Transcriptional Activation of the Human Brain-derived Neurotrophic Factor Gene Promoter III by Dopamine Signaling in NT2/N Neurons

Received for publication, November 12, 2002, and in revised form, May 8, 2003
Published, JBC Papers in Press, May 8, 2003, DOI 10.1074/jbc.M211539200

Hung Fang‡, Joanne Chartier, Caroline Sodja, Angele Desbois, Maria Ribecco-Lutkiewicz, P. Roy Walker, and Marianna Sikorska
From the Neurobiology Program, Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada

We have identified a functional cAMP-response element (CRE) in the human brain-derived neurotrophic factor (BDNF) gene promoter III and established that it participated in the modulation of BDNF expression in NT2/N neurons via downstream signaling from the D1 class of dopamine (DA) receptors. The up-regulation of BDNF expression, in turn, produced neuroprotective signals through receptor tyrosine kinase B (TrkB) and promoted cell survival under the conditions of oxygen and glucose deprivation. To our knowledge this is the first evidence showing the presence of a functional CRE in the human BDNF gene and the role of DA signaling in establishing transcriptional competence of CRE in postmitotic NT2/N neurons. This ability of DA to regulate the expression of the BDNF survival factor has a profound significance for the nigrostriatal pathway, because it indicates the existence of a feedback loop between the neurophin, which promotes both the maturation and survival of dopaminergic neurons, and the neurotransmitter, which the mature neurons ultimately produce and release.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family, structurally related to nerve growth factor, neurotrophin-3, and neurotrophin-4/5. Its biological activity is mediated by tyrosine kinase receptor B (TrkB) and its downstream signaling (1). The gene is highly expressed and widely distributed in the central nervous system, and it plays a significant role in the maintenance of function and survival of neurons (for review, see Ref. 2). Evidence accumulated in recent years suggests that BDNF is also involved in the modulation of synaptic activity in the adult brain, producing long-lasting changes in synaptic structure and function (for reviews, see Refs. 3–5).

There is a growing interest in BDNF as a potential therapeutic agent for neurodegenerative diseases, because its deficiency was found in brains of both Alzheimer’s and Parkinson’s patients (6–10). Indeed, BDNF treatment has been shown not only to potentiate synaptic transmission in vivo (11, 12) but also to increase neuronal survival and augment some behavioral changes in animal models (13, 14). However, more recent data indicate that BDNF can also induce behavioral sensitization by causing an overexpression of dopamine D3 receptors and could, actually, contribute to the amplification of pathophysiological associations with conditions such as epilepsy, drug addiction, schizophrenia, and Parkinson’s disease (15, 16). Clearly, further work is required to resolve some of these potential side-effects.

In contrast to a large body of work on the temporal and spatial patterns of BDNF expression in neurodevelopment and neurodegeneration, relatively little is known about the transcriptional regulation of the human BDNF gene. This is partially due to the fact that the genomic structure of the human gene has not yet been fully elucidated. The gene was first localized to chromosome 11p13 and predicted to consist of multiple exons (17), but the existence of multiple transcripts, derived from different exons, was demonstrated only recently by Aoyama et al. (18) in human neuroblastoma cells. A more detailed transcript mapping of an 810-kb region of chromosome 11p13-14 further defined its genomic localization, although no additional information on the actual structure of the gene itself was presented (19).

The existence of multiple human BDNF transcripts (18) is consistent with a genomic structure similar to that of the rat gene, which consists of four short 5’ exons, each controlled by a distinct promoter, and one 3’ exon encoding the mature BDNF protein (20, 21). In the rat, the four promoters direct expression of the BDNF gene in a tissue-specific manner, i.e. promoters I and II are active preferentially in neurons, whereas promoters III and IV are active both in neurons and in a limited number of non-neuronal tissues such as lung and heart (20, 21). Thus far, only a limited characterization of a 3.2-kb genomic fragment of the human gene containing some structural elements of a promoter was reported (22).

Recently, two transcription factors, CREB and a calcium-responsive factor, were identified as positive regulators of the rat promoter III (23–25), and the neuron-restrictive silencer factor as a negative regulator of promoters I and II (20, 26). It is still not known whether the regulatory machinery controlling the rat BDNF gene is conserved in humans.

Several studies indicate a direct involvement of neurotransmitters such as glutamate, GABA, acetylcholine, serotonin, and dopamine in the transcriptional regulation of BDNF gene (27–31) but the molecular components involved in this

† To whom correspondence should be addressed: Neurobiology Program, Institute for Biological Sciences, National Research Council of Canada, M-54, 1500 Montreal Rd., Ottawa, Ontario K1A 0R6, Canada. Tel.: 613-990-0891; Fax: 613-941-4475; E-mail: hung.fang@nrc.ca.
‡ The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received for publication, November 12, 2002, and in revised form, May 8, 2003
Published, JBC Papers in Press, May 8, 2003, DOI 10.1074/jbc.M211539200

Hung Fang‡, Joanne Chartier, Caroline Sodja, Angele Desbois, Maria Ribecco-Lutkiewicz, P. Roy Walker, and Marianna Sikorska
From the Neurobiology Program, Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada

