The Human NGFI-B Gene Gives Rise to Two Isoforms with Different Expression Profiles

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ABSTRACT

NGFI-B is an immediate-early gene that belongs to the steroid/thyroid receptor superfamily. In this study, we cloned and characterized a human NGFI-B isoform designated NGFI-B2, which has a unique 5'-untranslated region and which lacks C-terminal amino acid sequences corresponding to the putative ligand-binding or dimerizing domain. The NGFI-B and NGFI-B2 mRNAs are differentially expressed in human tissues. To study the transcriptional regulation of these isoforms, we examined the effects of forskolin and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) on their expression in human neuroblastoma and breast cancer cells. NGFI-B, but not NGFI-B2 transcripts were rapidly induced, suggesting that NGFI-B2 is not associated with the immediate-early response. The expression and regulation of the NGFI-B isoforms suggested that they are expressed by separate promoters, and that they play different roles in biological processes.

NGFI-B (9) [also known Nur77 (4), N10 (15), NAK1 (10) and TR3 (1)] is an immediate-early gene that was originally identified by virtue of its rapid activation by serum stimulation of quiescent fibroblasts. It is also activated by such diverse signals as membrane depolarization and nerve growth factor (NGF) in PC12 pheochromocytoma cells and by chemically induced seizure in hippocampal neurons (17). NGFI-B belongs to the steroid/thyroid receptor superfamily and it potently activates transcription through interactions with DNA at sequence-specific sites (18). Studies of NGFI-B expression suggest that it plays important roles in the central and peripheral nervous systems and in steroidogenic organs (3, 13). Furthermore, since the expression of "dominant negative" NGFI-B or

NGFI-B antisense transcripts prevents the activation-induced apoptosis of T-cells, NGFI-B might be involved in control of the activation-induced apoptosis of thymocytes and T-cell hybridomas (7, 19).

In this paper we show that the human NGFI-B gene gives rise to two distinct mRNAs, which were differentially expressed in tissues, and induced by stimulation in human neuroblastoma and breast cancer cells.

MATERIALS AND METHODS

Cloning of the isoform

A cDNA fragment encoding the DNA-binding domain of human NOR-1 was labeled with DIG-dUTP (Boehringer Mannheim) by PCR. A human skeletal muscle cDNA library in λ gt11 (Stratagene) was screened with the labeled fragment and we isolated a cDNA encoding a sequence similar to those of nuclear receptors. The cDNA was sequenced by means of dideoxy-nucleotide chain-termination.

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Reverse-transcriptase-PCR

Transcripts of NGFI-B2 and NGFI-B were detected by means of reverse transcriptase-PCR (RT-PCR). All cDNAs were synthesized using 1 μ g of total RNAs and reverse transcriptase (Superscript II, BRL) with the P1 primer (5'-GCAGCCGTACACCTGGAAGTCC-3'; for NGFI-B or NGFI-B2) or an oligo-dT primer [for glyceraldehyde 3-phosphate dehydrogenase (G3PDH)]. The 5' primers for amplification were 5'-CTTCGTGCGGTTGTCTGGGAC-3' for NGFI-B2 (nt 120-140; Fig. 1) and 5'-GCACAGAAGAACTTCGGGAGCG-3' for NGFI-B [nt 18-39 (1)]. The 3' oligonucleotide primer was from their common transactivating domain and it contained a sequence (5'-AGCATGGCTGGACTGTTCTGG-3') located immediately upstream of the primer used for reverse transcription. The single strand cDNA products were denatured and amplified by 40 PCR cycles. For control G3PDH cDNA amplification, the cDNA products were amplified by 35 PCR cycles with two primers (5'-CTTCACCACCATG-GAGAAGGCTGG-3', 5' primer; 5'-TCCAGGGGTCTTACTCCTTGGAGG-3', 3' primer). Each cycle consisted of denaturation at 94 °C for 30 sec, annealing at 55 °C for 75 sec and extension at 72 °C for 120 sec. The PCR products were confirmed by DNA sequencing.

