element required for Me2SO
Similar Promoter Elements Are Required for Expression in NG108 Neuroblastoma Cells—

NG108 neuroblastoma cells display high constitutive levels of high affinity NT-binding sites (30). To determine whether similar promoter elements are required for constitutive expression and to examine possible regulation by Me2SO, these cells were transfected with a series of promoter constructs using the same methods as were used for N1E-115 cells. Me2SO treatment reproducibly increased expression of the wild type promoter construct (5'-640) by 2–3-fold (Fig. 7A). The results obtained in these cells were similar to those obtained in N1E-115 cells. The 5'- and 3'-deletion mutants define a region between −589 and −507 that is required for full induction, and the analysis of selected clustered point mutations indicates that the CACCC box (URmut-1) and the center of the mPAL (mPalmut-2) are critical sequence elements required for induction (Fig. 7A). The only real difference was that mPal mut-6 had a modest negative effect on Me2SO induction in N1E-115 cells but decreased basal expression in NG108 cells by about one-half, and several mPal mutants that reduce basal expression in N1E-115 cells had no effect on expression in NG108 cells (mPalmut-1, -3, and -5). These results indicate that similar promoter elements are required for expression in these two neuroblastoma cell lines and provide further evidence that the CACCC box and the core of the mPAL are critical elements required for Me2SO induction.

Distinct Promoter Requirements for Expression in Mouse Embryo Fibroblasts—

To determine whether the NTR-1 promoter is expressed in a cell type-specific manner, the same series of constructs that were tested in NG108 cells were also transfected into mouse embryo fibroblasts that do not express the endogenous Ntr-1 gene (data not shown). Preliminary experiments revealed that Me2SO treatment of these cells had no effect on the expression of the wild type promoter, indicating that Me2SO induction is cell type-specific. The wild type promoter (5'-640) was active in these cells; however, this basal expression was dependent on largely different promoter elements compared with the neuroblastoma cell lines. There were several major differences. First, the Sp1-like sequence that is removed in the 5'-589 promoter construct appears to be unimportant for expression in MEF cells (Fig. 7B). Second, deletion of the region between the Sp1-like site and the mPAL (5'-548) essentially abolished expression in MEF cells but had only a modest effect in NG108 cells (Fig. 7B). The CACCC box appears to be critical for basal expression in these cells (Fig. 7B), in contrast to the situation in the neuroblastoma cell lines where the CACCC box is required for Me2SO induction and makes at most a modest contribution to basal expression (data not shown). The mPAL was also found to be important for basal expression in MEF cells; however, the core of the mPAL appears to be extremely important for basal expression in these cells (Fig. 7B, mPal mut-2) but again is mainly involved in Me2SO induction in the neuroblastoma cell lines. These results indicate that substantially different sequence elements are responsible for basal expression in MEFs as compared with neuroblastoma cells. The endogenous Ntr-1 gene must normally be repressed in fibroblasts so that the sequence elements identified in transient transfection experiments are masked in

**Fig. 6. Sequences required for Me2SO induction and basal expression of the Ntr-1 gene.** Clustered point mutations were introduced into the regulatory region using the PCR overlap extension technique (36) and cloned upstream of the luciferase reporter gene. All mutant promoters were completely sequenced to verify the mutation and to confirm that no other substitutions had occurred. The mutant promoters were analyzed as described in Fig. 5. The mean values are plotted with the S.E. indicated by error bars (n = 5–8). Mutational analysis of the mPAL region (A) and the region immediately upstream (B) are shown. Schematic diagrams of the promoter regions analyzed and the specific substitutions are shown below the graphs.
Factors Binding to the Regulatory Region—DNase I footprint analysis was used to identify protein-binding sites in the Ntr-1 gene regulatory region. Nuclear extracts were prepared from N1E-115 cells that were either grown under control conditions or treated with 1.5% Me₂SO for 48 h.³²P-End-labeled promoter
Sequences Required for Ntr-1 Gene Activation