We have identified a functional cAMP-response element (CRE) in the human brain-derived neurotrophic factor (BDNF) gene promoter III and established that it participated in the modulation of BDNF expression in NT2/N neurons via downstream signaling from the D1 class of dopamine (DA) receptors. The up-regulation of BDNF expression, in turn, produced neuroprotective signals through receptor tyrosine kinase B (TrkB) and promoted cell survival under the conditions of oxygen and glucose deprivation. To our knowledge this is the first evidence showing the presence of a functional CRE in the human BDNF gene and the role of DA signaling in establishing transcriptional competence of CRE in postmitotic NT2/N neurons. This ability of DA to regulate the expression of the BDNF survival factor has a profound significance for the nigrostriatal pathway, because it indicates the existence of a feedback loop between the neurophin, which promotes both the maturation and survival of dopaminergic neurons, and the neurotransmitter, which the mature neurons ultimately produce and release.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family, structurally related to nerve growth factor, neurotrophin-3, and neurotrophin-4/5. Its biological activity is mediated by tyrosine kinase receptor B (TrkB) and its downstream signaling (1). The gene is highly expressed and widely distributed in the central nervous system, and it plays a significant role in the maintenance of function and survival of neurons (for review, see Ref. 2). Evidence accumulated in recent years suggests that BDNF is also involved in the modulation of synaptic activity in the adult brain, producing long-lasting changes in synaptic structure and function (for reviews, see Refs. 3–5).

There is a growing interest in BDNF as a potential therapeutic agent for neurodegenerative diseases, because its deficiency was found in brains of both Alzheimer’s and Parkinson’s patients (6–10). Indeed, BDNF treatment has been shown not only to potentiate synaptic transmission in vivo (11, 12) but also to increase neuronal survival and augment some behavioral changes in animal models (13, 14). However, more recent data indicate that BDNF can also induce behavioral sensitization by causing an overexpression of dopamine D3 receptors and could, actually, contribute to the amplification of pathophysiological associations with conditions such as epilepsy, drug addiction, schizophrenia, and Parkinson’s disease (15, 16). Clearly, further work is required to resolve some of these potential side-effects.

In contrast to a large body of work on the temporal and spatial patterns of BDNF expression in neurodevelopment and neurodegeneration, relatively little is known about the transcriptional regulation of the human BDNF gene. This is partially due to the fact that the genomic structure of the human gene has not yet been fully elucidated. The gene was first localized to chromosome 11p13 and predicted to consist of multiple exons (17), but the existence of multiple transcripts, derived from different exons, was demonstrated only recently by Aoyama et al. (18) in human neuroblastoma cells. A more detailed transcript mapping of an 810-kb region of chromosome 11p13-14 further defined its genomic localization, although no additional information on the actual structure of the gene itself was presented (19).

The existence of multiple human BDNF transcripts (18) is consistent with a genomic structure similar to that of the rat gene, which consists of four short 5’ exons, each controlled by a distinct promoter, and one 3’ exon encoding the mature BDNF protein (20, 21). In the rat, the four promoters direct expression of the BDNF gene in a tissue-specific manner, i.e. promoters I and II are active preferentially in neurons, whereas promoters III and IV are active both in neurons and in a limited number of non-neuronal tissues such as lung and heart (20, 21). Thus far, only a limited characterization of a 3.2-kb genomic fragment of the human gene containing some structural elements of a promoter was reported (22).

Recently, two transcription factors, CREB and a calcium-responsive factor, were identified as positive regulators of the rat promoter III (23–25), and the neuron-restrictive silencer factor as a negative regulator of promoters I and II (20, 26). It is still not known whether the regulatory machinery controlling the rat BDNF gene is conserved in humans.

Several studies indicate a direct involvement of neurotransmitters such as glutamate, GABA, acetylcholine, serotonin, and dopamine in the transcriptional regulation of the BDNF gene (27–31) but the molecular components involved in this
regulation have not yet been revealed. However, DA exerts its effects through G protein-coupled transmembrane receptors, of which the D1-like class can stimulate adenylyl cyclase and increase production of cyclic AMP (cAMP) which, in turn, activates the downstream protein kinase A (PKA) signaling, it is possible that these events are involved in the transcriptional response of BDNF gene to DA via the transcription factor CREB.

In this study, we have derived the genomic structure of human BDNF gene from the Human Genome by a direct sequence and structure comparison with the rat gene and proceeded to characterize the molecular mechanisms of its transcriptional activation in Ntera2/D1 teratocarcinoma (NT2) cells. The NT2 cells can be differentiated into post-mitotic NT2/N neurons by a treatment with retinoic acid (32, 33). We have established the presence of CRE-like element in promoter III of the gene and examined the role of DA signaling in its regulation. Recently, it has been established that NT2/N neurons express functional DA receptors of both D1-like and D2-like classes and are responsive to dopamine (34–36).

