MOLECULAR AND CELLULAR BIOLOGY, July 1995, p. 3870–3881 Vol. 15, No. 7
0270-7306/95/$04.00+0

Characterization of Promoter Elements Required for Cell-Specific Expression of the Neurotensin/Neuromedin N Gene in a Human Endocrine Cell Line

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Received 9 January 1995/Returned for modification 8 March 1995/Accepted 26 April 1995

Expression of the gene encoding neurotensin/neuromedin N (NT/N) is mostly limited to the brain and specialized enteroendocrine cells (N cells) of the distal small intestine. We have analyzed the NT/N DNA sequences upstream of the RNA start site that direct cell-specific expression using a novel human endocrine cell line, BON, that resembles intestinal N cells in several important aspects, including NT/N precursor protein processing, ratios of different NT/N mRNA forms, and high levels of constitutive expression of the NT/N gene. Transient transfection assays with plasmids with progressive 5′ deletions of the rat NT/N promoter identified the proximal 216 bp of 5′ flanking sequences as essential for high-level constitutive NT/N expression in BON cells. In addition, a detailed mutational analysis defined multiple regions within the proximal 216 bp that contribute to cell-specific NT/N expression. These elements include a proximal cyclic AMP response element (CRE)/AP-1-like motif (TGACATCA) that binds c-Jun, JunD, CRE-binding (CREB), and ATF proteins, a near-consensus glucocorticoid response element, and a distal consensus AP-1 site that binds c-Fos, Fra-1, and JunD. In addition, elements contained within two 21-bp imperfect direct repeats play an important role in NT/N expression in BON cells and may bind novel factors that act as positive regulators of NT/N expression. DNase I footprinting and gel shift analyses demonstrate that the sites identified by mutational analysis, and at least one additional site, specifically bind BON cell nuclear proteins in vitro. We speculate that a complex pattern of regulation requiring interaction between a proximal CRE/AP-1-like motif and other upstream control elements play an important role in the high-level constitutive expression of NT/N in the human endocrine cell line BON. In addition, the BON cell line provides a unique model to further characterize the factors regulating cell-specific NT/N expression and to better understand the mechanisms responsible for the terminal differentiation of the N-cell lineage in the gut.

Neurotensin (NT), a tridecapeptide released from specialized enteroendocrine cells (N cells) of the small bowel (33), has numerous physiologic functions in the gastrointestinal (GI) tract, including stimulation of pancreatic secretion (8) and colonic motility (75), as well as the inhibition of gastric and small bowel motility (2). In addition, NT facilitates fatty acid translocation from the intestinal lumina of rats (6) and stimulates growth of various GI tissues, including pancreas (34), colon (29), and small bowel (20, 30, 31, 44, 79) tissues. Collectively, these studies identify NT as an important contributory hormone for the maintenance of both gut structure and function.

The primary sequence of the gene encoding NT and the structurally related hexapeptide neuromedin N in rats and humans has been determined elsewhere (10, 49). In the GI tract, NT/neuromedin N (NT/N) gene expression is primarily confined to the terminally differentiated N cell in adult small bowel (68). Similarly to other gut hormones (14, 51, 78), NT/N expression has also been identified in endocrine tumors of the pancreas (18, 74), thus suggesting a common ancestral lineage between N cells of the gut and pancreatic endocrine cells, a hypothesis which is further supported by the finding that enteroendocrine and pancreatic endocrine cells arise from the embryonic gut endoderm (53). Expression of the NT/N gene is developmentally regulated in the intestines of both rats and humans in a distinctive temporal and spatial-specific distribution (27, 32, 62). The level of NT/N expression in the small bowel is initially low in the fetus but rapidly increases after birth to assume the typical adult topographical distribution of increasing NT/N expression along the longitudinal axis of the small bowel. In the human colon, NT/N is expressed in the fetus during midgestation when the colon bears morphologic and functional similarity to the small bowel; NT/N expression is not apparent in the colon of the newborn or the adult (32). These studies indicate that the NT/N gene will provide a useful molecular paradigm to better define the complex differentiation pathway leading to small bowel and colonic phenotypes and to investigate the factors regulating establishment and maintenance of certain cell lineage-specific patterns along the duodenum-to-colon axis of the gut.

The factors regulating induction of the NT/N gene have been examined by using rat medullary thyroid carcinoma (rMTC) (22, 81) and rat pheochromocytoma (PC12) cell lines (40). The NT/N gene is not detectably expressed in untreated PC12 cells, and little or no increase in expression is observed in cells treated with single agents. In contrast, NT/N gene expression and NT peptide production are induced several hundredfold in response to combined treatment with nerve growth factor (NGF), dexamethasone, lithium, and the adenylate cyclase activator forskolin (23, 50, 76). Transfection experiments

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TABLE 1. Sequences of synthetic oligonucleotides used in this study

Oligonucleotide[a]	Sequence[b]	Reference(s)
WT1 (−60/−36)	5′-CCTGTACAGTCATGACATCACCCTG-3′	49, 50
WT2 (−122/−93)	5′-GTGGGGGATGAGATGAGGGACAAAGAGAAA-3′	49, 50
WT3 (−96/−67)	5′-GAAAGGAGGGGGGATGGAGATGAAGGCAGA-3′	49, 50
WT4 (−150/−125)	5′-GATCGTCACTTTCAATCAAGGTTCAT-3′	49, 50
WT5 (−170/−151)	5′-CAACAGCTGCAATTAGGGAA-3′	49, 50
WT6 (−198/−175)	5′-ACTCTTTCCGTGAGTCAGAAACCC-3′	49, 50
C/EBP	5′-GCTGCAGATTGCGCAATCTGCAGCA-3′	1
SEB	5′-GCCGGACGACAGCTGGGGGGGCGG-3′	78
IEB	5′-AGGTAGGCAGATGGCGAGAGGGGC-3′	46

[a] Rat NT/N (wild type), SEB, and IEB.
[b] Underlined sequences for WT1 correspond to the CRE/AP-1-like motif.

have demonstrated that all of the *cis*-regulatory elements required for NT/N induction in PC12 cells are contained in the proximal 200 bp of 5′ flanking sequences of the rat NT/N gene and include a consensus AP-1 site, two near-consensus cyclic AMP response elements (CREs), and a near-consensus glucocorticoid response element (GRE) (50). These elements function cooperatively to integrate multiple environmental stimuli into a concerted transcriptional response. In contrast to NT/N gene induction, the regulatory sequences required for the cell-specific expression of NT/N are not known, in part because of the lack of a suitable enteroendocrine cell model. Although notable attempts have been made to isolate enteroendocrine cells from the canine gastric and intestinal mucosa (9, 73), these primary cultures are insufficiently pure and difficult to maintain for more than a few days, thus rendering these cells unsuitable for many molecular biologic approaches.

In our present study, we have utilized the novel BON cell line to delineate the regulatory elements required for cell-specific expression of the rat NT/N gene. BON cells, like the terminally differentiated N cells of the small bowel, express high levels of NT/N mRNA, synthesize and secrete NT peptide, and process the NT/N precursor protein in a fashion identical to that of normal intestine, with neuromedin N present in its large molecular form and NT present as the small peptide (17, 28), thus providing an ideal model to discern the mechanisms underlying the high-level constitutive NT/N expression that is characteristic of the intestinal N-cell phenotype. A detailed mutational analysis defined multiple regions within the proximal 200 bp of 5′ flanking sequence that contribute to constitutive NT/N expression in BON cells. Certain elements are critical for both cell-specific expression in BON cells and induction in PC12 cells, for instance, a proximal region (positions −58 to −38) of the NT/N promoter that contains a CRE/AP-1-like motif (TGACATCA) which is identical to a c-*jun* autoregulatory element (3). However, in marked contrast, a distal consensus AP-1 site that plays a pivotal role for NT/N induction in PC12 cells has a much smaller effect on cell-specific expression in BON cells. Furthermore, elements contained within two purine-rich imperfect direct repeats (IDRs) play a prominent role in BON cells but a comparatively minor role in induction of NT/N in PC12 cells. DNase I footprinting and gel shift experiments confirm that the sites identified by mutational analysis (and at least one additional site) specifically bind BON cell nuclear proteins in vitro. Therefore, our results indicate that a different array of *cis*-regulatory motifs, localized in the proximal 120 bp of the 5′-flanking sequence, are required for high-level constitutive NT/N expression in BON cells and include, most importantly, a proximal CRE/AP-1 site that binds c-Jun, JunD, CRE-binding (CREB), and ATF proteins and a purine-rich IDR region that may bind novel transcription factors.