Fig. 8. DNase I footprint analysis of the mouse Ntr-1 gene regulatory region. DNase I footprinting assays were performed using an EcoRI/BamHI mouse NTR-1 promoter fragment 32P-end-labeled at either the BamHI (A) or EcoRI (B) site and nuclear extracts (180 μg) from either control (−) or MeSO-induced (48 h, +) N1E-115 cells. A series of reactions containing increasing amounts of DNase I were performed, and equal counts (2,000 cpm) were loaded on a sequencing gel for analysis. The products of a reaction in which the probe was treated with DNase I in the absence of protein (NP) were also analyzed. Lanes in which the extent of probe digestion was similar were compared and are depicted. Chemical sequencing reactions (lanes G/A, G) were used to generate markers for the alignment of footprinted regions on the promoter sequence. DNase I protections are indicated by either open circles (MeSO-inducible), gray circles (partially inducible), or black circles (constitutive). Footprinted regions are indicated schematically by boxes shaded like the circles and are numbered 1–6. The mouse NTR-1 promoter sequence in the region analyzed is depicted to the right of the autoradiographs and promoter schematics, and the positions of NTR-1 promoter sequence in the region analyzed is depicted to the right of the autoradiographs and promoter schematics.

Fragments of the autoradiographs and promoter schematics, and the positions of NTR-1 promoter sequence in the region analyzed is depicted to the right of the autoradiographs and promoter schematics. 

cell extracts is not cell type-specific. PC12 cells express no detectable NTR-1 mRNA (data not shown). A cluster of constitutive hypersensitive sites (indicated by arrows in Fig. 8A) was observed just downstream of a putative Sp1 site, indicating that this region is occupied in both MeSO-induced and control cells. The significance of FP5 and FP6 is uncertain since deletion of this region has no effect on either basal expression or MeSO induction. However, the MeSO-inducible footprints FP3 and FP4 lie over functionally important sequences.

To examine FP-1 in more detail, the EcoRI/BamHI promoter fragment was labeled at the EcoRI site and subjected to footprint analysis using MeSO-induced and control N1E-115 cell nuclear extracts (Fig. 8B). The footprint was found to consist of a region of MeSO-induced protections (FP1A) and an adjacent region that was constitutively protected (FP1B). The constitutive footprint at least partially overlaps a half-CRE site (CGTCA), and many CREs bind cAMP-response element family proteins constitutively (48). The MeSO-inducible FP1A is within a region that the 5′-622 deletion construct indicates is required for full basal expression in N1E-115 cells but does not affect MeSO induction (Fig. 5B); thus, the significance of this site is uncertain. Since there is a relative paucity of strong DNase I cleavage sites, it remains possible that additional proteins may bind to this region.

To characterize further the factors binding to the promoter region, gel shift experiments were performed using a probe that spans the mPAL and CACCC box (−589 to −507). Nuclear extracts were prepared from N1E-115 cells that were either treated with 1.5% MeSO for 72 h or grown under control conditions and incubated with the 32P-labeled promoter fragment, and the resulting complexes were analyzed on non-denaturing acrylamide gels (Fig. 9A). At least four complexes (Fig. 9A, complexes 1–4) were identified that were specifically competed by cold wild type probe fragment (longer exposure times were required to detect complexes 3 and 4, Fig. 9A, bottom panel). To examine whether the functional sequence elements defined in the transfection experiments described above were required for the formation of these complexes, competition experiments were performed with wild type and mutant promoter fragments. An oligonucleotide corresponding to the collagenase TRE (49) was used as a nonspecific competitor. Competition with a cold mutant promoter fragment containing clustered point mutations in the CACCC box (mut-1 in Fig. 6B) identified two complexes that require this sequence for binding (Fig. 9A, 4th lane, complexes 1 and 2). Comparison between the induced and control lanes indicates that MeSO treatment results in the specific induction of complex 1 (Fig. 9A, compare 2nd and 6th lanes). Close DNA contacts in the inducible complex were analyzed using a methyltransferase interference assay (Fig. 9B). Complexes were formed by mixing partially methylated 32P-labeled probe (the same probe used in the experiment depicted in Fig. 8A) with nuclear extract from MeSO-induced N1E-115 cells, separated on a native acrylamide gel, and the regions corresponding to complex 1/2 and free probe were excised for analysis of methylated G residues on sequencing gels. Several bands were underrepresented in complex 1/2 compared with free probe, specifically the Gs that are complementary to last three Cs in the CACCC sequence (Fig. 9B). Analysis of the other strand was not informative most likely due to the absence of G residues in the region containing the CACCC sequence. These results indicate that the MeSO-inducible complex makes specific DNA contacts in the CACCC element, since complex 1 is the major component of the complex 1/2 band in MeSO-induced cell extracts (see Fig. 9A).