MATERIALS AND METHODS

Experimental Cell System—Human embryonic teratocarcinoma Ntera2/D1 (NT2) cells (Stratagene, La Jolla, CA) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Bethesda, MD) supplemented with 10% fetal calf serum (FCS, Wisent Inc., St. Bruno, PQ), and 40 μg/ml gentamicin sulfate (Sigma Cell Culture, St. Louis, MO). The cells were differentiated into post-mitotic NT2/N by the procedure described by Pleasure et al. (33). Accordingly, the cells were treated for 4 weeks with 10 μM all-trans-retinoic acid (RA, Sigma Cell Culture) followed by replating, for an additional 3 weeks with DNA synthesis inhibitors (1 μM cytosine-β-arabinofuranoside and 10 μM 5-iododeoxyuridine, Sigma).

Mouse Neuro-2a (N2a) neuroblastoma cells (ATCC CCL-131) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% FCS (Wisent Inc.) and used for transfections at density of 50% confluence.

Experimental Treatments—Both undifferentiated and post-mitotic NT2/N neurons were treated with 0.2 mM Bt2cAMP for up to 3 h and harvested for RNA and protein extractions as described below. Stimulation of D1 class DA receptors with SKF-81297 agonist was performed as follows. The NT2/N neurons were placed in fresh medium 10–12 h prior to the treatment, and 20 μM SKF-81297 (freshly prepared) was then added to the cultures for periods of time up to 3 h. We have shown previously that this concentration of SKF-81297 was the most effective in increasing the intracellular cAMP level (36). In some experiments 10 μM SKF-81297 for up to 3 h. The cells were subsequently harvested for the RNA extraction.

The cells were also subjected to oxygen-glucose deprivation (OGD) according to the following protocol. Post-mitotic NT2/N neurons, cultured in 12-well plates, were pretreated for 30 min with either 0.2 mM Bt2cAMP, 10 μM DA, or 20 μM SKF-81297. Subsequently, the cells were washed once with glucose-free DME medium, placed in glucose-free DME supplemented with 10% FCS and containing the same concentrations of Bt2cAMP, DA, or SKF-81297, and were exposed to OGD as described below. In some experiments 2 μM/ml anti-TrkB antibody was added to the cultures for 1.5 h prior to the addition of Bt2cAMP and the OGD treatment. The cells were incubated for 2 h at 37 °C in a Gas Pak 100 chamber containing Gas Pak Plus gas generator envelopes (BD Biosciences). The generator envelopes catalytically reduce the oxygen concentration while providing a humidified atmosphere with 5% CO2. Control cells were subjected to the same 2-h OGD period but without any pretreatments. Cell viability was assessed at the end of the OGD treatment (0 time) and after a 24-h recovery period under normoxic conditions. At the same time points the cells were also harvested for RNA and protein extractions.

Cell Viability Assays—The 5-carboxyfluorescein diacetate (CFDA) assay was used as an indicator of live cells and Hoechst 33342 dye staining to identify the dead cells. For the CFDA assay the cells were washed with PBS and incubated at 37 °C for 30 min with 5 μg/ml 5-CFDA, AM (Sigma) in Earle’s balanced salt solution (Sigma). Fluorescence was quantified using a CytoFluorTM 2300/2350 fluorescence measurement system (Millipore, Bedford, MA) with an excitation filter at 480/20 nm and an emission filter at 530/25 nm. For the Hoechst staining assay the cells were rinsed off the flasks with medium and pelleted by centrifugation. The collected cells were fixed in suspension with 3% paraformaldehyde in PBS buffer (0.13 M NaCl, 5 mM Na2HPO4, 1.6 mM KH2PO4). The cell suspensions were drawn through 26-gauge needles to disperse them and were stained with 0.2 μg/ml Hoechst 33342 dye in PBS. An aliquot of each sample was mounted on glass slides, and these were viewed on an Olympus BX50 fluorescence microscope with an Olympus ×40, numerical aperture 1.0, Planapo oil immersion objective.

RNA Extraction, RT-PCR, and Real-time Quantitative PCR—RNA was extracted with TriReagent according to the manufacturer’s protocol (Molecular Research Center, Inc., Cincinnati, OH) and was reverse-transcribed into single-stranded cDNA with 400 units of Moloney retrotranscriptase (Invitrogen, Bethesda, MD) using 0.3 μg of random primers (Invitrogen) in a 40-μl reaction containing 2 μg of total RNA. The cDNA reaction mixtures were diluted 5-fold, and ∼3 μl of diluted cDNA was used for each PCR amplification. Each 25-μl PCR reactions contained 1 × reaction buffer (Invitrogen), 2 μM of primers, 0.2 mM dNTP, 1.5 mM MgCl2, and 2.5 units of Taq polymerase (Invitrogen). PCR amplification for both CREB and BDNF was carried out for 25 cycles and for β-actin for 19 cycles, which were in the linear range of amplification. Each cycle consisted of the following steps: 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min. Total RNA in the samples was normalized to the amount of β-actin. The PCR products were separated by electrophoresis on agarose gels. Ethidium bromide-stained gels were photographed using the Ultra.Lum gel documentation system (Clarenmont, CA) and quantified, when applied, using Scion image software (Frederick, MD).