MATERIALS AND METHODS

Materials. Restriction, ligation, and other DNA-modifying enzymes were purchased from Promega (Madison, Wis.) or Stratagene (La Jolla, Calif.). DNase I (DPRF) was purchased from Worthington Biochemical Corporation (Freehold, N.J.). Nucleotides and poly(dI · dC) were purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, N.J.), and radioactive compounds were obtained from Du Pont-New England Nuclear (Boston, Mass.). Tissue culture media and reagents were obtained from Gibco-BRL (Grand Island, N.Y.). All other reagents were of molecular biology grade and were obtained from either Sigma (St. Louis, Mo.) or Amresco (Solon, Ohio). Elutip-d columns were purchased from Schleicher & Schuell (Keene, N.H.). Thin-layer chromatography (TLC) plates were purchased from Whatman (Hillsboro, Oreg.), En^{3}Hance spray was purchased from Du Pont (Boston, Mass.), and autoradiography film was purchased from Kodak (Rochester, N.Y.). Oligonucleotides containing consensus CRE, AP-1, and Sp1 binding sites were obtained from Promega. Oligonucleotides corresponding to rat NT/N wild-type promoter sequences, C/EBP (1), the secretin E box (SEB) (78), and the insulin E box (IEB) (46) were synthesized, and the coding strands are indicated in Table 1. Specific antibodies against the AP-1 and CREB/ATF/CREM proteins were from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Antisera against C/EBPα, C/EBPβ, and C/EBPδ were provided by Steven L. McKnight (Tularik, Inc., San Francisco, Calif.).

Cell culture. The human carcinoid cell line BON, which was established in our laboratory (26, 65), was cultured in Dulbecco's modified Eagle's medium and F12K in a 1:1 ratio supplemented with 5% (vol/vol) fetal calf serum. MIA PaCa-2 cells, a human pancreatic adenocarcinoma cell line (80), and HeLa cells, a human cervical cancer cell line, were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum. AKR-2B cells, a normal mouse fibroblast cell line (61), were cultured in McCoy's medium with 5% fetal calf serum. All cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Plasmid constructions. Rat NT/N gene 5′ deletion, linker scanner, and internal deletion chloramphenicol acetyltransferase (CAT) constructs have been described previously (50). To characterize the functional significance of the rat NT/N promoter region from −168 to −155 (protected region III), mutations were introduced into this region by oligonucleotide-directed mutagenesis. Briefly, the NT/N promoter region was cloned into M13mp19 and transfected into a Dam$^-$ JM101 strain (JM101-201) of *Escherichia coli* to generate single-stranded template DNA. Phosphorylated oligonucleotides were individually annealed with template DNA, extended with unmodified T7 DNA polymerase (11), and treated with T4 DNA ligase, and the resulting double-stranded DNA was transformed into JM101-201. The oligonucleotides were designed such that successive 6-bp promoter sequences were converted to either *Sma*I, *Bam*HI, or *Cla*I restriction sites. Mutant phages were identified by digestion of replicative form DNA with appropriate restriction enzymes and were sequenced. Mutant promoters were excised by digestion with *Hin*dIII and *Sac*I and were cloned upstream of the luciferase gene in *Hin*dIII-*Sac*I-digested pXP2 (63). The construction of the pXP2 luciferase reporter plasmid driven by the wild-type rat NT/N promoter fragment has been previously described (50). Plasmids for transfection were prepared by alkaline lysis and then by two successive bandings on cesium chloride density gradients by standard methods (70). At least two plasmid DNA preparations were used for each experiment to ensure reproducibility.

Transient transfection, CAT, luciferase-, and β-galactosidase assays. For the transfection studies with the rat NT/N promoter CAT constructs, cells were seeded at 2×10^6 in 100-mm-diameter culture dishes 1 day prior to transfection. A DNA mixture containing 25 μg of transient-expression vector was precipitated by the calcium phosphate method (38, 39). To control for differences in trans-

fection efficiency, cells were cotransfected with 5 μg of the plasmid pCH110 (42), which contains the bacterial β-galactosidase gene fused to the simian virus 40 early promoter. The cells were refed 2 to 3 h prior to the transfection. The medium was removed 4 h later, and the cells were treated for 2 min with 15% (vol/vol) glycerol in phosphate-buffered saline (PBS), washed twice with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline, and refed. The cells were harvested 48 h after the addition of DNA. Cell extracts were prepared by freeze-thawing of the transfected cells three times in 100 μl of 0.25 M Tris-HCl (pH 7.5) and by centrifugation for 10 min at 4°C. Protein concentrations were determined by the method described by Bradford (13). CAT activity in the cell extracts was assayed as described by Gorman et al. (38) with acetyl coenzyme A and [^{14}C]chloramphenicol. The reaction products were separated by TLC. TLC plates were dried, sprayed with En^{3}Hance, and visualized by autoradiography. Acetylated reactions were quantified by liquid scintillation counting of the excised TLC spots.

For luciferase assays, BON cells were plated at a density of 0.7×10^6 cells in 60-mm dishes 36 h prior to transfection. Rat NT/N promoter-luciferase gene constructs (7.5 μg) were cotransfected with 2.0 μg of pCH110 by calcium phosphate coprecipitation. Forty-eight hours after transfection, the cells were rinsed with PBS and lysed directly on the dish with 250 μl of a 1× cell culture lysis reagent (Promega). Luciferase activity in 20 μl of extract was assayed with a luciferase assay system (Promega). Light emissions were integrated for the initial 10 s of emission by using a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

β-Galactosidase assays were carried out as follows. A 20-μl volume of supernatant was added to 0.6 ml of reaction mixture (0.1 M sodium phosphate [pH 7.8], 10 mM KCl, 50 mM β-mercaptoethanol, 1 mM $MgCl_2$). The reaction mixture was warmed to 37°C, 120 μl of ortho-nitrophenol-β-D-galactopyranoside (2 mg/ml) was added, the mixture was incubated at 37°C. The reactions were allowed to proceed until a visible color developed and then were stopped by the addition of 0.3 ml of Na_2CO_3 and extrapolated to $A_{420/h}$. Either CAT or luciferase promoter activity resulting from transfection of each test construct was normalized to expression of β-galactosidase. For experiments using the 5′ deletion NT/N-CAT plasmids, CAT activities (means ± standard deviations [SD] after normalization for β-galactosidase) for four to eight independent transfections (BON cells) and three independent transfections (AKR-2B, HeLa, and MIA PaCa-2 cells) were expressed as percentages of activity of pSV2CAT (38) measured in parallel sets of dishes. For the mutational analyses performed with BON cells, either CAT or luciferase activities were expressed as percentages of the wild type (positions −216 to +56 [−216/+56]) and are means ± SD of at least four separate transfections.

Preparation of nuclear extracts and in vitro DNase I footprinting. Crude nuclear extracts were prepared from BON cells according to the method described by Shapiro et al. (71). The extracts were quick-frozen and stored in aliquots at −80°C and used within 2 months of extraction.

The rat NT/N promoter fragment (−216/+56) was end labeled on either the noncoding or the coding strand with [α-^{32}P]dATP by a fill-in reaction using DNase I polymerase I Klenow fragment. The appropriate end-labeled fragments were obtained by secondary digestion with a suitable restriction enzyme and fractionation on a 5% polyacrylamide gel. The gel was exposed to X-ray film for 3 min at room temperature, and the radioactive insert band was cut from the gel, eluted in 0.3 M NaAc overnight, and then purified by Elutip-d columns and then by ethanol precipitation. DNase I digestion assays were carried out on ice in a final volume of 50 μl containing 8 mM HEPES (pH 7.9), 40 mM KCl, 10.5% glycerol, 2% polyvinyl alcohol, 10 mM Tris (pH 8.0), 0.6 mM EDTA, 0.7 mM dithiothreitol, and 3 μg of poly(dI · dC) with various amounts of BON nuclear protein. The extract was preincubated for 10 min, after which 25,000 cpm of end-labeled probe was added and the mixture was incubated for an additional 20 min at room temperature. Freshly diluted DNase I in 10 mM $MgCl_2$ was added, and digestion was allowed to proceed for 30 s at 20°C. The amount of DNase I was adjusted empirically for each extract to produce an even pattern of partial cleavage products. Reactions were stopped by the addition of 150 μl of stop solution (5 mM EDTA, 0.5% sodium dodecyl sulfate, 8 M urea). Samples were then extracted with phenol-chloroform, and the DNA was precipitated from the aqueous phase with 2 volumes of ethanol. The DNA pellets were dried and resuspended in sequencing stop buffer (0.1% xylene cyanol, 0.1% bromophenol blue, 10 mM Na_2 EDTA, 95% deionized formamide), and the suspension was incubated at 95°C for 3 min and loaded onto a 6% polyacrylamide–8 M urea sequencing gel. Areas protected from digestion were determined by parallel running of DNA sequencing ladders.