The CACCC box is a binding site for a number of zinc finger
transcription factors, including Sp1-related and Krüppel-like proteins (50–52). To examine whether complexes 1 and 2 were Sp1-related factors, competition and antibody detection experiments were performed using the same probe (Fig. 9C). A competitor oligonucleotide containing a consensus Sp1 site effectively competed for binding of complex 2 but not complex 1, indicating that complex 2 has a binding specificity similar to Sp1. To determine whether complex 2 contains Sp1, a specific Sp1 antibody was added to the binding reaction at two different concentrations (1–4 μl), and for comparison a Jun B-specific antiserum was added to control reactions. Complex 2 was abolished by addition of 4 μl of Sp1 antiserum but was not affected by the same amount of JunB antiserum, indicating that complex 2 contains Sp1 (Fig. 9C). These results provide evidence that complex 2 is due to binding of Sp1 to the CACCC box; however, complex 1 appears to be due to a distinct factor(s), perhaps a Krüppel-like protein, that is specifically induced during MeSO-induced neuronal differentiation of N1E-115 cells.

**DISCUSSION**

Several Sequences, Including a CACCC Box, Are Required for MeSO Induction of Ntr-1 Gene Expression—DNA transfection experiments were used to define promoter elements required for Ntr-1 gene induction in neuroblastoma cells. Previous work has shown that MeSO-stimulated neuronal differentiation of N1E-115 neuroblastoma cells is accompanied by increased expression of high affinity neurotensin-binding sites (28). We demonstrate here that MeSO treatment induces the expression of the endogenous Ntr-1 gene from 3- to 5-fold and has a similar effect on the expression of a luciferase reporter gene driven by NTR-1 promoter sequences. MeSO also increased
Ntr-1 gene expression in NG108 neuroblastoma × glioma hybrid cells that have previously been shown to express high affinity NT-binding sites (30). This was a selective effect since the expression of a luciferase reporter gene controlled by the Rous sarcoma virus-long terminal repeat was not affected by Me₃SO treatment of either neuroblastoma cell line. In contrast, Me₃SO had no effect on NTR-1 reporter constructs transfected into fibroblasts. Mutational analysis of the NTR-1 promoter has defined an 80-bp region encompassing a CACCC box and large GC-rich palindrome as critical for Me₃SO induction. The CACCC box is bound by an Me₃SO-induced complex in N1E-115 cells. Several regions within the mPAL are required for full Me₃SO induction; however, the central core appears to be the most critical Me₃SO-responsive sequence. The transcriptional start site was mapped to the 5' end of the mPAL to a region that closely matches the initiator element that was first identified in the terminal deoxynucleotidyltransferase gene (32). The regulatory sequences identified through transfection analysis here are highly conserved between mouse, rat, and human (Fig. 1B), indicating that they are functionally important in vivo. These results provide evidence that a conserved region surrounding the transcriptional start site is critical for Ntr-1 gene activation during a program of neuronal differentiation initiated by Me₃SO treatment in N1E-115 cells.