Real-time Q-PCR was performed with 10 ng of cDNA, purified on a QIAquick Spin column (Qiagen, Hilden, Germany) and quantified with an Oligo GREEN single-stranded DNA quenching reagent mix (Molecular Probes, Eugene, OR), using the ABI GeneAmp 5700 sequence detection system (ABI, Foster City, CA) and Quantitate SYBR Green PCR kit (Qiagen). PCR primers were the same as those used for RT-PCR and are listed in Table I. Thermal cycling was initiated with a 2-min incubation at 50 °C, followed by a first denaturation step of 15 s at 94 °C and then 40 cycles of 94 °C for 15 s and 55 °C for 1 min (annealing and extension). The real-time PCR amplification data were collected continuously and analyzed with the Sequence Detection System (ABI, Foster City, CA). The copy number of target sequences in the tested samples was inversely proportional to and, hence, can be deduced from the Ct (the threshold cycle) level at which a significant increase in fluorescence signal was first detected. The amount of amplified products of the treated samples was normalized to the untreated controls and was calculated using the term, 2 −ΔΔCt.

Preparation of Protein Extracts—Cells were trypsinized, centrifuged, washed once with PBS, and lysed with radiolabelling precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% deoxycholate, 1% Triton X-100, 1 mM Na3VO4, and 1 × Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN)). The lysates were vortexed, incubated for 15 min on ice, centrifuged at 13,000 rpm (IEC/MicroMax) for 20 min, and the supernatants (total cell lysates) were used for further analysis.

To prepare nuclear protein extracts, cells were lysed in 2 μM KH2PO4/KOH buffer, pH 6.55, containing 0.15 M NaCl, 1 mM EGTA, 5 mM MgCl2, 0.1 mM DTT, 0.3% Triton X-100, and 1 × Protease Inhibitor Cocktail (Roche Diagnostics). Nuclei were collected by centrifugation and washed once with the cell lysis buffer, and nuclear proteins were extracted with 20 μM HEPES buffer, pH 7.9, containing 25% v/v glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, and 1 × Protease Inhibitor Cocktail.

Western Blotting—Protein extracts (50 μl/gane) were boiled in Laemmli/SDS sample buffer (2 × buffer: 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromphenol blue) for 5 min, separated by electrophoresis on 10% SDS-PAGE, and electrotransferred onto Hybond-C extra membranes (Amersham Biosciences, Piscataway, NJ). The membranes were first incubated for 1 h at room temperature with 5% nonfat milk powder in TBST and then washed 3 times with TBST for 5 min each. The membranes were incubated with the following antibodies: 1:1000 dilution of rabbit anti-phospho-CREB (Santa Cruz Biotechnology, Santa Cruz, CA; anti-phospho-CREB (anti-p-CREB), an affinity-purified rabbit polyclonal antibody raised against a synthetic phosphopeptide corresponding to residues
123–136 of rat CREB coupled to keyhole limpet hemocyanin (Upstate Biotechnology, Lake Placid, NY); affinity-purified rabbit polyclonal anti-BDNF antibody (Santa Cruz Biotechnology).

Electrophoretic Mobility Shift Assay—A 19-bp probe corresponding to the CRE-like element (boldface letters) of the human BDNF promoter III was synthesized as complementary oligonucleotide strands (5’-GA-CACGCGAATTCAGGA-3’, 5’-GGTGCCCTTGACGCGC-3’). Complementary oligonucleotides containing mutated CRE were also applied (5’-GACGCACTGCAGGAAGGAC-3’, 5’-GGTGCCCTTGACGCGC-3’). The strands were annealed and labeled with [32P]dCTP using a nucleotide fill-in method catalyzed by the Klenow enzyme (Amersham Biosciences). The protein binding assay was carried out in a 30-μl reaction mixture containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 40 mM KCl, 5 mM DTT, 10% glycerol, 30 or 60 ng of poly(dI-dC), 3 μg of bovine serum albumin, 6 μg of nuclear protein extract, and about 2 ng of [32P]-labeled probe (50–60,000 cpm/assay). The mixture was incubated for 20 min at room temperature, and the reaction was stopped by an addition of 1/10 volume of 1× sample loading buffer (250 mM Tris-HCl, pH 7.5, 40% glycerol, and 0.2% bromphenol blue). In the competition assays, an excess (50×) of unlabeled probe was added to the reaction mixture 30 min prior to the addition of the labeled oligonucleotides. In the antibody supershift assays, reaction mixtures were preincubated for 30 min at room temperature with 1 μl of rabbit polyclonal anti-CREB antibody (Rockland Inc., Gilbertsville, PA) or 1 μl of anti-ATF1 antibody (F1–1, Santa Cruz Biotechnology, Santa Cruz, CA) prior to the addition of the labeled probe. DNA-protein complexes were resolved by electrophoresis on a non-denaturing 6% polyacrylamide gel run in 50 mM Tris, 1 mM EDTA, and 300 mM glycine running buffer (pH 8.6–8.7) and were visualized by autoradiography on Kodak BioMax MS film.