Cell culture and RNA isolation

The human neuroblastoma cell line, NB-OK-1, and the breast cancer cell line, MCF7, were maintained in RPMI 1640 medium containing 10% fetal bovine serum (Mitsubishi Kasei). Cells (approximately 1×10^7 cells/10 cm dish) were incubated in fresh medium for 18 h, then stimulated with 10 μ M forskolin (Calbiochem) and 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Wako Chemicals) dissolved in dimethyl sulfoxide. Total RNA fractions were isolated from the cells 3 h later, using the acid guanidinium thiocyanate-phenol-chloroform method.

RESULTS AND DISCUSSION

Isolation of NGFI-B cDNA isoform

We previously isolated NOR-1, a member of the Nur77/NGFI-B family (11, 12). To further characterize the members of this family, we searched

for variants of their gene products. By means of hybridization at low stringency with probes encoding sequences corresponding to the DNA-binding domain of human NOR-1, we isolated a novel cDNA variant of NGFI-B, designated NGFI-B2, from a human skeletal muscle λ gt11 cDNA library.

Fig. 1 shows the cDNA sequences of human NGFI-B2. The size of the cDNA insert is 1813 nucleotides and it contains a major open reading frame of 978 nt starting with an initiation codon at 182 nt. From ATG (at 180 nt) until 1057 nt, it was identical to NGFI-B, but the 5' and 3' non coding regions completely differed (Fig. 2). The divergent points of the cDNA were identical to the boundary between exons (2), indicating that this variant is an isoform generated by alternative splicing. The NGFI-B2 reading frame was followed by a 3' untranslated region of 654 nucleotides. No canonical AATAAA sequence was found, indicating that the sequence of 1813 nucleotides lacked information for a 3' end. An ATTTA motif, which may facilitate selective mRNA degradation or destabilization by removal of the poly(A) tail (16), is present in most Nur77/NGFI-B family members (1, 4, 9, 15), whereas the NGFI-B2 3'-sequences did not contain this motif. This suggested that stability of the NGFI-B2 mRNA may differ from that of other members of the Nur77/NGFI-B family. The predicted NGFI-B protein sequence revealed that it had sequences identical to that of NGFI-B at the transactivating domain, but lacked the C-terminal amino acid sequences corresponding to the putative ligand-binding or dimerizing domain. A receptor for 9-*cis* retinoic acid, RXR, forms heterodimers with NGFI-B, and its growth factor-induced immediate-early gene products are implicated as modulators of the retinoic acid signaling pathway (15). Since NGFI-B2 protein lacked the putative dimerizing domain, it may compete with the NGFI-B-RXR heterodimer. Furthermore, while the steroid/thyroid receptor superfamily members contain two zinc-fingers, NGFI-B2 had only one of the C2-C2 type. This suggested that NGFI-B2 does not bind to the NGFI-B response element (NBRE; 5'-AAAAGGTCA-3').