EMSAs and methylation interference analysis. Synthetic oligonucleotides corresponding to the top and bottom strands of wild-type NT/N sequences were synthesized. A single strand was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and then annealed to its complementary nonradiolabeled oligonucleotide. Electrophoretic mobility shift assay (EMSA) mixtures contained 30,000 cpm (~0.5 ng) of ^{32}P-end-labeled oligonucleotide and 10 to 20 μg of nuclear protein in a final volume of 20 μl of 12.5 mM HEPES (pH 7.9)–100 mM KCl–10% glycerol–0.1 mM EDTA–0.75 mM dithiothreitol-0.2 mM phenylmethylsulfonyl fluoride–1 μg of bovine serum albumin with 3 μg of poly(dI · dC) as nonspecific competitors. The reaction was incubated for 20 min at room temperature. Competition binding experiments were performed by first incubating the competitor fragment, in molar excess, with the nuclear protein extract and binding buffer for 10 min on ice. The labeled probe was then added, and incubation continued for 15 min at room temperature. The reaction mixtures were loaded onto 6% nondenaturing polyacrylamide gels and resolved by electrophoresis at 180 V for 2 to 3 h. For antibody studies, 1 to 2 μl of antiserum was added during the preincubation for 20 min at room temperature prior to the addition of labeled probe. The reaction mixtures were then allowed to incubate for an additional 30 min at room temperature before electrophoresis on a 6% nondenaturing polyacrylamide gel for 4 to 5 h. The gels were subsequently dried and autoradiographed at −70°C with an intensifying screen.

For methylation interference analysis, the oligonucleotides were partially methylated with dimethyl sulfate as described by Maxam and Gilbert (57) for 5 min at room temperature. For a typical preparative binding reaction, the usual conditions were scaled up fivefold and binding and gel electrophoresis were as described above. After electrophoresis, the gel was exposed wet for 1 h, and the complex and free fragment bands were excised, eluted, and precipitated according to the method described by Maxam and Gilbert (57). Prior to ethanol precipitation, the solution was extracted sequentially with phenol and chloroform. The pellet was rinsed thoroughly with cold 70% ethanol, dried, and then redissolved in 100 μl of 1 M piperidine. Base cleavage reactions were carried out for 30 min at 90°C, and then the piperidine was removed by lyophilization. After two additional rounds of lyophilization from water, the products were analyzed by electrophoresis through 15 to 20% polyacrylamide gels in the presence of 8 M urea and then by autoradiography at −70°C.

RESULTS

***cis*-Regulatory elements located in the proximal 216 bp of the NT/N promoter control cell-specific expression of the rat NT/N gene.** To delineate the regions of the NT/N promoter that are important for constitutive expression, transient-transfection assays with a series of NT/N reporter genes containing sequential deletions of 5′-flanking DNA cloned upstream of the structural gene encoding CAT were performed. Expression of these constructs in BON cells, a human pancreatic endocrine tumor cell line which constitutively expresses the NT/N gene (28), was compared with that in three cell lines (MIA PaCa-2, HeLa, and AKR-2B) which do not express the NT/N gene (data not shown). As little as 216 bp of 5′-flanking sequence of the rat NT/N promoter was sufficient for maximal reporter gene expression in BON cells, whereas the other cell lines failed to support expression (Fig. 1). Deletion to −173, which removed a distal consensus AP-1 site located at positions −188 to −182, decreased CAT activity in BON cells by approximately 50%. A further deletion to −120 restored transcriptional activity to about the same level as that of the −216 construct, suggesting the presence of a minor negative regulatory element between nucleotides −173 and −120. Progressive deletions from −120 to −62 resulted in stepwise decreases in promoter activity, indicating the presence of several positive regulatory elements. The lowest level of activity in the series of deletion plasmids was observed with NT/N −43/+56, a minimal promoter plasmid in which a proximal CRE/AP-1-like element (−48 to −41) is truncated but which still retains the NT/N TATA box. These results suggest that multiple *cis*-regulatory elements within the region from −216 to −43 contribute to cell-specific expression in BON cells.

Mutational analysis of the rat NT/N promoter identifies multiple *cis* elements required for maximal expression. To identify regions within the proximal 216 bp of the rat NT/N promoter that are essential for its function, a series of linker scanner and internal deletion mutant plasmids were transfected into BON cells. CAT activities generated by the plasmids were determined and were compared with the activity of the wild-type (−216/+56) NT/N promoter fragment (Fig. 2A). Mutations that alter a distal AP-1 site (TGAGTCA), defined by M1 (−189/−182) and M2 (−182/−173), reduced CAT expression by 50%. Linker scanner mutant M3 (−145/−136), which alters a CRE-like half-site (CGTCA), reduced CAT expression minimally (approximately 25%). The internal deletion mutant M4 (−145/−125), which alters the distal CRE-like

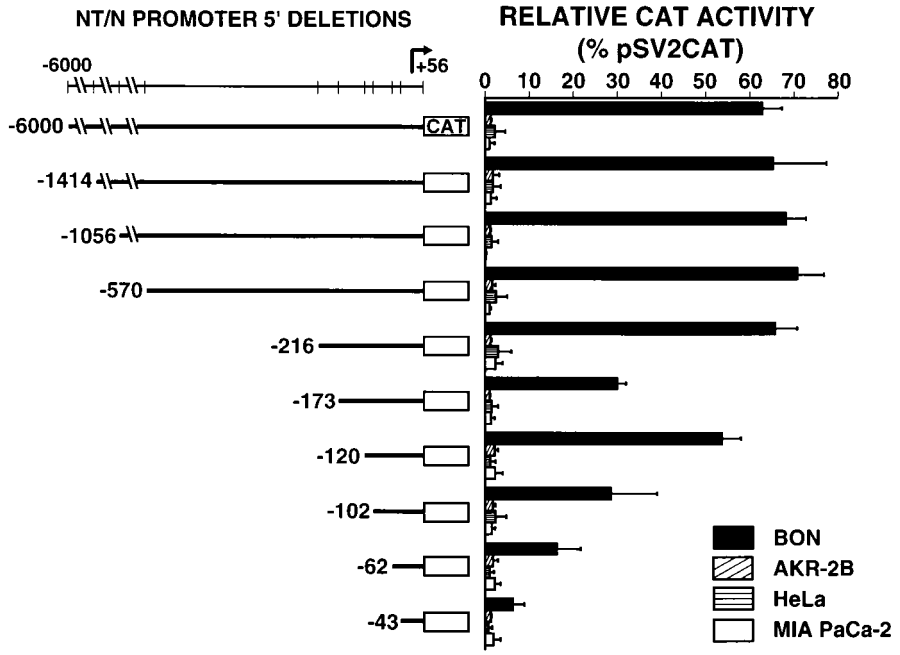


FIG. 1. NT/N transcriptional activity in the NT/N-expressing BON cell line and NT/N-nonexpressing cell lines AKR-2B, HeLa, and MIA PaCa-2. CAT activities (means ± SD) for four to eight independent transfections (BON cells) and three independent transfections (AKR-2B, HeLa, and MIA PaCa-2 cells) are expressed relative to the activity of pSV2CAT transfected in parallel sets of dishes after normalization for differences in transfection efficiency by the β-galactosidase plasmid (pCH110).

motif along with 10 additional downstream nucleotides, increased CAT expression to a level slightly higher than that of the wild-type NT/N promoter fragment, which suggests the binding of a minor repressor protein to this region. Collectively, the results obtained from the mutated NT/N promoter constructs M1 to M4 correlate very well with the changes in CAT activity using the 5′ deletion plasmids shown in Fig. 1.

Examination of mutations that alter sites within the proximal 120 bp of the NT/N promoter (M5 to M13) identified two critical regions that are essential for NT/N expression in BON