Plasmid Constructs—Genomic DNA prepared from NT2 cells was used as PCR template to amplify the 548-bp fragment of human BDNF promoter III with a forward primer, 5’-TACTAGCTCTTATACGTTGTTTGCGTGTGTG-3’, and a reverse primer, 5’-TGCTGAGGAGGAAGGACACGGTGGCAAGAGGTGTG-3’, and a reverse primer, 5’-ATCTCGAGCGCTTTCAGTGCACGCTA-3’. The fragment was cloned into pGL3-Basic luciferase reporter vector (Promega, Madison, WI) with a primer, 5’-TATCATAT-GAGCAGCCGCTTCAAGGACACGGTGGCAGGAC-3’, in which the CRE site was mutated (underlining indicates CRE-site changes). Both pGL3-PIII and pGL3-PIII_CREm plasmids were sequenced to confirm the correctness.

Cell Transfection and Luciferase Activity Assay—Transient transfections were carried out on neuroblastoma N2a cells using Lipofect-AMINETM 2000 (Invitrogen Canada, Inc., Burlington). The transfection efficiency of NT2 cells was too low to obtain any meaningful data. Briefly, cells growing in 6-well plates were co-transfected with 5 μg of pGL3 reporter plasmid and 2 μg of pSV-β-Gal (Promega) per well following the manufacturer’s protocols. Two hours after transfection, cells were placed in serum-free DMEM, and 24 h later they were treated with 0.2 mM 3B,5-CAMP for 2 h. The cells were then washed twice with cold Ca2+/Mg2+-free PBS and lysed with 500 μl/well of Glo lysis buffer (1×) (Promega). One hundred microliters of cell lysate was used for Bright-GloTM luciferase assay system (Promega). The luciferase activity was measured using a luminescence counter (1450 MicroBeta Trilux, Wallac). Thirty microliters of cell lysate was used for analysis with the β-galactosidase Enzyme Assay System (Promega). The activity of β-galactosidase was detected by absorption at 420 nm using a spectrometer (Spectra Max 340, Molecular Devices, Sunnyvale, CA).

Data Analysis—Data were analyzed and plotted using Jandel Sigmaplot 2000 software.

RESULTS

Genomic Structure of Human BDNF—We used two web-based programs, BLAT and the Human Genome Browser (both written by Jim Kent, University of California at Santa Cruz), to deduce the entire structure of the human BDNF gene. The BLAT search was performed with rat sequences GI557909, GI557910, GI557911, and GI557912 corresponding to exons I–IV, respectively (21). The search identified four DNA sequences on human chromosome 11 in the working draft of the genome (April 1, 2001 freeze) that shared similarities with the rat exons. The Human Genome Browser tool identified several BDNF mRNAs (X91251, X60201, and M61176) encoded by some 11 sequences 5′ upstream of the coding region (Fig. 1A).

Subsequently, we included human sequences GI557945, GI14090245, GI14090247, GI14090249, GI14090251, and GI14090253, which were sequenced, were identified by BLAT search of the draft assembly of the human genome, using rat sequences GI557909, GI557910, GI557911, and GI557912 and including human sequences GI14090245, GI14090247, GI14090249, GI14090251, and GI14090253. The sequences transcribed into mRNAs (X91251, X60201, and M61176) are also shown. The alignment was performed with the Genome Browser tool written by Jim Kent (University of California at Santa Cruz). Exons are shown as black boxes; lines with arrowheads pointing in the direction of transcription represent introns. A, a schematic representation of the entire gene. Exons are shown as boxes and introns as lines. The region corresponding to the coding sequence is represented by the filled box, and arrows indicate transcription initiation sites. Exons are numbered with Roman numerals; introns are numbered with Arabic numbers. The locations of exon V-specific primers (F1/R1) and primers amplifying exons III–V (F2/R2) are also indicated.
sponding to the rat exons I–V. It is possible that the gene contained an additional exon IVi, as suggested by Aoyama et al. (18), and its presence is also noted in Fig. 1B. The overall structure of the human gene was very similar to that of the rat, although the entire gene spanned more than 64 kb (Fig. 1B).

Identification of a CRE-like Sequence in Promoter III of the Human Gene—It is well known that the expression of the rat BDNF gene is directed by four distinct promoters of which the activation of promoter III involves CRE and CaRE1 elements activated by the transcription factors CREB and calcium-response factor (20, 21, 23–25). Subsequently, we used the rat promoter III and exon III sequence (GI 557911) (21) to search against the human genome (Human Genome browser, April 1, 2001 freeze) and identified the corresponding human promoter III sequence. The two sequences were further aligned using the ClustalW program (www2.ebi.ac.uk/clustalw). As shown in Fig. 2, the alignment revealed that the human promoter III contained a CRE-like sequence, 5'-GCACGTCA-3' (boxed), and a potential transcription start site is indicated by a star sign.