NGFI-B2 and NGFI-B expression in human tissues

We investigated NGFI-B2 and NGFI-B expres-

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GCTCGCTCAGCTGCTGCCAGCCTCGGCTGTGAGGATAG 39
GCTGGCTGGGCAGCACGTCTCTCCACAGGGTCCCTGAGACCACAGGAAGAGCCCCAACCAATCTTG 110
GGATTCTCCCTTCGTGCGGTTGTCTGGGACCTTTTCCAGGGTCAAAGCAGATCGTGAGGAGGAAGCTGAG 181
[ATG] CCC TGT ATC CAA GCC CAA TAT GGG ACA CCA GCA CCG AGT CCG GGA CCC CGT 235
GAC CAC CTG GCA AGC GAC CCC CTG ACC CTT GAG TTC ATC AAG CCC ACC ATG GAC 289
CTG GCC AGC CCC GAG GCA GCC CCC GCT GCC CCC ACT GCC CTG CCC AGC TTC AGC 343
ACC TTC ATG GAC GGC TAC ACA GGA GAG TTT GAC ACC TTC CTC TAC CAG CTG CCA 397
GGA ACA GTC CAG CCA TGC TCC TCA GCC TCC TCC TCG GCC TCC TCC ACA TCC TCG 451
TCC TCA GCC ACC TCC CCT GCC TCT GCC TCC TTC AAG TTC GAG GAC TTC CAG GTG 505
TAC GGC TGC TAC CCC GGC CCC CTG AGC GGC CCA GTG GAT GAG GCC CTG TCC TCC 559
AGT GGC TCT GAC TAC TAT GGC AGC CCC TGC TCG GCC CCG TCG GCC TCC ACC CCC 613
AGC TTC CAG CCG CCC CAG CTC TCT CCC TGG GAT GGC TCC TTC GGC CAC TTC TCG 667
CCC AGC CAG ACT TAC GAA GGC CTG CGG GCA TGG ACA GAG CAG CTG CCC AAA GCC 721
TCT GGG CCC CCA CAG CCT CCA GCC TTC TTT TCC TTC AGT CCT CCC ACC GGC CCC 775
AGC CCC AGC CTG GCC CAG AGC CCC CTG AAG TTG TTC CCC TCA CAG GCC ACC CAC 829
CAG CTG GGG GAG GGA GAG AGC TAT TCC ATG CCT ACC GCC TTC CCA GGT TTG GCA 883
CCC ACT TCT CCA CAC CTT GAG GGC TCG GGG ATA CTG GAT ACA CCC GTG ACC TCA 937
ACC AAG GCC CGG AGC GGG GCC CCA GGT GGA AGT GAA GGC CGC TGT GCT GTG TGT 991
GGG GAC AAC GCT TCA TGC CAG CAT TAT GGT GTC CGC ACA TGT GAG GGC TGC AAG 1045
GGC TTC TTC AAG GTA CCG CGC AGC CCC AGG TGG GGC CTT TTG TTG GAA ATG GAG 1099
AGA GGC TGG CCT CAT CCC ATT GGG ACC TGT GGT CTC CCC CTG GGT TCT CCT CCT 1153
AGC [TAA] GTCCTGTCCTGCAGGGTGGGATCAGCCCTGCCAGGTGGGCCGCCTTCTGGAGACCCGTAGAT 1222
GCCAGGGCTGGAAGCTTTTCATTTGCCGGGACACTCGGGCCCATGGGATTGCACAGAGCTGGAGGGAGGGT 1293
GAGATAGGGGCAGATAGGAGCTGCAGGGGTGCCTGGCGAGCCTCTGGTTTTCTCTGCTCCTCTGCCTGTC 1364
CTCTCCCAACTCAAGGTTCTAGTGGAAGGGTGCCCCAGGCTCTCATGTTCTTGGCGTGAGATGAAAGGA 1435
TCCTGCGGAGGGTTTGGTTCTTGAGGGCTGGGGTGGACTTGGGAACAGGCTGTGTGTTTGTCCAGCGAT 1506
GGTGCCTGCTTAGCTTCCGTCCCCACCCCCAGCCCTTGGCCCTCTCTGTCTGCCCTAGGGAGAAGGC 1577
AGGTGGACAAGGGCCCATGAAAAATACAGGTGTCTAGACTGCCAGGGAGACCCTGGCCCCCAGTAGTGT 1648
GTCTGGGGACTTCTCAGAGCGAGAAACCTCCCCCAATGTCTTCAAGACTTTTCTCTCCCCCCCCCA 1719
ACCCCGTCTCTCCCTCCCTTGCACCCAAATGTAGAAAAATAGCTGTGAACAGAGAGCGCTTTTGTCTGC 1790
AATGGCAGCAGGATCTGGACGGT 1813

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Fig. 1 Nucleotide sequence of human NGFI-B2 cDNA. The numbers on the right refer to the nucleotide sequence. The initiator and terminator are boxed. A region found in both NGFI-B2 and NGFI-B is shadowed.

