Vol. 182, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1992 Pages 325-332

CHARACTERIZATION OF THE 5'-FLANKING REGION OF THE HUMAN BRAIN-DERIVED NEUROTROPHIC FACTOR GENE

Asae Shintani, Yoshitaka Ono, Yoshihiko Kaisho and Koichi Igarashi

Biotechnology Research Laboratories, Takeda Chemical Industries, Ltd., Yodogawa-ku, Osaka 532, Japan

Received November 26, 1991

SUMMARY: The 5'-flanking region of the human brain-derived neurotrophic factor (BDNF) gene was isolated from a human placental genomic library using the cDNA fragment for the 5'-noncoding region of human BDNF as a probe. A 3.2Kbp genomic fragment containing the 5'-flanking region, the first exon and a portion of the first intron was isolated and sequenced. The transcriptional initiation site, identified by S1 nuclease mapping, was located 26bp downstream from the TATA-like sequence. Several expression plasmids, in which the BDNF promoter regions were fused to the chloramphenicol acetyltransferase (CAT) gene, were constructed. Transient expression in human glioma Hs683 cells demonstrated that a fragment of about 0.5Kbp from the transcriptional initiation site was sufficient for promoter activity.

Neurotrophic factors are believed to play an essential role in the growth, survival, and differentiation of neurons in the nervous system. Thus far three members of the target derived neurotrophic molecules have been identified: nerve growth factor (NGF) (1,2), brain-derived neurotrophic factor (BDNF) (3,4) and neurotrophin-3 (NT-3) (5,6)/nerve growth factor-2 (NGF-2) (7). Comparison of the primary structures deduced from the nucleotide sequences reveals that they have strictly conserved domains, and therefore, they are thought to be derived from closely related ancestral genes (8). However, the regional distribution, cellular localization and developmental regulation differ slightly among these three. Therefore, analysis of the promoter regions of the corresponding genes is important for elucidation of the regulation of gene expression. Only the promoter region for NGF gene,

0006-291X/92 $1.50

however, has been cloned (9). We report here the isolation of a human BDNF genomic clone and the identification of the transcriptional initiation site. We also demonstrated the 5'-flanking region having the promoter activity using the expression of transfected BDNF promoter-CAT fusion genes.

MATERIALS AND METHODS

Library screening and DNA sequencing: Two oligonucleotide primers were synthesized on the basis of pig BDNF gene (3). The sense primer was 5'-GACCATCCTTTTCCTTACT-3' (corresponding to the nucleotide number 168 to 186) and the antisense primer was 5'-CACTATCTTCCCCTCTTAAT-3' (corresponding to the nucleotide number 907 to 926). PCR amplification was performed using these primers and pig genomic DNA (94°C 1min, 60°C 2min, 72°C 3min, 30 cycles). After the amplification, the product was digested with Sau3AI, and a 540bp DNA fragment was recovered. This 540bp DNA fragment, labeled by the random-oligolabelling reaction, was used as a probe. A human glioma (Hs683) cDNA library (Clontech Laboratories, USA) was screened using nitrocellulose filters. Hybridization was carried out for 16h at 65°C in 6xSSC (1xSSC contains 150mM NaCl and 15mM sodium citrate), 5xDenhalt's solution (1xDenhalt's solution contains 0.02% polyvinylpyrrolidone, 0.02% Ficoll and 0.02% bovine serum albumin), 0.1% SDS and 100μg/ml heat denatured salmon sperm DNA. The filters were washed for 5min three times at room temperature in 2xSSC-0.1% SDS and then once for 90min at 65°C in 1xSSC-0.1% SDS. The filters were autoradiographed onto Kodak X-AR films at -40°C with intensifying screens.

The 280bp DNA fragment containing the human BDNF cDNA 5'-noncoding region, obtained by digesting the cloned cDNA with EcoRI and DraIII, was labeled by the oligolabelling reaction and used as a probe. A human placental genomic DNA library was screened using nitrocellulose filters under the condition described above.

Nucleotide sequences were determined by the enzymatic chain termination method using Sequenase (U.S.B., USA).