Expression Pattern of BDNF Gene during Differentiation of NT2 Cells—We used two separate sets of primers to distinguish transcripts generated by activation of different promoters. Accordingly, the F1/R1 primers were located in exon V and would amplify all BDNF transcripts, whereas the F2/R2 primers would amplify messages containing exons III and V, generated by promoter III (Table I and Fig. 1B). The expression of the gene, as measured by the level of exon V transcripts, was relatively low in both the undifferentiated and the 3- and 4-week RA-treated cells (Fig. 3, lanes 1–3; top panel), but it significantly increased and remained high in the post-mitotic NT2/N neurons treated with DNA synthesis inhibitors for 1–3 weeks (lanes 4–6, top panel). The expression pattern of exons III–V mRNA was very similar. The level of these transcripts was also much lower in the undifferentiated and RA-treated cells (Fig. 3, lanes 1–3, middle panel) than in neurons maintained in culture in the presence of mitotic inhibitors (lanes 4–6, middle panel), suggesting that the activity of promoter III...
Alternative splicing of the CREB gene generates several isoforms, of which CREBα and CREβ are the most abundant and functionally most significant in mammalian cells. We used a pair of primers that amplified both CREBα and CREβ. The amplified products could be distinguished by their size on agarose gels (Fig. 5A). The CREBα mRNA remained unchanged during the entire differentiation paradigm (Fig. 5A, upper panel), but the expression CREβ increased significantly following exposure of the cells to RA (compare lanes 1 with lanes 2 and 3) and remained elevated in the post-mitotic neurons (lanes 4 and 5).

The CREB protein was also present in the undifferentiated, RA-only-treated, and mature NT2/N cells (Fig. 5B, upper panel). Furthermore, the level of CREB phosphorylated at Ser-133 (p-CREB), which is considered to be transcriptionally competent, was very similar in the RA-treated cells (Fig. 5B, lane 2, lower panel), in which the promoter III was inactive (Fig. 3).
entiated cells (Fig. 3), they responded to Bt2cAMP treatment for
expressed BDNF mRNA at levels higher than they did in undiffer-
right panel that the one or more additional molecular factors were involved
pattern of promoter III-driven gene transcription, suggesting
change in CREB phosphorylation either (Fig. 6
post-mitotic NT2/N neurons (Fig. 6
the time period studied. A very different picture was seen in the

There was no induction of the message (Fig. 6
C
expression levels were normalized to that of the

Materials and Methods.

Effects of Bt 2cAMP on BDNF Expression—The data pre-
above raised a question of when promoter III became
regulated and differentiated cells were treated with Bt2cAMP, and
NT2 cells differentiation. To establish this, both undifferen-
ted and differentiated cells were treated with Bt2cAMP, and
its effects on CREB phosphorylation and promoter III-driven tran-
scription were analyzed 2 h later (Fig. 6). Clearly, Bt2cAMP had no
influence on BDNF transcription in undifferentiated cells (Fig. 6A).
There was no induction of the message (Fig. 6A, left panel) and no
change in CREB phosphorylation either (Fig. 6A, right panel) over
the time period studied. A very different picture was seen in the
post-mitotic NT2/N neurons (Fig. 6B). Although these cells already
expressed BDNF mRNA at levels higher than they did in undiffer-
entiated cells (Fig. 3), they responded to Bt2cAMP treatment fur-
ther by both the up-regulation of CREB phosphorylation (Fig. 6B,
right panel) and the expression of BDNF (Fig. 6B, left panel). The
up-regulation of BDNF expression in response to Bt2cAMP was
also verified by real-time PCR (Q-PCR) (Fig. 6B, left panel) and
confirmed by Western blot (Fig. 6B, right panel). These results
indicated that the undifferentiated NT2 cells possibly lacked one or
more regulatory molecules essential for coupling the stimulus to
the transcriptional apparatus.

To determine the functionality of the promoter III CRE ele-
ment (5′-GCACGTCA-3′), a 548-bp genomic DNA sequence
containing about 210 bp of 5′ flanking region and exon III (Fig.
2) was inserted into plasmid pGL3-basic, which contained a
firefly luciferase reporter gene. In parallel a substitution muta-
tant, in which the CRE sequence was changed to 5′-GACCT-
GCA-3′ to eliminate the CREB binding, was also generated.
Each reporter plasmid was transiently transfected into mouse
N2a neuroblastoma cells. 24 h after transfections, the cells
were treated with 0.2 mM Bt2cAMP for 2 h. As shown in Fig. 6C,
the expression of luciferase gene was driven by the CRE-con-
taining promoter III fragment, even in the absence of the
stimulation, and the reporter gene expression was significantly
higher following the exposure of the cells to cAMP. Further-
more, the substitution mutations within the CRE not only
severely affected the basal activity of promoter III (by ~5-fold)
but also eliminated its responsiveness to Bt2cAMP. These find-
ings strongly suggested that the CRE element was an impor-
tant mediator of BDNF exon III transcription.

Activation of BDNF Expression by Dopamine Signaling—In
neuronal cells the neurotransmitter DA triggers an elevation of
intracellular cAMP and activation of PKA pathway through the
D1 class of receptors. We have shown recently that the post-
mitotic NT2/N cells express functional DA receptors of both D1
and D2 classes capable of modulating the intracellular level of
BDNF transcription (Fig. 7). Consistently, the treatment resulted both in the increased levels of exon III
containing BDNF transcripts in N2a cells exposed to cAMP for
2 h (2B) and in the increased levels of exon III
containing BDNF transcripts in N2a cells exposed to cAMP for
2 h (2B). The SKF-81297 treatment was completely ineffective in inducing
the BDNF message in the presence of the PKA inhibitor H89 (Fig. 7C). Changes in BDNF expression in response to these treatments were also verified by Q-PCR. This data clearly demonstrated that the transcriptional activation of promoter III through the D1 class of receptors required downstream PKA signaling.