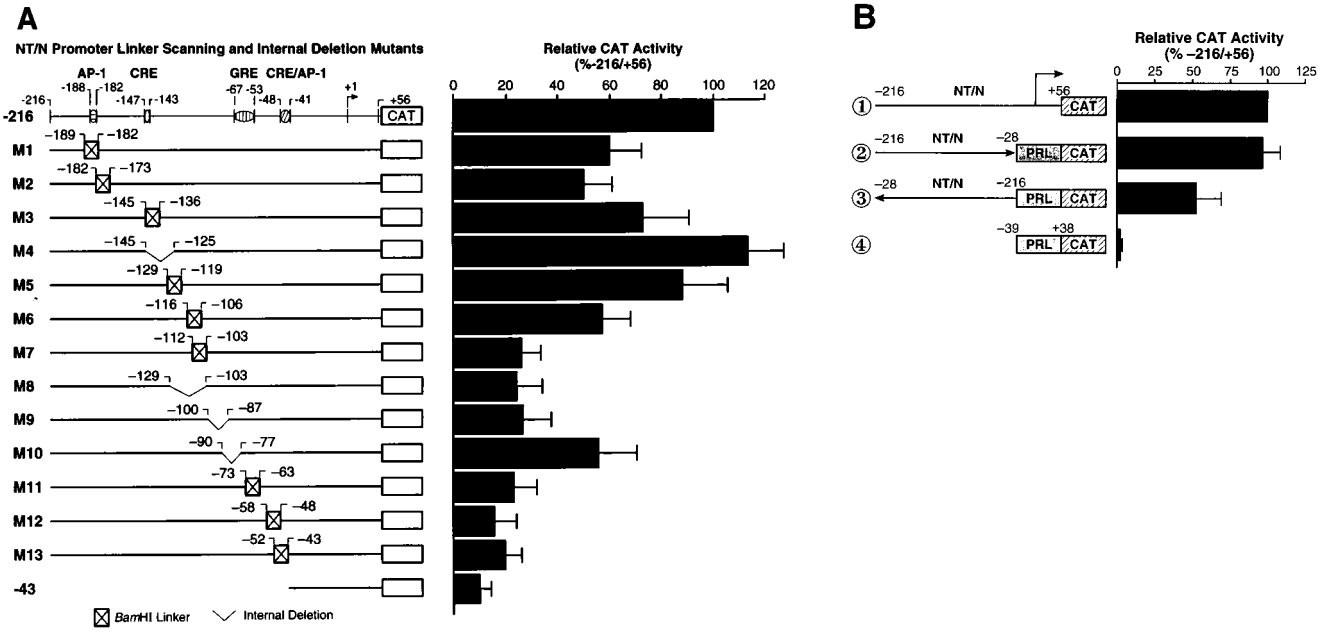


FIG. 2. Mutational analysis in BON. (A) Relative CAT expression generated by the wild type (−216/+56) or linker scanner and internal deletion NT/N-CAT mutants (M1 to M13) after transfection in BON cells. The activity of −43/+56 NT/N CAT was measured for comparison and is also depicted. CAT activities are expressed as percentages of that of the wild type and are means ± SD of four separate transfections after normalization for β-galactosidase expression. (B) Plasmids containing the rat NT/N control region (−216/−28) in either orientation upstream of a minimal heterologous prolactin (PRL) promoter and transfected into BON cells. Relative CAT activities are expressed as percentages of that of the wild-type (−216/+56) NT/N fusion plasmid and are means ± SD of four separate transfections after normalization for β-galactosidase expression.

cells (Fig. 2A). The first region, delineated by the linker scanner mutant M7 (−112/−103) and two mutants with internal deletions, M8 (−129/−103) and M9 (−100/−87), contains two 21-bp purine-rich IDRs. The second region, defined by linker scanner mutants M11 (−73/−63), M12 (−58/−48), and M13 (−52/−43), contains a GRE-like sequence located at positions −67 to −53, that differs by one nucleotide from a functional tyrosine aminotransferase gene GRE (45); a CRE/AP-1-like motif located at −48 to −41, which is identical to a c-*jun* autoregulatory element (3); and a CRE half-repeat (GTCA) immediately upstream at −52 to −49. Selective mutations in both regions produced a 75 to 80% reduction in CAT activity compared with that of the wild-type (−216/+56) NT/N promoter fragment.

We then determined whether the NT/N 5′-flanking sequences upstream of the TATA element could direct expression in BON cells when placed in either orientation in front of a heterologous rat prolactin minimal promoter (Fig. 2B). CAT activity was similar to that of the wild-type rat NT/N promoter when the sequences were placed in the proper orientation, indicating that the *cis*-regulatory sequences necessary for basal expression of NT/N are located 5′ to the rat NT/N TATA box. However, when the sequences were placed in reverse orientation, CAT activity decreased by 50%, suggesting that these sequences do not function in a strictly orientation-independent fashion as has been described for other enhancer regions (7, 16, 25, 59, 67).

DNase I footprinting analysis identifies four protected regions in the rat NT/N promoter. To map binding sites for *trans*-acting factors potentially involved in NT/N gene expression, we performed DNase I footprinting assays using a 272-bp fragment of 5′-flanking, noncoding region of the rat NT/N gene (−216/+56). Incubation with increasing amounts of BON nuclear protein resulted in the appearance of four regions of protection (Fig. 3A). As shown in Fig. 3B, region I (−58/−38) contains a CRE/AP-1-like sequence (TGACATCA) at −48 to −41 and the proximal portion of a putative, near-consensus GRE. Region II (−150/−124) contains a CRE-like site (CGTCA) (60) located at nucleotides −147 to −143. Region III (−168/−155) contains a potential E-box binding site (CAGCTG) (46, 77, 78) and a sequence from −162 to −158 (GCAAT) that is similar to that of the binding site for C/EBP (12, 35, 69). Region IV (−198/−175) contains a consensus AP-1 site (TGAGTCA) at −188 to −182. No protection was noted at sites between nucleotides −116 and −87, despite the deleterious effect of mutations in this region; however, proteins that specifically bind to this region were detected by EMSAs (see below).

A complex of proteins that include c-Jun, JunD, ATF-1, ATF-2, and CREB binds to NT/N region I. To better define the footprinted region I of the NT/N promoter, DNase I footprinting assays were repeated by using the coding strand of the rat NT/N promoter (−216/+56) (Fig. 4A). Region I (−58/−38) was well delineated with increasing amounts of BON nuclear protein. The addition to the binding reaction mixture of a synthetic oligonucleotide (WT1), corresponding to nucleotides −60 to −36 of the NT/N promoter, resulted in the disappearance of the footprint. EMSAs with BON nuclear extracts and the ^{32}P-labeled NT/N promoter fragment WT1 were then utilized to define the proteins that interact with footprinted region I (Fig. 4B). Multiple DNA-protein complexes which were efficiently abolished with excess unlabeled WT1 and an oligonucleotide containing consensus CRE sites were detected. Furthermore, an oligonucleotide containing consensus AP-1 binding sites effectively competed for binding to labeled WT1 with complete inhibition of complex B and partial competition for the remaining complexes. In contrast, none of the complexes

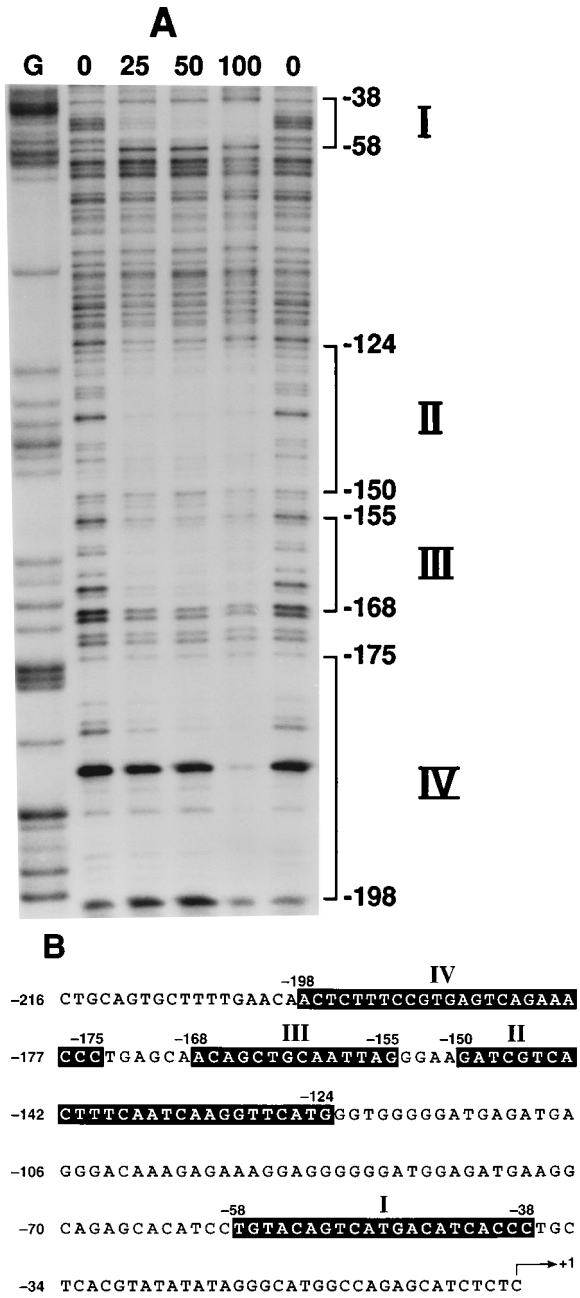
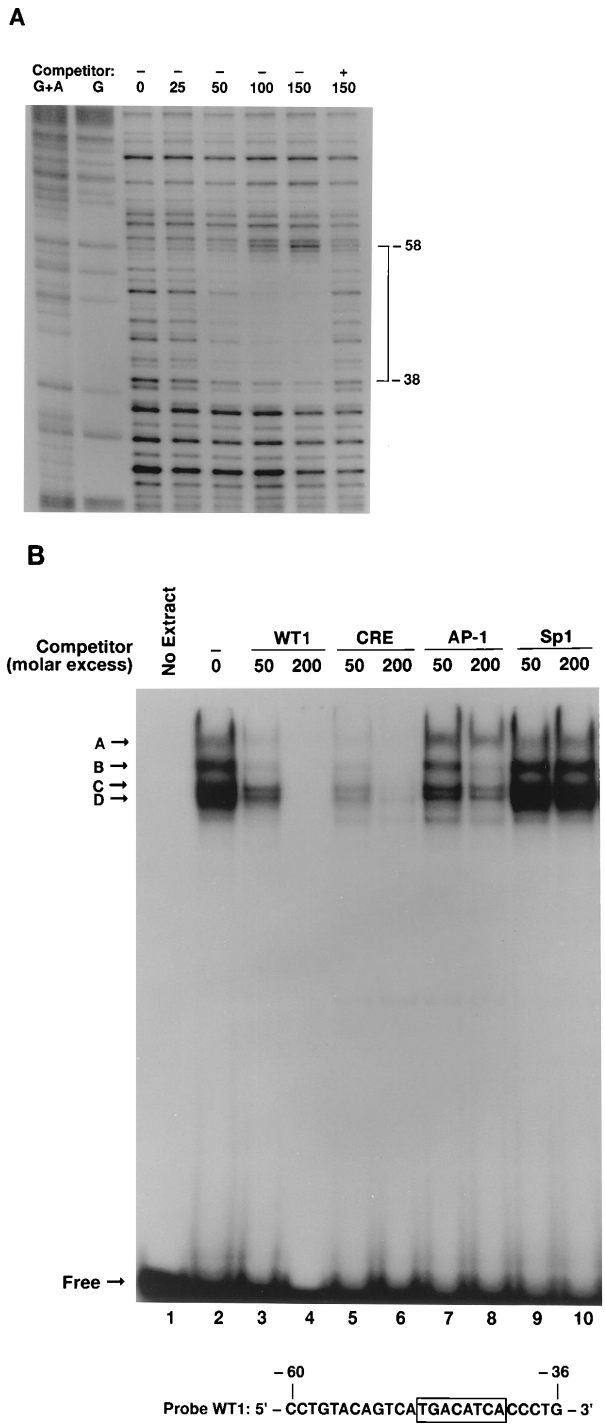