S1 nuclease mapping: Total RNA from human glioma Hs683 cells was isolated by the guanidinium isothiocyanate/CsCl method (10). The antisense single stranded DNA was synthesized by the reaction of the E. coli DNA polymerase (Klenow fragment) using a 19 mer oligonucleotide (5'-GCTTTAATGAGACACCCACC-3', +152 to +171), end-labeled with r-[^{32}P]ATP, as a primer. The synthesized DNA was digested with PstI, and the probe DNA (0.53Kbp fragment spanning -358 to +171) was isolated from a 6% polyacrylamide 8M urea gel. Total RNA (100μg) and probe were dissolved in hybridization buffer (40mM PIPES, pH6.4, 0.4MNaCl, 1mM EDTA, 80% formamide) and heated at 80°C for 10min. The hybridization mixture was kept at 50°C for 3h. The hybrids were analyzed on a 6% polyacrylamide urea sequencing gel after digestion with 360 units of S1 nuclease at 37°C for 30min.

Plasmid construction and CAT assay: pCAT-Basic, pCAT-Enhancer, and pCAT-Control plasmid were purchased from Promega (USA). pCAT-Basic plasmid contains only the bacterial CAT gene, while pCAT-Enhancer plasmid contains the CAT gene and the SV40 enhancer element. pCAT-Control plasmid containing CAT gene and the SV40 promoter/enhancer element was used as a positive control. For the construction of human BDNF promoter-CAT fusion plasmids, the ends of each DNA fragment were converted to HindIII sites by T4

polymerase and HindIII linkers (pCAAGCTTG, Takara Shuzo, Japan). Several deletions of the 5'-flanking region of the BDNF gene were produced using Kilo-Sequencing deletion Kit (Takara Shuzo, Japan). Fusion constructs were transfected to human glioma Hs683 cells by the calcium phosphate precipitation method (11). Forty-eight hours after transfection, cells were harvested and CAT activities were measured (12). CAT enzyme reactions were performed using 0.25μCi[^{14}C]chloramphenicol, 5mM acetyl-CoA and cell extract at 37°C for 4h. Acetylated reaction products were resolved by TLC analysis using a TLC plate(Merck, Germany) and a 95% $CHCl_3$ / 5% MeOH mobile phase and were then autoradiographed.

Plasmid pTB1328, which was constructed by insertion of PLAP489 isolated from pGem-4Z/PLAP489(SEAP)(13) into the EcoRI site of pTB701(14), was co-transfected to provide an internal control for differences in transfection efficiency between different precipitates. Alkaline phosphatase activity was measured by the increase in light absorbance at 405nm which accompanies the hydrolysis of p-nitrophenylphosphate(13). The conditioned medium was removed from the transfected plate and heated at 65°C for 5min. The medium was then clarified by centrifugation in a microfuge at 14000xg for 5min. Ten-microliter aliquots of medium were then adjusted to 1xSEAP assay buffer (1.0M diethanolamine, pH9.8, 0.5mM $MgCl_2$, 10mM L-homoarginine) in a final volume of 200μl and prewarmed to 37°C for 10min in a 96-well flat-bottom culture dish (Nunc, Denmark). Twenty microliters of 120mM p-nitrophenylphosphate dissolved in SEAP assay buffer was then added with mixing, and the A_{405} of the reaction mixture was read.

RESULTS

Cloning and sequencing of human BDNF genomic DNA

In order to isolate the 5'-flanking region of the human BDNF gene, a human placental genomic DNA library was screened using 280bp of EcoRI-DraIII fragment from the 5'-noncoding region of human BDNF cDNA as a probe. Four positive clones were isolated from $2x10^6$ plaques. A 1.5Kbp EcoRI fragment found in all the clones was hybridized with the probe by Southern blotting analysis. The nucleotide sequences of the 1.5Kbp EcoRI fragment and 1.6Kbp of the upstream region were determined as shown in Fig.1. The sequence from +29 to +294 was exactly the same as that of the 5'-noncoding region of human BDNF cDNA. The consensus sequence of the splice donor site was found at the position from +294 to +295. This indicates that the sequence downstream from +295 is the first intron of the human BDNF gene. Furthermore, TATA box like- and CAAT box like-sequences were found at the position from -26 to -19 and from -49 to -46, respectively. It is known that the TATA box is located approximately 30bp upstream from the transcriptional initiation site. If this TATA box like-sequence is functional as a core promoter, the BDNF transcriptional initiation site should be located about 30bp downstream from this sequence. Thus, we tried to identify the transcriptional initia-