Fig. 2 Comparison of NGFI-B2 and NGFI-B cDNAs. The boundaries of NGFI-B exons are shown at the bottom. The boxes and bars are represent the major open reading frames and zinc-finger motifs, respectively. The shadowed box indicates the DNA-binding domain.

sion in various tissues by means of RT-PCR. We detected NGFI-B in skeletal muscle, fetal brain and placenta at a similar intensity, whereas NGFI-B2 was found only in skeletal muscle (Fig. 3). PCR amplification with primers designed from the G3PDH cDNA sequence as a control was not changed in any of the tissues examined. The tissue-specific expression of NGFI-B2 and NGFI-B suggested that they have different functions. Other members of the Nur77/NGFI-B family, NOR-1 and RNR-1, also have an isoform, but they are concomitantly expressed with their original transcripts (unpublished results). The NGFI-B gene might have a high level of transcriptional complexity.

Fig. 3 Reverse-transcriptase-PCR analysis of NGFI-B isoforms. NGFI-B2, NGFI-B and G3PDH cDNAs, synthesized using RNAs from the indicated sources, were amplified by means of PCR. The products were resolved by electrophoresis on agarose gels.

NGFI-B2 and NGFI-B induction in human cells

To determine whether or not NGFI-B2 mRNA is induced by stimulation, we examined the effect of TPA and forskolin in human neuroblastoma (NB-OK-1) and breast cancer (MCF7) cells. NGFI-B was used as a positive control because it is identified as a TPA and/or forskolin inducible gene in neuroblastoma cells (8). TPA and forskolin rapidly induced the transcripts of NGFI-B, but not of NGFI-B2, in both NB-OK-1 and MCF7 cells (Fig. 4). PCR amplification of the G3PDH cDNA sequence as a control was not changed in any samples. Although all members of the Nur77/NGFI-B family (NGFI-B, RNR-1 and NOR-1) are defined as immediate-early genes (8), our results suggested that NGFI-B2 may not be associated with immediate-early responses. However, we cannot rule out the possibility that a small amount of NGFI-B2, which was not detected by RT-PCR, is induced in those cells, or that NGFI-B2 is induced in other cell types. The differential transcription regulation of NGFI-B isoforms and the two unique 5'-ends also suggest that this gene has two promoters, similar to those described for the RARs and RXRs (5, 6).

The complexity of the organization and expression of the NGFI-B gene is reminiscent of that of other key developmental genes. This complexity may serve to generate several regulatory factors from a single gene through alternative splicing and multiple promoters. Since there are multiple isoforms of NOR-1 and RNR-1, the functional differences between these isoforms of the Nur77/NGFI-B family should be determined.

In conclusion, we identified and characterized a human NGFI-B2 cDNA isoform, which encodes a common core amino acid sequence corresponding to the transactivating domain, and which has a unique 5'-untranslated region and lacks a putative ligand-binding domain. The expression and regulation of NGFI-B isoforms suggested that these variants play different roles in biological processes. Since the NGFI-B gene is implicated in important biological processes including apoptosis, G_0/G_1 transition and neural development, detailed studies of NGFI-B isoforms are crucial to determine the molecular mechanisms of these processes.

Fig. 4 Effect of forskolin and TPA on gene expression of NGFI-B2, NGFI-B and G3PDH in human neuroblastoma (NB-OK-1) and breast cancer (MCF7) cells. Cells were stimulated with forskolin ($10 \mu\text{M}$) and TPA (100nM) for 3 h. Total RNA fractions were amplified by RT-PCR. [-]: non-treated control, [+]: exposed to forskolin and TPA.

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FOOTNOTE

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence database with the following accession number D85245.