FIG. 3. In vitro DNase I footprint analysis of the NT/N gene promoter. (A) A rat NT/N gene fragment from nucleotides −216 to +56 was labeled on the noncoding strand, incubated with nuclear extracts from BON cells, and digested with DNase I. Areas protected from digestion are bracketed with the corresponding nucleotide positions determined by parallel running of a DNA sequencing ladder (G reaction). The numbers at the top of the autoradiogram are the amounts of nuclear extract (in micrograms of protein) used in each reaction mixture. (B) Sequence of the rat NT/N promoter fragment from −216 to +1 and DNase I-protected regions I to IV (shown enclosed in dark boxes).

was affected by an oligonucleotide containing Sp1 binding sites, which provides additional evidence that these complexes resulted from sequence-specific binding. To further confirm interaction with CREB-like and AP-1 binding proteins, the oligonucleotides containing the consensus CRE and AP-1 sites, which were used as competitors in Fig. 4B, were labeled and used in EMSAs with BON nuclear extract. Unlabeled



WT1 competed for binding to both the CRE- and AP-1-labeled oligonucleotide (data not shown). Taken together, these data suggest that both CREB-like and AP-1 proteins bind specifically to probe WT1.

To further elucidate the proteins binding to footprinted region I of the rat NT/N promoter, antisera specific for AP-1 and CREB/ATF/CREM family members (obtained from Santa Cruz Biotechnology) were used in EMSAs (Fig. 4C). Supershifted complexes were noted by using antisera to c-Jun (lane 3), JunD (lane 4), ATF-1 (lane 7), ATF-2 (lane 8), and CREB (lane 9). Complex A was made up predominantly by ATF-2 and c-Jun proteins with lesser reactivity to ATF-1. Complex B was partially supershifted by antibodies specific to JunD and ATF-2. Complex C was partially supershifted in the presence of anti-CREB, anti-ATF-1, and anti-JunD. Finally, complex D was partially supershifted by anti-JunD. In contrast, antibodies to JunB, c-Fos (Fig. 4C) and FosB, Fra-1, Fra-2, ATF-3, ATF-4, and CREM (data not shown) did not form a supershift or prevent formation of the DNA-protein complex. Taken together, these results identify multiple proteins which include at least c-Jun, JunD, ATF-1, ATF-2, and CREB binding to the proximal NT/N promoter element.

JunD, c-Fos, and Fra-1 proteins bind to the distal consensus AP-1 site (region IV) of the NT/N promoter. We then identified the proteins that bind to footprinted region IV of the rat NT/N promoter. As a first step, an oligonucleotide (WT6) corresponding to positions −198 to −175 of the rat NT/N promoter was used in EMSAs with nuclear extracts from the BON cell line (Fig. 5A). A single DNA-protein binding complex was demonstrated which was effectively inhibited by both unlabeled WT6 and an oligonucleotide containing consensus AP-1 binding sites. In contrast, a nonspecific oligonucleotide corresponding to Sp1 consensus sites failed to disrupt binding of BON nuclear extracts to WT6. These results confirm binding of AP-1 proteins to this distal promoter element.

Preincubation with antisera to the Jun and Fos families of transcription factors were then performed to determine the specific proteins binding to this region (Fig. 5B). Antisera specific to JunD (lane 5), c-Fos (lane 7), and Fra-1 (lane 9), but not c-Jun, JunB, FosB, or Fra-2, produced a supershifted pattern with BON nuclear extract; however, the amounts of immune complexes were small, especially with antibodies to c-Fos and Fra-1. Doubling the amount of antisera in the supershift assays exhibited no change in the intensities of the supershifted bands (data not shown), indicating that the amounts used were sufficient. Collectively, these results identify JunD, c-Fos, and Fra-1 as part of the protein complex that

FIG. 4. Identification of the AP-1 and CREB/ATF proteins that bind to region I (−60/−36) of the NT/N promoter. (A) To better delineate protected region I, the coding strand of the NT/N promoter fragment (−216/+56) incubated with nuclear extracts from BON cells. (The numbers on top of the autoradiogram are the amounts of nuclear extract [in micrograms of protein] used in each reaction mixture.) The area protected from digestion is bracketed with the corresponding nucleotide positions determined by parallel running of DNA sequencing ladders (G+A and G reactions). A 100-fold molar excess of an oligonucleotide corresponding to an NT/N promoter fragment from −60 to −36 was used as a competitor (last lane). (B) Competitive binding analysis by gel retardation assays with BON nuclear extracts (10 μg) and the wild-type (WT1) NT/N promoter fragment (−60/−36) as a probe. (Boxed sequences designate the CRE/AP-1 site.) Unlabeled WT1 and oligonucleotides containing consensus CRE, AP-1, and Sp1 sites (Promega) were used as competition DNAs at 50- and 200-fold molar excesses. At least four specific complexes were noted and were labeled A through D. (C) Nuclear extracts (10 μg per lane) from BON cells were preincubated with antisera (2 μl) to the AP-1 and CREB/ATF proteins (Santa Cruz) prior to the addition of labeled WT1. Supershifted complexes were noted for c-Jun (lane 3), JunD (lane 4), ATF-1 (lane 7), ATF-2 (lane 8), and CREB (lane 9). IgG, immunoglobulin G.

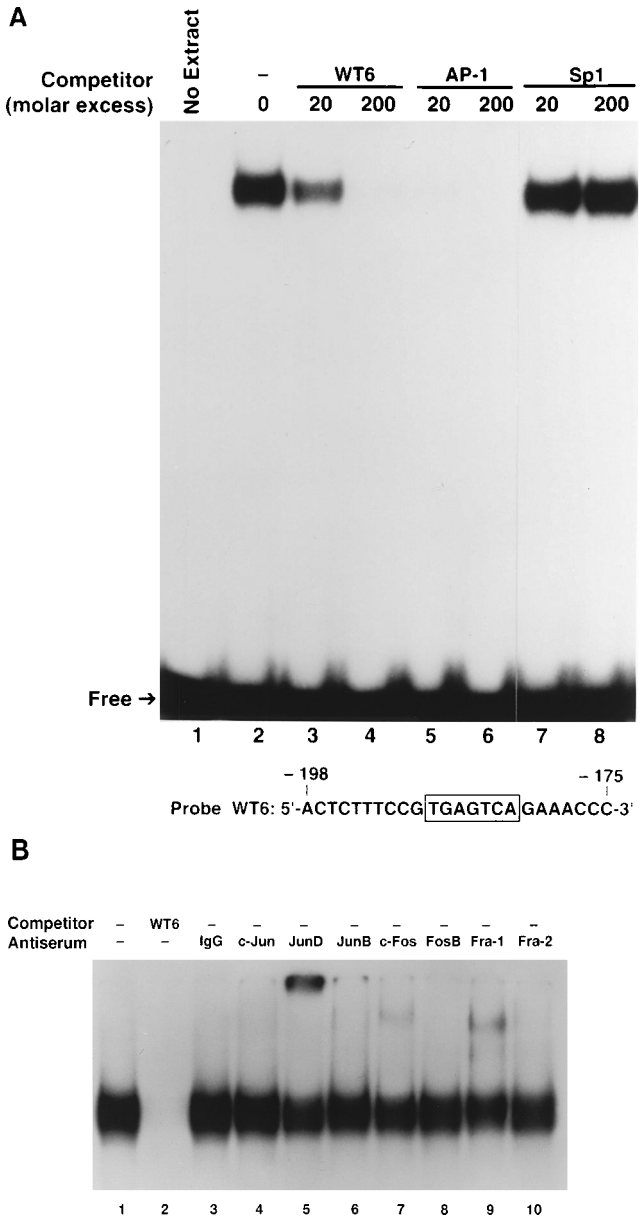


FIG. 5. Identification of the AP-1 proteins that bind to region IV (−198/−175) of the NT/N promoter. (A) Competitive binding analysis by gel retardation assays with BON nuclear extracts (10 μg) and the wild-type (WT6) NT/N promoter fragment (−198/−175) as a probe. (Boxed sequences designate the consensus AP-1 site.) Unlabeled WT6 and oligonucleotides containing consensus AP-1 and Sp1 sites were used as competition DNAs at 20- and 200-fold molar excesses. (B) Nuclear extracts from BON cells were preincubated with unlabeled WT6 (300-fold excess) or specific AP-1 antiserum (Santa Cruz) as indicated. A supershifted complex for JunD (lane 5), c-Fos (lane 7), and Fra-1 (lane 9) is noted. IgG, immunoglobulin G.

binds to region IV of the rat NT/N promoter. Given the fact that only a relatively small portion of the complex is actually supershifted with a battery of antisera to known AP-1 proteins suggests the possibility of additional AP-1 factors that may be unique to the BON endocrine cell line.