The CACCC box was first identified through sequence comparisons of β-globin genes and has been shown to be important for expression in erythroid cells. This site binds a number of related zinc finger transcription factors, including Sp1- and Kruppel-related proteins (50, 53). The majority of the characterized Kruppel-related genes are expressed in tissue-restricted patterns. Gene targeting approaches have revealed that the erythroid Kruppel-like factor is essential for high level erythroid-specific β-globin gene expression (54, 55) and that lung Kruppel-like factor is essential for T-cell development (56), indicating that specific Kruppel-like proteins play key roles in transcriptional control and terminal differentiation. The β-globin CACCC boxes are required for activation during Me₃SO-induced differentiation of mouse erythro leukemia cells (57), possibly through the phosphorylation of erythroid Kruppel-like factor (58). The CACCC box in the NTR-1 promoter is clearly required for Me₃SO induction and is bound by both constitutive and Me₃SO-inducible complexes in N1E-115 neuroblastoma cells. The major constitutively expressed complex is closely related to Sp1 (Fig. 9C, complex 2). The inducible complex clearly has a binding specificity different from that of the Sp1-related proteins and makes close contacts within the CACCC box, similar to Kruppel-like proteins (see for example Ref. 53). These data suggest a model in which the induction of one or more Kruppel-like factors is required for Ntr-1 gene activation during N1E-115 cell differentiation. Intriguingly, both gut-enriched (52) and neuron-enriched (59–61) Kruppel-like factors have been described, and these are the major sites of Ntr-1 gene expression in vivo (15, 31).

The mutational analysis of the mPAL revealed that it most likely consists of several independent cis-active elements that influence Me₃SO-induced and basal expression. Several mutations along the length of the mPAL reduced Me₃SO inducibility in both N1E-115 and NG108 cells (Figs. 6A and 7A). Alteration of sequences near the center of the mPAL greatly reduced Me₃SO responsiveness (Figs. 6A and 7A, mPal mut-2). This sequence is a perfect palindrome consisting of alternating G and C residues and is conserved in both the rat and human genes; however, comparisons with known transcription factor-binding sites did not reveal any close similarities, indicating that either it is a novel binding site or functions as a DNA structural element. Mutation of an additional sequence located at the 5' end of the palindrome (mPal mut-3) also decreased Me₃SO activation in both N1E-115 and NG108 cells but appears to affect a distinct functional element. This sequence is nearly identical to the consensus initiator element, and mPal mut-3 also reduced basal activity in N1E-115 and MEF cells but surprisingly not NG108 cells (Figs. 6B and 7B). The major transcriptional start site was also mapped to this location making it likely that this is a functional initiator. DNase I footprint analysis indicates that factor binding to this site is regulated by Me₃SO in N1E-115 cells (Fig. 8A). This could be due to either increased expression of a factor that binds to this site or cooperative interactions with the Me₃SO-inducible CACCC box-binding factor. Thus, the mutational analysis indicates that there are at least two distinct cis-regulatory elements within the mPAL that are required for full Me₃SO responsiveness, and DNase I footprint analysis indicates that Me₃SO-inducible complexes bind in the mPAL core region. These results coupled with the results discussed above for the CACCC box indicate that Me₃SO induction of Ntr-1 gene expression requires cooperative interactions between multiple sites, including the CACCC box, the mPAL core, and the initiator element.

Different Promoter Sequences Required for Basal Expression in Neuroblastoma and MEF Cells—There were several differences in the sequences required for basal expression in the three cell lines, although the results obtained in N1E-115 and NG108 cells were similar. A notable difference was that the CACCC box and the central region of the mPAL are required for high level basal expression in MEF cells but are principally involved in Me₃SO responsiveness in the neuroblastoma cell lines. The contribution of the CACCC box to basal expression in MEFs is most likely due to the expression of one or more CACCC box-binding proteins in these cells, for instance BKLF is expressed in fibroblasts (62). A CACCC box has been shown to be important for β-globin gene expression in transient expression assays in non-erythroid cell lines (63), although this element mediates cell-specific expression through the binding of erythroid Kruppel-like factor in erythroid cells (54, 55, 57). The endogenous Ntr-1 gene is most likely repressed in most cell types and only accessible to Kruppel-like proteins and perhaps other CACCC box-binding proteins in neurons and a restricted set of other cell types. The induction of these proteins during neuronal differentiation or in response to environmental cues could underlie Ntr-1 gene activation in specific neuronal populations.