AGCTTGGTGAACGTGGGTAAATTATTTCCTGGCAGAGGATCGGATTCTTCTTTTATAAAACGGGTAAATAATTTCTGTCACTAGTCTTTA -2284

GAAGTTCTAAAATAGCTAATGTTAGTGAATTCATTTTGCTAACTGTAAACCCTTAGGCAAATTGAACTGAGTATGTAATAATATTATATA

TTCAGTTCAACAGCACATTCTTGGTAACCACAAGAGGGTCCAGGAAAGGAAACTGTTTATAAATATTTCCCTTTAGCAAAATTAATGTTG -2104

GAGTCTTTAGGGAAATTCTTACAGCAATAGTCTTCGCAATTATTAGGTCAAACCCCTTTGAGATTACAGAAAAACGCACACACACAGAAA

GCTGCCTGCAGAATTTGGGTGTGGGCTTGGTGGGAGATTCCTCTGATACCCAGTGTTGTACCCCCAAGAGGTGTTTCTCAAAGTGTGACT -1924

TCAGATTGTCTGCATTCGAATTGCTTGTGGATTTATTAAAATTATAACTCCTGGGCCCTGCCCCACCCCTACTAAATCACAATTTCAGGA

GGAGGGACCTTCATTTTAACACTCACCCAGGTGATTTTTATGCTCCGAGGAGGTCCAGGGACTCCAAGTTAAGTACGGTACTGCTGTCTT -1744

ATTCTTTATTCTAAATTTTAATGGTCTGCACAAATTGGTTGAACTAATGAGAAGAAAATTCAGCTTTAAAGCAGAAACACAGGTAGACGG

TTGACAGAGTTCATCAAATGGATAATTGAAAATGTCCTCTGGACCCTAGCCATATAAGTTCTCTTCAAGGGTCTTGGCTACAGGCAAATG -1564

AGAACCCGAAAGGCTATTTGCTATTTTGCTGCGGGCAGTGGTGGGGGTGGAGGGCGGGGGAGGATTAACTGAGCCAGTTCTGCCCCCACC

CTCGAATCACCTACCCCCACTCTGGTTAAAGCAGAAGACTTTTTATTTATCTTGGCTGCCCTGGTTCGTTATTAAAAGGGTTAGCTTATA -1384

CGTGTGTTTGCTGGGGCTGGAAGTGAAAACATCTGCAAAAGCATGCAATGCCCTGGAACGGAACTCTTCTAATAAAAGATGTATCATTTT

AAATGCGCTGAATTTTGATTCTGGTAATTCGTGCACTAGAGTGTCTATTTCAGAGGCAGCGGAGGTATCATATGACAGCGCACGTCAAGG -1204

CACCGTGGAGCCCTCTCGTGGACTCCCACCCACTTTCCCATTCACCGCGGAGAGGGCTGCTCTCGCTGCCGCTCCCCCCGGCGAACTAGC

ATGAAATCTCCCTGCCTCTGCCGAGATCAAATGGAGCTTCTCGCTGATGGGGTGCGAGTATTACCTCCGCCATGCAATTTCCACTATCAA -1024

TAATTTAACTTCTTTGCTGCAGAACAGAACCAGTACATACCGGGCACCAAAGACTCGCGCCCCCTCCCCCCTTTAATTAAGCGAAGGGAA

CGTGAAAAAATAATAGAGTGTGGGAGTTTTGGGGCCGAAGTCTTTCCCGGAGCAGCTGCCTTGATGGTTACTTTGACAAGTAGTGACTGA -844

AAAGGTGGGTTTGTTTTCTTTCTTTCTCTTTCCGTTTTTTCTGTTTGGTCGGCTAGAAAGCGTGTGGCTTTAGCGAGGTCTGTCATTGCC

TGGGCTTCCTGGCTGGAACAAGTAACTTGGTGTAACGTTATCTGGGGGCGTTCATCAATAAAAAATGCTGTTATTATCTTGATTGAATTC -664

CTATTAGGCAAACTCTAGAGAGGTCAGTGCGCGAACTCTGTTTAAGCCGGCGTGTTTAAGGCAGCAGAGTAAACCAATAGCCCCCATGCT

CTGTGCGATTTCATTGTGTGCTCGCGTTCGCAAGCTCCGTAGTGCAGGAAGGTGCGGGAAGGTGTGTCTGTGGCCCGGGAAACGCACGCC -484

CTCTCCCAGAGAACTTGGGTGCTGGGATGGGGAGGAAGGGGAGAGTTGAAAGCTAGGGGAGCGAGACCTCGGGGCGTGCGATTCTCACTC

GCTCCCTCCCGCCCCAGCGCCCACAGCCGGGGTTTCTGCAGAGGGAGAGGGACGCGGGGTTCCCCGGGGCTGAGGCTGGGGCTGGAACAC -304

CCCTCGAAGCCGCGGGCGTCCTGTCCAAGGCGCCCCAGGAGGGCGCAGGACTCGCAGGGCGATGTCGCGGGGCCCTAGGGGAGGAGGTGA