Distinct patterns of binding to the IDR regions of the NT/N promoter. Although no areas of protection were detected over the two IDR regions by using DNase I footprinting assays, the functional significance of these sequences for NT/N expression was established by both deletion and mutation analyses (Fig. 1 and 2). Therefore, to determine whether proteins bind these sites, we used overlapping 30-bp oligonucleotides covering this region in EMSAs and methylation interference analyses (Fig. 6). Incubation of BON nuclear extract with probe WT2 (−122/−93), corresponding to the distal IDR, produced a specific retarded DNA-protein complex that was abolished with excess unlabeled WT2; however, oligonucleotide WT3 (−96/−67), which corresponds to the proximal IDR, only partially competed for binding to WT2 (Fig. 6A). When the WT3 oligonucleotide was used as a probe, a faster-migrating ill-defined complex was detected as well as a specific more slowly migrating DNA-protein complex (Fig. 6B). Both complexes were effectively inhibited by increasing concentrations of unlabeled WT3; only the upper complex was partially inhibited by an excess of unlabeled oligonucleotide WT2. In addition, an oligonucleotide containing the nonspecific Sp1 binding sites failed to alter the binding pattern for either WT2 or WT3 (data not shown). These observations suggest the binding of specific proteins to both IDR regions of the NT/N promoter.

To then determine the locations of the binding sites within the IDR regions, we used the technique of methylation interference. End-labeled DNA fragments corresponding to the GA-rich coding strands of WT2 and WT3 were partially methylated at purine contact points (Fig. 6C and D). As shown in Fig. 6C, methylation of four G residues on the coding strand of WT2 interferes with protein binding, thus illustrating that the contacted regions are located in the sequences from −111 to −104 (GATGAGGG) of the distal IDR. Mutation of this region, as noted by linker scanner mutant M7 (−112/−103) (Fig. 2A), reduces NT/N promoter activity by approximately 75%, which strongly suggests that the protein binding this element acts as a positive factor for NT/N transcription. Analysis of the proximal IDR indicated that a similar sequence (GGATGGAG) is required for complex formation (Fig. 6D). These results serve to further confirm binding of protein factor(s) to the IDR regions and, in addition, have localized the binding of the proteins to similar sequences contained within the IDRs.

To examine whether the complexes binding to the WT2 and WT3 probes were cell specific, nuclear extracts that had been prepared from several additional cell lines in which the NT/N gene is not expressed were analyzed (Fig. 6E). No specific complexes corresponding to those detected in BON cell extracts were formed by using HeLa and SKBR-3 (a human breast cancer cell line) nuclear extracts, although MIA PaCa-2 (human pancreatic cancer) extracts formed a faint complex with the WT2 probe that comigrated with the BON cell complex. Binding complexes with mobilities different from that noted for BON cell extract were also identified in the NT/N-nonexpressing cell lines. Each of the extracts used in this analysis contained intact nuclear proteins, as evidenced by the detection of specific complexes to a binding site which recognizes the ubiquitous Sp1 transcription factor (data not shown). This analysis shows that the patterns of binding proteins in these cell lines differed from the NT/N-expressing BON cells. Finally, a computer search analysis of known *cis*-acting elements revealed partial sequence similarity of this rat NT/N promoter region to GATA-binding sites that have been described mainly for genes of hematopoietic cells (64) and, more recently, for the intestine (5) and to a purine-rich GAGA sequence located in the rat insulin proximal promoter (48); however, protein binding to labeled WT2 or WT3 was not affected by oligonucleotides containing either GATA or GAGA consensus binding sites (data not shown). Taken to-

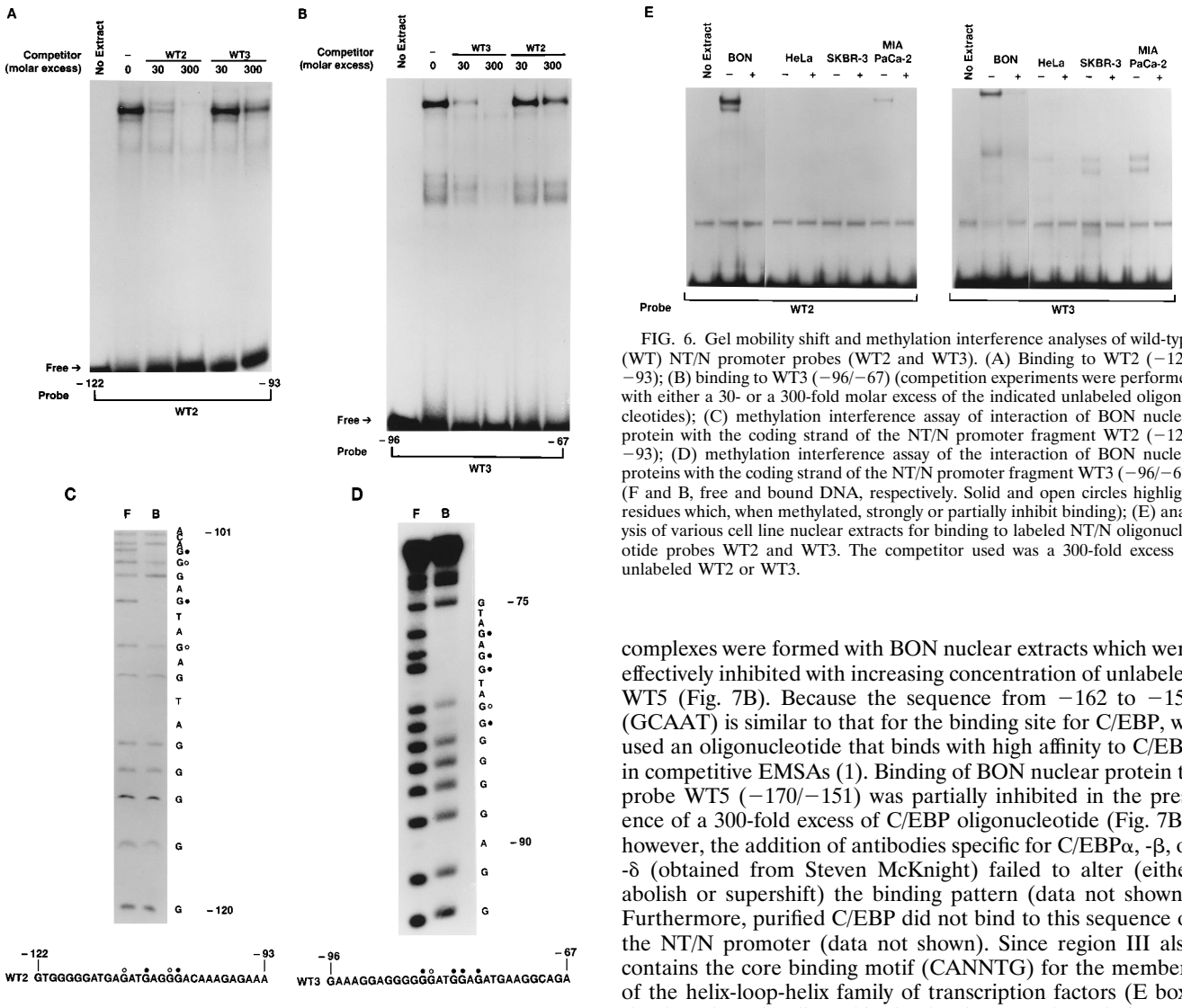


FIG. 6. Gel mobility shift and methylation interference analyses of wild-type (WT) NT/N promoter probes (WT2 and WT3). (A) Binding to WT2 (−122/−93); (B) binding to WT3 (−96/−67) (competition experiments were performed with either a 30- or a 300-fold molar excess of the indicated unlabeled oligonucleotides); (C) methylation interference assay of interaction of BON nuclear protein with the coding strand of the NT/N promoter fragment WT2 (−122/−93); (D) methylation interference assay of the interaction of BON nuclear proteins with the coding strand of the NT/N promoter fragment WT3 (−96/−67) (F and B, free and bound DNA, respectively. Solid and open circles highlight residues which, when methylated, strongly or partially inhibit binding); (E) analysis of various cell line nuclear extracts for binding to labeled NT/N oligonucleotide probes WT2 and WT3. The competitor used was a 300-fold excess of unlabeled WT2 or WT3.

gether, these findings suggest the possibility of novel factors binding to these purine-rich sequences of the NT/N promoter and acting as positive regulators of NT/N expression.