Neuroprotective Effects BDNF against OGD—To establish whether BDNF expression in the post-mitotic NT2/N neurons could promote their survival, the cells were pre-treated for 30 min with 0.2 mM Bt2cAMP, 10 µM DA, or 20 µM SKF-81297 to up-regulate the BDNF expression (Fig. 7), and, subsequently, they were subjected to the OGD treatment to activate the cell death process. According to the data obtained from two independent tests, the CFDA viability assay (Fig. 8A) and counting of Hoechst dye-stained condensed nuclei (Fig. 8B), the 2-h OGD treatment reproducibly resulted in 35–40% of cell loss within 24 h. Based on the morphological assessment of Hoechst-stained nuclei the OGD treatment triggered neuronal apoptosis (Fig. 8B). The cAMP pretreatment was evidently neuroprotective, because only ~10% of cells died under the same OGD regimen and within the same time frame. Similar results were obtained for cells exposed to either DA or SKF-81297 treatments, no significant viability loss was detected at 24 h after the OGD (Fig. 8A). Cell analysis at the end of the OGD treatment showed much higher p-CREB and BDNF message levels in Bt2cAMP treated than in control cells (Fig. 8C), indicating that this neuroprotective pathway might involve BDNF itself. To further confirm this, we blocked the interaction of BDNF molecule with its receptor TrkB by preincubating cells with anti-TrkB antibody prior to the Bt2cAMP/OGD treatment. As shown in Fig. 8A, the anti-TrkB antibody completely reversed the neuroprotective effects of Bt2cAMP, presumably by eliminating the downstream signaling of BDNF.

**DISCUSSION**

We have performed a computer-aided sequence and structure analysis of the human BDNF gene to obtain information about the promoters and cis-acting DNA regulatory elements involved in its transcriptional regulation in NT2-derived neuronal cells. These cells were generated using the previously described differentiation protocol, and they represent a well-characterized model of neuronal cultures (32, 33). The differentiated NT2/N neurons have been shown to co-express multiple phenotypes, including GABAAergic, catecholaminergic, cholinergic, and peptidergic (38–42). They also express pro-
teins involved in exocytosis, such as synaptophysin and chromogranin (33), and form both glutamatergic excitatory and GABAergic inhibitory synapses (42, 43). All these features suggest that they are able to establish functional interactions with each another and, therefore, are a convenient in vitro model of human neuronal cells.

We have established that the overall structure of the human gene is very similar to that of the rat, with at least five exons and four introns (Fig. 1) clearly pointing out the complexity of structure and transcriptional regulation. However, to date there has been only one study on a genomic fragment containing some structural elements of the human gene promoter (22). According to the sequence alignment performed in this study (Fig. 1A), this fragment represents promoter IV of the human gene.

Multiple promoters have been shown to regulate rat BDNF gene expression, but the activity-dependent transcription appears to be controlled mainly by the promoter III (23–25). However, it has been reported recently that a CRE located in the rat BDNF gene promoter I also plays a role in its activation, although, the activity of promoter I is ~8-fold lower than that of the promoter III (44). Our unpublished data indicated that neither exon I nor exon II of human BDNF gene was expressed in NT2 cells, hence these promoters did not contribute to the BDNF message level in our system (data not shown). The present study was undertaken to establish whether the same structural and molecular components that regulate rat BDNF gene promoter III were also involved in the control of the human gene. The human promoter III contained the same pyrimidine-rich sequence identified by Timmusk et al. (20) as the initiator of the rat exon III transcription and no identifiable TATA box, although it has been reported by Nakayama et al. (21) that the rat promoter III contains a potential TATA element, TATAAT, located between the CaRE and CRE elements. This finding, however, is somewhat controversial, because this element is not found in the sequence published by Timmusk et al. (20). The latter authors report a TATCAT sequence at this location, which is also present in the human promoter (Fig. 2). It is possible, therefore, that the conclusion of Nakayama et al. (21) results from a sequencing error.

The human promoter III contained both the CaRE and CRE elements (Fig. 2), confirming that the structural elements critical for activity-dependent regulation of transcription in rat (23–25) were also conserved in the human gene promoter. The CRE sequence, 5'-GGACGTCA-3', located upstream from the exon III transcription initiation site, differed slightly from the palindromic 5'-TGACGTCA-3' consensus sequence (37). However, the two bases C and G at the center of the element (boldface letters) that discriminate between CREB binding and members of the AP-1 family (45, 46) were conserved. Indeed, in the in vitro EMSA the binding of CREB but not the closely related CREB family member ATF1 to this CRE sequence was established (Fig. 4).

Dopamine signaling is mediated in neuronal cells by the activation or inhibition of adenyl cyclase. DA exerts its action through a subset of specific membrane receptors classified as seven-transmembrane domain G-protein-coupled receptors. Thus far, five distinct DA receptors, D1 to D5, have been identified and characterized, and based on their biochemical and pharmacological properties the receptors have been subdivided into two families, D1-like (the D1 and D5) and D2-like (D2a, D2b, and D3). The D1-like receptor subtypes are positive and D2-like are negative regulators of the cAMP pathway (for reviews, see Refs. 47 and 48). We have shown previously that the NT2/N cells express functional D1R and D2R receptors, and their activation results in an increase of ~2-fold in the cAMP level (36).