GGACAGGCCCCGGGGGAGCGGGGAGTTCCGGGCGCCCCTCGGTTTCCCCGCGCGAGGAAAAGACGCGGCGTTCCCTTTAAGCGGCCGCCT -124

CGAACGGGTATCGGTAGCGCGGGCGAGCGGGGAGCGGGGGGGCGGGGGGCGGGGGGGGGGGCGGCGCCGTTTGACCAATCGAAGCTCAACC

GAAGAGCTAAATAATGTCTGACCCGGGCGCAAGGCGCAGCCTGGAGCTCCGGGTCCCCGCCGCTGCCGCCGCCGCGCCCGGGCGCACCCG +57

CCCGCTCGCTGTCCCGCGCACCCCGTAGCGCCTCGGGCTCCCGGGCCGGACAGAGGAGCCAGCCCGGTGCGCCCCTCCACCTCCTGCTCG

GGGGGCTTTAATGAGACACCCACCGCTGCTGTGGGGCCGGCGGGGAGCAGCACCGCGACGGGGACCGGGGCTGGGCGCTGGAGCCAGAAT +237

CGGAACCACGATGTGACTCCGCCGCCGGGGACCCGTGAGGTTTGTGTGGACCCCGAGgtaggcaagcgctgggaatggggcttggtgcag

gagctgcccgtccgcgggagagagttgactgggggatcccccaccccaaagttgtgggacgaggccatgtctccttctttcctcccctcc

ggtagaagggacgatttggagttactcttggggagttttctcccccatcccacaacccagaaggtcagccggcaccaccagggaaaaagg

gacccggggaagtcacgaagtagaggagggaaggcctggaggagacccagagctgcgtgatgggagcaaagacggagacccggggatccc

tcgcagccctcccccagcccaggagtagtcgagagagacttaggggccagagctgtcgagggtcctgactgaggggagggtgctggggc

taggctaggaatccttccaggggtgggtggtccccgcgccgacttgcgggggagtgggagggaagcttgcgccttcagcccgcatccc

ttccccggagctgcacacggctacctgctcccaggaattgagactgaagtggacttacaagtccgaagccaagtgagcttggaaaacttg

ggaggcgaattc

Figure 1. Nucleotide sequence of the cloned human BDNF genomic DNA. Nucleotide numbering is relative to the transcriptional initiation site which is denoted by the asterisk. TATA and CAAT boxes are boxed. The primer sequence for S1 nuclease mapping is underlined. The typical sequence containing the SP1 binding sites is underscored with a bold line. The intron sequences are represented by lower-case letters.

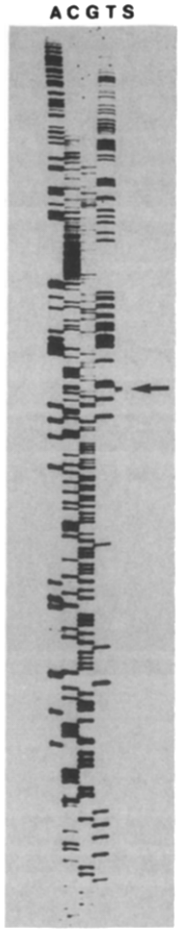

Figure 2. Transcriptional initiation site of human BDNF gene as determined by S1 nuclease mapping. The detectable band is indicated by the arrow. The DNA sequencing ladder is used to correctly estimate the size of this band.

tion site by S1 nuclease protection mapping. As shown in Fig.2, one fragment, 171 nucleotides in length, was protected from S1 nuclease digestion by human glioma Hs683 cell RNA. We concluded, therefore, that the transcription is initiated from the asterisk position as indicated in Fig.1, and the TATA box like-sequence at 26bp upstream from the transcriptional initiation site might be functional.