Competitive binding analyses of protected regions II and III of the NT/N promoter. DNase I footprint analysis indicated binding of BON nuclear proteins to site II (−150/−124) and site III (−168/−155) of the NT/N promoter; EMSAs were used to further characterize the nuclear proteins that bind these two regions. A single specific complex was formed with BON nuclear extract using an oligonucleotide (WT4) corresponding to nucleotides −150 to −125 of the NT/N promoter (Fig. 7A). Region II contains a CRE-like half site (CGTCA) that, when altered, curtails forskolin-mediated NT/N gene induction in PC12 cells (50); however, a consensus CRE oligonucleotide competed poorly for complex formation, suggesting that sequences outside the CGTCA motif are required for efficient complex formation (Fig. 7A). Furthermore, antibodies to CREB, ATF-1, and ATF-2 failed to supershift the complex, indicating that these proteins do not bind to region II (data not shown).

EMSAs were then used in an attempt to better characterize binding to protected site III of the NT/N promoter. Multiple complexes were formed with BON nuclear extracts which were effectively inhibited with increasing concentration of unlabeled WT5 (Fig. 7B). Because the sequence from −162 to −158 (GCAAT) is similar to that for the binding site for C/EBP, we used an oligonucleotide that binds with high affinity to C/EBP in competitive EMSAs (1). Binding of BON nuclear protein to probe WT5 (−170/−151) was partially inhibited in the presence of a 300-fold excess of C/EBP oligonucleotide (Fig. 7B); however, the addition of antibodies specific for C/EBPα, -β, or -δ (obtained from Steven McKnight) failed to alter (either abolish or supershift) the binding pattern (data not shown). Furthermore, purified C/EBP did not bind to this sequence of the NT/N promoter (data not shown). Since region III also contains the core binding motif (CANNTG) for the members of the helix-loop-helix family of transcription factors (E box) that have been implicated in the developmental regulation of a wide variety of genes including the GI hormones insulin (46), secretin (78), and gastrin (77), we also used oligonucleotides with E box sequences from the secretin (SEB) or insulin (IEB) gene promoter; the binding of nuclear protein to WT5 was not inhibited in the presence of excess E-box oligonucleotides. Therefore, our results indicate that the proteins binding to this region are unlikely to be members of the E-box transcription factor family or C/EBP, although the possibility exists that other CCAAT box-binding proteins such as CP1, CTF/NF-1, and CBF (19, 55) bind region III.

Finally, to assess the possible functional significance of the proteins binding to protected region III of the NT/N promoter, a series of sequential 6-bp mutations spanning this promoter region were cloned upstream of the luciferase gene and analyzed by transfection into BON cells (Fig. 7C). Mutations within this region (con2 and con3) moderately reduced NT/N promoter activity to approximately 40 to 50% of the wild-type (−216/+56) vector, whereas mutations involving the distal three nucleotides of region III (con1) and the intervening nucleotides between regions II and III (con4) produced no reduction in promoter strength. Taken together, our findings indicate that, similarly to protected region IV, proteins binding to region III of the NT/N promoter contribute to the overall

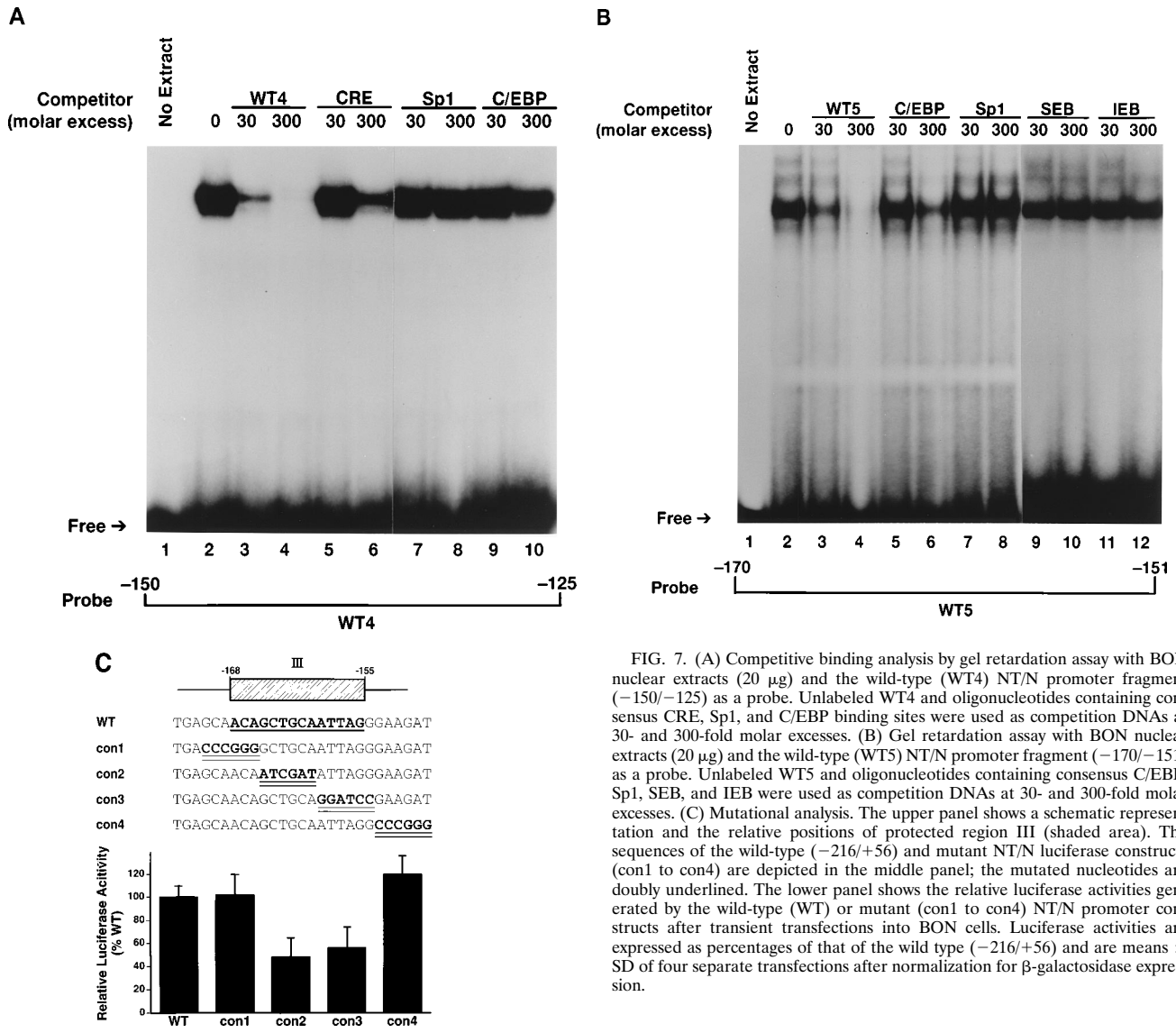


FIG. 7. (A) Competitive binding analysis by gel retardation assay with BON nuclear extracts (20 μg) and the wild-type (WT4) NT/N promoter fragment (−150/−125) as a probe. Unlabeled WT4 and oligonucleotides containing consensus CRE, Sp1, and C/EBP binding sites were used as competition DNAs at 30- and 300-fold molar excesses. (B) Gel retardation assay with BON nuclear extracts (20 μg) and the wild-type (WT5) NT/N promoter fragment (−170/−151) as a probe. Unlabeled WT5 and oligonucleotides containing consensus C/EBP, Sp1, SEB, and IEB were used as competition DNAs at 30- and 300-fold molar excesses. (C) Mutational analysis. The upper panel shows a schematic representation and the relative positions of protected region III (shaded area). The sequences of the wild-type (−216/+56) and mutant NT/N luciferase constructs (con1 to con4) are depicted in the middle panel; the mutated nucleotides are doubly underlined. The lower panel shows the relative luciferase activities generated by the wild-type (WT) or mutant (con1 to con4) NT/N promoter constructs after transient transfections into BON cells. Luciferase activities are expressed as percentages of that of the wild type (−216/+56) and are means ± SD of four separate transfections after normalization for β-galactosidase expression.

high-level expression of NT/N noted in the BON endocrine cell line. Modest reductions in NT/N promoter activity were demonstrated with the introduction of specific mutations into both of these sites; however, the most significant reductions in NT/N promoter activity occurred with mutations of sites contained within the proximal 120 nucleotides upstream of the transcriptional start site.

DISCUSSION

The findings in our present study identify crucial *cis*-regulatory elements of the NT/N gene that are required for cell-specific transcriptional activity in the novel endocrine cell line BON. These elements include a pivotal area (region I) of the proximal NT/N promoter that binds ubiquitous AP-1 and CREB/ATF proteins and a purine-rich region, located upstream from the proximal CRE/AP-1 site, which functions as an essential component of the NT/N enhancer and may, in fact, bind proteins unique to NT/N-producing cells. Furthermore, we show that a distal consensus AP-1 site binds JunD, c-Fos, and Fra-1 proteins and contributes to maximal NT/N expression, albeit at a level much lower than those of the more proximal elements.