Thus, the activation of the D1 class of receptors by SKF-81297 agonist led to the up-regulation of promoter III-driven BDNF transcription in the post-mitotic NT2/N neurons, signifying the requirements for functional D1R signaling and the CRE element of the promoter in this process (Fig. 7A). This activation did not occur in the presence of PKA inhibitor H-89 (Fig. 7C), suggesting activation of PKA was involved. Consistently, the stimulation of promoter III by cAMP was evident in the post-mitotic NT2/N neurons but not in undifferentiated or RA-only-treated cells (Fig. 3), indicating clearly that this process required additional intracellular components, notably D1 class receptor signaling, capable of coupling the stimulus to transcription.

It has been documented in previous studies (49–51) that BDNF modulates the survival and differentiation of mesencephalic dopaminergic neurons, including those that degenerate in Parkinson’s disease, and promotes the development of dopaminergic networks in rodent retina (52). In our study, coupling of the downstream D1R signaling with CRE-mediated gene expression in the post-mitotic NT2/N neurons produced a BDNF-dependent neuroprotection against oxidative stress (the OGD treatment, Fig. 8). Similar conclusions can be discerned from the studies on the role of DA in the development and maturation of mouse striatal GABAergic neurons (53–55). In this cell system DA was also shown to up-regulate BDNF expression through the D1-like receptors (31).

In summary, we have put together the full structure of the human BDNF gene and have identified the cis-regulatory elements of the promoter III. Subsequently, we focused our attention on the CRE element and established that it participated in the modulation of BDNF expression in NT2/N neurons via downstream signaling from the D1 class of DA receptors via cAMP, PKA, and CREB. The up-regulation of BDNF expression, in turn, produced neuroprotective signals promoting cell survival under the oxidative stress generated by the OGD conditions. Significantly, our data pointed to the existence of a feedback loop between the neurophin that promotes the maturation and survival of dopaminergic neurons and the neurotransmitter, which the mature neurons ultimately produce and release. Clearly, this ability of DA to regulate the expression of the survival factor has a profound significance for the nigrostriatal network. For, as documented in the Parkinsonian brain (9, 10, 56), the loss of dopaminergic neurons and the impairments of IA input would contribute to decreased BDNF expression and, consequently, to the uncoupling of cell death/survival controlling mechanisms.

REFERENCES

1. Barbacid, M. (1995) Ann. N. Y. Acad. Sci. 768, 442–458
2. Murer, M. G., Yan, Q., and Raisman-Vozari, R. (2001) Prog. Neurobiol. 63, 71–124
3. Jankowsky, J. L., and Patterson, P. H. (1999) Mol. Cell Neurosci. 14, 273–286
4. Lu, B., and Chow, A. (1999) J. Neurosci. Res. 58, 76–87
5. McElister, A. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13600–13602
6. Phillips, H. S., Hains, J. M., Armanini, M., Laramie, G. R., Johnson, S. A., and Winslow, J. W. (1991) Neuron 7, 695–702
7. Murray, K. D., Gall, C. M., Jones, E. G., and Isaacson, P. J. (1994) Neuroscience 60, 37–48
8. Nairn-Saito, M., Wakabayashi, K., Tsuji, S., Takahashi, H., and Nawa, H. (1996) Neuron 17, 2925–2928
9. Nagai, M., Tagari, A., Kanda, T., Mizuno, Y., Komure, O., Kuno, S., Ichinose, H., and Nagatsu, T. (1999) Neurosci. Lett. 270, 45–48
10. Parain, K., Murer, M. G., Yan, Q., Fauchex, B., Agid, Y., Hirsch, E., and Raisman-Vozari, R. (1999) Neuron 16, 557–561
11. Kang, H., and Schuman, E. M. (1995) Science 267, 1658–1662
12. Messaoudi, E., Bardes, K., Srebro, B., and Bramham, C. R. (1998) J. Neurophysiol. 79, 496–499
13. Altar, C. A., Boylan, C. B., Jackson, C., Hershenson, S., Miller, J., Wiegand, S. J., Lindsay, R. M., and Hyman, C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11347–11351

* H. Fang, J. Chartier, C. Sodja, A. Desbois, M. Ribecbo-Lutkiewicz, P. Roy Walker, and M. Sikorska, unpublished data.
Transcriptional Activation of the Human Brain-derived Neurotrophic Factor Gene Promoter III by Dopamine Signaling in NT2/N Neurons
Hung Fang, Joanne Chartier, Caroline Sodja, Angele Desbois, Maria Ribbecc-Lutkiewicz, P. Roy Walker and Marianna Sikorska

J. Biol. Chem. 2003, 278:26401-26409.
doi: 10.1074/jbc.M211539200 originally published online May 8, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211539200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 56 references, 12 of which can be accessed free at
http://www.jbc.org/content/278/29/26401.full.html#ref-list-1