Promoter activity of human BDNF promoter

A chloramphenicol acetyltransferase (CAT) assay was performed

to confirm that this 5'-flanking region contained a functionally active promoter. Plasmid pTB1428, in which a 2.4Kbp DNA fragment from -2374 to +244 was fused to the CAT gene, was transfected into Hs683 cells by the calcium phosphate precipitation method. As shown in Fig.3, the extract from pTB1428-transfected cells displayed positive enhanced CAT activities, suggesting that the 2.4Kbp DNA fragment contains the functional promoter. Next, to determine whether the enhancer or the silencer element is located in the 5'-flanking region of the human BDNF gene, several 5'-deletion mutants were constructed and CAT activities were determined. As shown in Fig.4, the CAT activities in the extracts from the transfected cells were not changed significantly, except in the case of the extract from the pTB1437 transfected cells (-308 to +11). This suggests that the 2.4Kbp of 5'-flanking region of

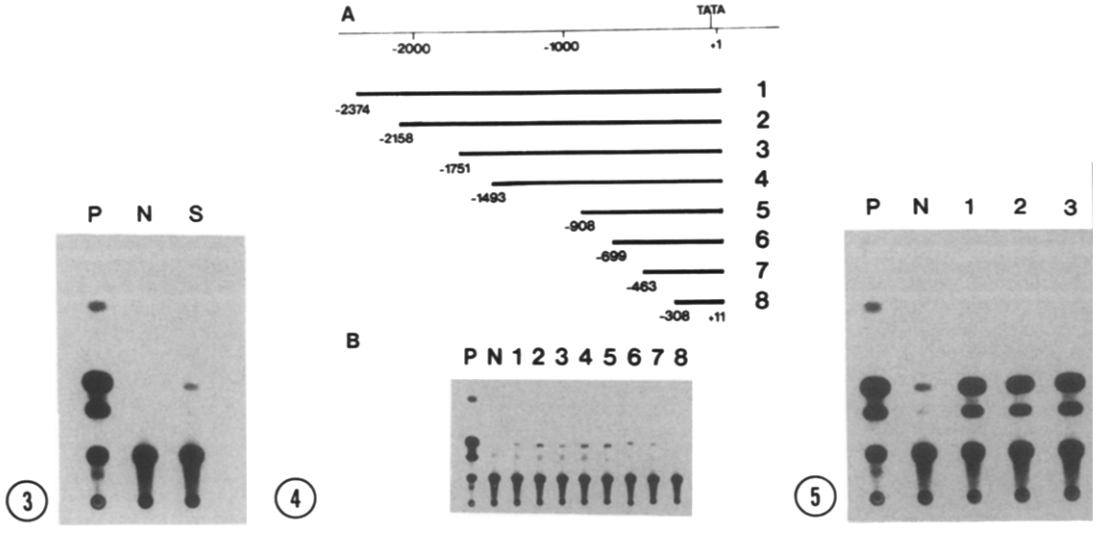

Figure 3. CAT activity in the extracts of Hs683 cells transfected with the fusion gene. P:pCAT-Control plasmid, N:pCAT-Basic plasmid, S:pTB1428 (-2378 to +244). Essentially identical results were obtained in at least three independent experiments.

Figure 4. (A) Schematic representation of the 5'-deletion mutants. (B) CAT activity for the 5'-deletion mutants. P:pCAT-Control plasmid, N:pCAT-Basic plasmid, 1:pTB1429 (-2374 to +11), 2:pTB1430 (-2158 to +11), 3:pTB1432 (-1751 to +11), 4:pTB1433 (-1493 to +11), 5:pTB1434 (-908 to +11), 6:pTB1435 (-699 to +11), 7:pTB1436 (-463 to +11), 8:pTB1437 (-308 to +11). Essentially identical results were obtained in at least three independent experiments.

Figure 5. CAT activity of plasmids containing the human BDNF 5'-flanking region and the SV40 enhancer element. P:pCAT-Control plasmid, N:pCAT-Enhancer plasmid, 1:pTB1438 (-699 to +11), 2:pTB1439 (-468 to +11), 3:pTB1440 (-308 to +11).

the human BDNF gene doesn't contain the apparent silencer element.