Protected region I, which is located between nucleotides −58 and −38 of the proximal rat NT/N promoter, is remarkably well-conserved in both the rat and the human NT/N genes (10) and contains a near-consensus CRE/AP-1-like motif (TGACATCA) that binds a complex of proteins which include c-Jun, JunD, CREB, ATF-1, and ATF-2. This proximal promoter element of the NT/N gene is identical to an autoregulatory element present in the proximal promoter of the c-*jun* protooncogene which binds AP-1 complexes (3) and CREB and CREM (CRE modulator) dimers (52, 56). Both CREB and CREM binding produces repression of c-*jun*-positive autoregulation exerted by the c-*jun* product (56). In addition, three other CRE-like enhancer elements with the same G-to-A substitution as that found in the NT/N promoter have been described. An element (δA) found in the T-cell receptor α and β and CD3 promoters binds three different isoforms of ATF-2 and functions as an independent enhancer in T cells, mediating changes in gene expression during T-cell development (36, 37). In another example, an identical site is present in the human

beta interferon promoter that binds ATF-2 and is required for virus induction (24). Finally, the same TGACATCA site in the proximal promoter of the E selectin gene binds c-Jun and ATF (ATFa, ATF-2, and ATF-3) proteins either as homo- or heterodimers and cooperates with a nearby NF-κB site to regulate E selectin promoter activity (47, 66). Therefore, this multifunctional element, which is located in the proximal NT/N promoter, serves a pivotal role in the regulation of NT/N gene expression and the tissue-specific or developmental expression of a limited number of other genes.

The Jun, Fos, ATF, and CREB proteins belong to the leucine zipper family of proteins (15). Jun proteins can homodimerize or heterodimerize with proteins of the Fos family (15, 21). In addition, the different CREB/ATFs will selectively heterodimerize with each other or with other members of the leucine zipper family, such as c-Jun, to generate either stimulatory or inhibitory complexes (41, 54, 66). This functional cross-talk between different signaling pathways may be a mechanism whereby ubiquitous members of various transcription factor families can limit the expression of certain genes. Furthermore, there exist both qualitative and quantitative tissue type specificities in the relative distributions of the different AP-1- and CREB/ATF-binding proteins, suggesting that these factors play a role in tissue-specific or developmental gene expression (4, 41). With the binding of multiple AP-1 and CREB/ATF proteins to the TGACATCA element of the NT/N proximal promoter, the vast combinatorial possibilities of these transcription factors to regulate NT/N gene expression can be envisioned, thus providing a powerful system that limits NT/N expression to specific tissues during various stages of development.

In addition to the proximal NT/N promoter element, mutation of sites between nucleotides −116 and −87, containing two 21-bp purine-rich IDRs, resulted in an 80% loss of NT/N promoter activity in BON cells and, except for alteration of the CRE/AP-1-like sequence, represented the most deleterious mutation in the 5′-flanking region. In marked contrast, the IDRs play a relatively minor role in NT/N induction in PC12 cells, with mutations only slightly reducing the response to inducer combinations (50). Although a possible explanation for the results obtained in our study is the disruption of the spatial architecture of transcriptionally active complexes of the NT/N promoter created by the internal deletion mutants −129/−103 and −100/−87, evidence to support the importance of this region for NT/N expression in BON cells is provided by the finding of a similar reduction in CAT activity obtained by the linker scanner mutant −112/−103 and by the results of the 5′ deletion analysis demonstrating progressive reductions in promoter activity when nucleotides between −120 to −62 are removed. Furthermore, gel retardation studies with oligonucleotides to this purine-rich IDR region identified specific binding patterns with BON nuclear extract. Taken together, these results strongly support the functional significance of this purine-rich region of the NT/N promoter for cell-type-specific expression in BON cells.

DNA sequence analysis of this IDR region highlighted similarities to known transcription factor binding sites that include a near-consensus GATA site (5, 64) and a consensus GAGA box that plays a major role in transcription of the rat insulin I gene (48). Competitive binding assays using oligonucleotides containing either consensus GATA or GAGA sites, however, failed to inhibit binding of BON extract to labeled rat NT/N oligonucleotides, suggesting the possibility of novel proteins that bind to this purine-rich region of the rat NT/N promoter and that are essential for NT/N gene expression. This possibility is further corroborated by the differences noted in protein binding of NT/N-nonexpressing cell lines to labeled NT/N probes corresponding to this GA-rich promoter region compared with the binding pattern of BON cell extract. Although not specifically identified in our present study, the molecular nature of these binding proteins should be revealed through more extensive biochemical analysis and from eventual molecular cloning of the corresponding genes. Collectively, our findings indicate that the complex of proteins that assemble on the proximal 120 bp of the NT/N promoter contains both ubiquitous (AP-1 and CREB/ATF) and possibly novel transcription factors which may interact such that they culminate in the high-level expression of the NT/N gene in N cells of the gut.

Expression of the NT/N gene is developmentally regulated in a well-defined pattern along the jejunoileal axis (32). This tightly regimented expression pattern is not altered by ectopic placement of fetal small bowel segments or changes in luminal dietary contents (27). In marked contrast, NT/N expression can be rapidly induced in certain regions of the brain in response to various neuroleptic agents such as haloperidol, which increases c-*fos* gene transcription (58, 72). Similarly, in PC12, a cell line that differentiates into a neuronal phenotype after NGF treatment (40), NT/N gene expression is dramatically induced in response to the synergistic interaction of multiple inducing agents that include NGF (50). This increase in the level of NT/N expression is dependent on an intact consensus AP-1 site located at −188 to −182 which serves as the functional focal point for NT/N gene induction in PC12 cells. In our present study, we demonstrate that this AP-1 site binds a conglomerate of proteins that include c-Fos, Fra-1, and JunD but, in contrast to NT/N induction in PC12, plays a comparatively minor role in constitutive NT/N expression in BON cells. Therefore, our findings suggest that a different array of regulatory elements is required for the constitutive NT/N expression pattern in the gut, in which NT functions as an endocrine agent, compared with NT/N gene induction in the central nervous system, in which NT serves as a neurotransmitter or neuromodulator. Teleologically, this possibility would allow an agent (such as NT/N) to serve multiple and diverse functions in vivo, depending on the locations and the presence or absence of certain transcription factors.

An additional level of NT/N gene regulation could be provided by a near-consensus GRE, located immediately upstream of the proximal CRE/AP-1-like motif. This site binds purified GR (43) and is required for dexamethasone induction of NT/N in PC12 cells (50). Mutations altering this GRE-like sequence reduced CAT activity in BON cells to the same level as mutation of the proximal CRE/AP-1-like motif, thus suggesting a functional significance of this site for constitutive NT/N expression. Furthermore, it has become increasingly obvious that cell-specific control of eukaryotic gene transcription involves negative as well as positive regulation (7, 16, 25, 59, 67); therefore, another level of NT/N gene regulation could be provided by the binding of a repressor protein between nucleotides −145 and −125. Future studies will address possible interactions of the GR with AP-1 proteins in BON cells and the possible role of minor repressor elements in overall NT/N gene regulation.

In conclusion, transient-expression assays with a series of reporter gene constructions containing progressive 5′ deletions or specific mutations indicate that efficient cell-specific transcription of the NT/N gene requires the complex interaction of multiple *cis*-active elements located between nucleotides −216 and −28 of the 5′-flanking region, with the most important sites localized to the proximal 120 bp upstream from the transcriptional start site. These positive elements include a CRE/AP-1-like motif that binds multiple members of the AP-1 and

CREB/ATF transcription factor families and a purine-rich IDR region that may bind novel transcription factors. Collectively, our findings that multiple regulatory elements are required to achieve maximal levels of NT/N expression in BON cells are consistent with the regulation of expression in other genes (7, 16, 59) and, moreover, underscore the complexity of eukaryotic gene regulation. Furthermore, our study demonstrates that the BON cell line, which is identical to the intestinal N-cell in the expression and processing of NT/N, will provide a unique and attractive model for the further characterization of factors regulating cell-specific transcription of the NT/N gene and, in addition, may provide insights into the molecular mechanisms responsible for the stage-specific differentiation of the N-cell lineage in the gut.

ACKNOWLEDGMENTS

We thank Sam Wilson and Robert Carraway for helpful suggestions throughout the course of this work and for their critical reading of the manuscript. In addition, we thank Larry Janecka, Gene Dockal, and Eileen Figueroa for manuscript preparation.

This work was supported by grants AG10885 (B.M.E.), HL33307 (P.R.D.), and DK35608 and RO1 DK48345 (C.M.T.) from the National Institutes of Health; CB-571 from the American Cancer Society (C.M.T.); 15867 from the Shriners' Burns Institute (B.M.E.), and by the James E. Thompson Memorial Foundation.

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