There were also differences in the requirement for the Sp1-like sequence located between -590 and -603 and a sequence near the 5' end of the positive regulatory region for basal expression. The Sp1-related sequence was clearly important for basal activity in the neuroblastoma cell lines but not in MEF cells (compare 3'-590 and 3'-603 in Figs. 5B and 7B). Sp1 is constitutively expressed in N1E-115 cells (see Fig. 9A, complex 2), and nuclear extracts from both control and Me₃SO-induced cells create DNase I-hypersensitive sites just downstream of the Sp1-related sequence indicating that this site is constitutively occupied. Sequences near the 5' end of the positive control region were found to be important for basal expression in N1E-115 cells (Fig. 5B, compare 5'-640 and 5'-622) but not in NG108 or MEF cells (Fig. 7B). DNase I footprinting experiments indicate that this region binds both constitutive and Me₃SO-inducible factors (Fig. 8B), and sequence comparisons indicate that this region is similar to a neural specific regulatory element identified in the Drosophila dopacarboxylase
(Ddc) gene (64). The Ntr-1 gene is expressed at high levels in midbrain dopamine neurons (31), and the homology to the Droso-
phila neural element raises the intriguing possibility that this region may be required for expression in catecholamine-producing
neurons (N1E-115 cells produce catecholamines).

Although there were significant differences in the promoter elements required for basal expression, certain regions of the
mPAL are important in all the lines examined. The most crit-
sophila (66). Comparison of the promoter regions from the DA D 2
(37). Our own attempts to map the transcription start
site of the mouse gene is located at or near the initiator con-
sequence similarities suggest that these two genes that are co-
expressed in DA neurons could rely on similar regulatory stra-
egies; however, additional regulatory elements are also likely
to be required to generate the specific complex patterns of expression characteristic of these two genes.