Essentially similar results were obtained when the BDNF promoter-CAT fusion plasmids were introduced into non-neuronal mouse L cells (data not shown). Furthermore, when plasmid pTB1440 which contains the DNA fragment from -308 to +11 in the CAT-Enhancer plasmid was transfected into Hs683 cells, the cell extract displayed significant CAT activity (Fig.5).

These results indicate that the 0.3Kbp region upstream from the transcriptional initiation site is sufficient to support core promoter activity and that the enhancer element might be located at the position from -463 to -308.

DISCUSSION

In this paper we described the isolation and the structural analysis of the human BDNF genomic DNA including the promoter region. In the 5'-flanking region of the human BDNF gene, TATA and CAAT boxes were located -26bp and -49bp upstream from the transcriptional initiation site, respectively, and the tandem repeats of the consensus sequence for SP1 binding (GGGCGG) were found just upstream from the CAAT box. It is not yet clear whether these SP1 binding sequences are functional with respect to regulation of the gene expression of human BDNF. It should be noted, however, that such a G-rich region is present at a similar position, upstream from the TATA box like-sequence, in both the mouse and rat NGF promoter regions.

Although it is suggested that the enhancer element might be located at the position from -468 to -308, typical sequences corresponding to known enhancer elements could not be found. Therefore, there might be a novel cis-element in this region. We cannot exclude the possibility, however, that full expression of the gene may require other elements either sequences elsewhere in the DNA.

It is reported that mouse NGF promoter has many AP-1 binding sites, and particularly an intronic AP-1 binding site is necessary for NGF promoter activity (15). On the other hand, in the case of the human BDNF gene, no typical AP-1 binding sequence was found in either the 5'-flanking region or the first intron. Indeed, promoter activities could not be enhanced by the treatment with phorbol ester (data not shown). This might suggest that the expression of the BDNF gene is regulated by trans-acting factors different from those in the case of NGF gene.

BDNF, one of the neurotrophic factors, promotes the survival of neurons particularly in the central nervous system. Analysis of the transcriptional regulatory element of the BDNF promoter is important to understand the physiological function of BDNF in the central nervous system. In addition, the cloned BDNF promoter region reported in this paper will be useful for the identification of the specific trans-acting factors regulating the BDNF gene expression.

ACKNOWLEDGMENTS

We thank Dr. A. Kakinuma for his encouragement and Dr. R. Micanovic for supplying the plasmid pGem-4Z/PLAP489(SEAP).

REFERENCES

1. Thoenen, H., and Barde, Y. -A. (1980) Physiol. Rev. 60, 1284-1335.
2. Gnahn, H., Hefti, F., Heumann, R., Schwab, M.E., and Thoenen, H. (1983) Dev. Brain Res. 9, 45-52.
3. Barde Y. -A., Edgar, D., and Thoenen, H. (1982) EMBO J. 5, 549-553.
4. Leibrock, J., Lottspeich, F., Hohn, A., Hoffer, M., Hengerer, B., Masiakowski, P., Thoenen and Barde, Y. -A. (1989) Nature 341, 149-152.
5. Hohn, A., Leibrock, J., Bailer, K., and Barde, Y. -A. (1990) Nature 344, 339-341.
6. Maisonpierre, P. C., Belluscio, L., Squinto, S., Ip, N. Y. (1990) Science 247, 1446-1451.
7. Kaisho, Y., Yoshimura, K., and Nakahama, K. (1990) FEBS Lett. 266, 187-191.
8. Rosenthal, A., Gerddel, d. V., Nauyen, T., Lewis, M., Shin, A. (1990) Neuron 4, 767-773.
9. Zheng, M., and Heinrich, G. (1988) Mol. Brain Res. 6, 133-140.
10. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18,5294-5299.
11. Graham, F. L., and van der Eb, A. J. (1973) Virology 52, 456-467.
12. Widen, S. G., Kedar, P., and Wilson, S. H. (1988) J. Biol. Chem. 263, 16992-16998.
13. Barger, J., Hauber, J., Hauber, R., Geiger, R., and Cullen, B. R. (1988) Gene 66, 1-10.
14. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarash, K., and Nishizuka, Y. (1988) J. Biol. Chem. 263, 6927-6932.
15. Hengere, B., Lindholm, D., Heumann, R., Ruther, U., Wagner, E. F., and Thoenen, H. (1990) Proc.Natl. Acad. Sci. USA 87, 3899-3903.