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REFERENCES
1. Carraway, R. E., and Leeman, S. E. (1975) J. Biol. Chem. 250, 1907–1911
2. Reinecke, M. (1985) Progr. Histochem. Cytochem. 16, 1–175
3. Dobner, P. R., Barber, D. L., Villa-Komaroff, L., and McKiernan, C. (1987)
Proc. Natl. Acad. Sci. U. S. A. 84, 3516–3520
4. Kislauskis, E., Bullock, B., McNeil, S., and Dobner, P. R. (1988) J. Biol. Chem.
263, 4963–4968
5. Szegethy, E., and Beaudet, A. (1989) J. Comp. Neurol. 270, 128–137
6. Wouffe, J., and Beaudet, A. (1989) Brain Res. 479, 402–406
7. Pinnock, R. D. (1985) Brain Res. 338, 151–154
8. Jiang, Z.-G., Pessin, M., and North, R. A. (1994) J. Physiol. (Lond.) 474, 119–129
9. Shi, W.-X., and Bunney, B. S. (1991) Brain Res. 543, 315–321
10. Kalivas, P. W., Nemeroff, C. B., and Prange, A. J., Jr. (1981) Brain Res. 229, 525–529
11. Kalivas, P. W., Nemeroff, C. B., and Prange, A. J., Jr. (1982) Eur. J. Pharma-
col. 78, 471–474
12. Nemeroff, C. B., Biassette, G., Prange, A. J., Jr., Loosen, P. T., Barlow, T. S.,
and Lipton, M. A. (1977) Brain Res. 128, 485–496
13. Ervin, G. N., Birkeno, L. S., Nemeroff, C. B., and Prange, A. J., Jr. (1981)
Nature 291, 73–76
14. Kalivas, P. W., Nemeroff, C. B., and Prange, A. J., Jr. (1984) Neuroscience 11,
919–930
15. Tanaka, K., Masu, M., and Nakanishii, S. (1990) Neuropsychopharmacology 4, 847–854
16. Mazella, J., Botto, J.-M., Guillermont, A., Coppola, T., Sarret, P., and Vincent,
J.-P. (1996) J. Neurosci. 16, 5613–5620
17. Chalon, P., Vita, N., Kachoud, M., Guillermont, M., Bonnin, J., Delphe, B., Le
Fur, G., Ferrara, P., and Caput, D. (1996) FEBS Lett. 386, 91–94
18. Botto, J.-M., Guillermont, A., Vincent, J.-P., and Mazella, J. (1997) Neurosci.
Lett. 233, 193–196
19. Bolden-Watson, C., Watson, M. A., Murray, K. D., Isackson, P. J., and
Dorsa, D. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 543–547
20. Boudin, H., Pelaprat, D., Rostene, W., and Beaudet, A. (1996) J. Comp. Neu-
rosci. 7, 63–76
21. Herve, D., Tassin, J. P., Studler, J. M., Dana, C., Kitabgi, P., Vincent, J.-P.,
Dowekins, J., and Rostene, W. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6203–6207
22. Merchant, R. M., Hanson, G. R., and Dorsa, D. M. (1994) J. Pharmacol. Exp.
Ther. 269, 806–812
23. Betancur, C., Rostene, W., and Berod, A. (1997) Mol. Brain Res. 44, 334–340
24. Azizi, M., Nicot, A., Gally, D., Kitabgi, P., Berod, A., and Rostene, W. (1994)
Neurosci. Lett. 172, 97–100
25. Azizi, M., Boudin, H., Mahmudi, N., Pelaprat, D., Rostene, W., and Berod, A.
(1996) Mol. Brain Res. 42, 213–221
26. Amano, T., Richelson, E., and Nireberg, M. (1972) Proc. Natl. Acad. Sci.
U. S. A. 69, 258–263
27. Kimbi, Y., Palfrey, C., Spector, I., Barak, Y., and Littauer, U. Z. (1976) Proc.
Natl. Acad. Sci. U. S. A. 73, 462–466
28. Pousaitis, C., Mazella, J., Kitabgi, P., and Vincent, J.-P. (1984) J. Neurosci.
4, 1094–1100
29. Cusack, B., Stanton, T., and Richelson, E. (1991) Eur. J. Pharmacol. 206,
329–342
30. Nakagawa, Y., Higashida, H., and Miki, N. (1994) J. Neurosci. 14, 1653–1661
31. Nicot, A., Rostene, W., and Berod, A. (1994) J. Comp. Neurol. 341, 407–419
32. Smale, S. T., and Baltimore, D. (1989) Cell 57, 103–113
33. Somboek, J., Fritsch, E. P., and Maniatis, T. (1989) Molecular Cloning: A
Laboratory Manual, 2nd ed., pp. 2.108–2.111, Cold Spring Harbor Laboratory, Cold
Spring Harbor, NY
34. Bronster, A. R., Tate, J. E., and Habener, J. F. (1989) BioTechniques 7,
1116–1122
35. Harrison, R. J., McNeil, G. P., and Dobner, P. R. (1995) Mol. Endocrinol.
9, 981–993
36. Nordeen, S. K. (1988) BioTechniques 6, 454–458
37. Maeno, H., Yoshimura, R., Fujita, S., Su, Q., Tanaka, K., Wada, K., and
Kiyama, H. (1996) Mol. Brain Res. 40, 97–104
38. Le, P., Grosshan, K., Zeng, X. P., and Richelson, E. (1997) J. Biol. Chem. 272,
