Volume 266, number 1,2, 187–191 FEBS 08534 June 1990

Cloning and expression of a cDNA encoding a novel human neurotrophic factor

Yoshihiko Kaisho, Koji Yoshimura and Kazuo Nakahama

Biotechnology Research Laboratories, Takeda Chemical Industries Ltd, 17–85 Jusohonmachi 2-chome, Yodogawa-ku, Osaka 532, Japan

Received 18 April 1990; revised version received 26 April 1990

A cDNA encoding a novel human neurotrophic factor (designated nerve growth factor-2; NGF-2) was cloned from a human glioma cDNA library using a synthetic DNA corresponding to human nerve growth factor (NGF). The cloned cDNA encodes a polypeptide composed of 257 amino acid residues including a prepro-sequence of 138 residues and a mature region of 119 residues. The amino acid sequence of human NGF-2 exhibits 58% similarity with that of human NGF. Conditioned medium of COS-7 cells transfected with an expression plasmid for human NGF-2 cDNA supported the survival of sensory neurons isolated from dorsal root ganglia of embryonic chicks. A 1.5 kb of NGF-2 mRNA can be detected from an early development stage in rat brain, by Northern blotting analysis.

Novel neurotrophic factor; cDNA cloning; Gene family; Glioma cell

1. INTRODUCTION

Neurotrophic factors are believed to play an essential role in the growth, survival, and differentiation of neurons in the nervous system. So far, several well-characterized neurotrophic factors have been reported. One is nerve growth factor (NGF) which was isolated from the male mouse submaxillary gland [1]. This factor is essential for the survival and development of peripheral sensory and sympathetic neurons [2] and cholinergic neurons of the basal forebrain [3]. Another is brain-derived neurotrophic factor (BDNF) which is isolated from pig brain [4] and supports the survival of sensory neurons [5,6] but does not support the survival of sympathetic neurons [5]. The primary structure of BDNF deduced from the cDNA is structurally related to that of NGF [7].

To isolate other NGF related genes, we tried to screen a human glioma cDNA library under low stringent hybridization conditions using a synthetic DNA corresponding to human NGF as a probe. The cDNA thus cloned encoded a novel neurotrophic factor (designated nerve growth factor-2, NGF-2). We report here the cloning and expression of human NGF-2 cDNA, and the presence of NGF-2 mRNA in several tissues in the rat.

Correspondence address: Y. Kaisho, Biotechnology Research Laboratories, Takeda Chemical Industries Ltd, 17-85 Jusokonmachi 2-chome, Yodogawa-ku, Osaka 523, Japan

2. MATERIALS AND METHODS

2.1. *cDNA cloning*

A 0.38 kb DNA fragment encoding human NGF was synthesized according to the nucleotide sequence reported by Ullrich et al. [8]. The DNA fragment was labelled by the oligolabelling reaction and used as a probe. A λgt11 human glioma (Hs683) cDNA library (Clontech Laboratories) was screened using nylon filters (Amersham) and the probe. Hybridization was carried out for 16 h at 60°C in 6×SSC (1×SSC contains 150 mM NaCl and 15 mM sodium citrate), 5×Denhardt's solution (1×Denhardt's solution contains 0.02% polyvinylpyrrolidone, 0.02% Ficoll and 0.02% bovine serum albumin), 0.1% SDS, and 20 μg/ml sonicated denatured salmon sperm DNA. The filters were washed three times each for 5 min at 25°C in 2×SSC containing 0.1% SDS and then once for 90 min at 60°C in 1×SSC containing 0.1% SDS. The filters were autoradiographed onto Kodak X-AR films at −40°C with intensifying screens. Nucleotide sequence was determined by the dideoxy chain-termination method [9].

2.2. *Northern blotting analysis*

Total RNAs were isolated from human glioma Hs683 cells and from the indicated tissues of the rat by the method of Chirgwin et al. [10]. Poly $(A)^+$ RNAs were prepared by oligo(dT)-cellulose column chromatography (Pharmacia) [11]. Poly $(A)^+$ RNAs were electrophoresed on 1.5% agarose gel containing formaldehyde and transferred to a nitrocellulose filter (S & S). DNA fragments containing human NGF-2 cDNA, NGF gene cloned from a human genomic library, or rat NGF-2 gene cloned from a rat genomic library exhibiting 90% homology in nucleotide sequence with human NGF-2 cDNA were labelled and used as probes. Hybridization was carried out as described above. The filters were washed 3 times each for 5 min at 25°C in 2×SSC containing 0.1% SDS, and then once for 90 min at 68°C in 0.1×SSC containing 0.1% SDS. The filters were autoradiographed onto Kodak X-AR films at −40°C with intensifying screens.

2.3. *Biological assays*

COS-7 cells were transfected with each plasmid DNA using the

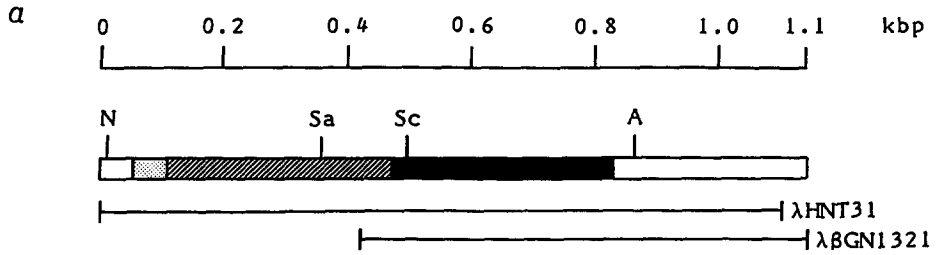
Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

calcium phosphate coprecipitation technique [12] and grown for 24 h. After medium was replaced with Dulbecco's modified Eagle's medium (DMEM) containing 0.5% fetal calf serum (FCS), the cells were grown for 48 h. The biological activity for NGF-2 was assayed using dorsal root ganglia from embryonic day-8 chicks (E8) as described by Davies and Lindsay [13].

3. RESULTS

3.1. *Cloning and sequencing of human NGF-2 cDNA*

Cloning of cDNA which hybridized with human NGF gene was carried out from a human glioma




```
b
                                                    -138                -130
                                                    MetSerIleLeuPheTyrValIlePheLeu
TGCCATGGTTACTTTTGCCACGATCTTACAGGTGAACAAGGTGATGTCCATCTTGTTTTATGTGATATTTCTC   73

                    -120                -110                -100
AlaTyrLeuArgGlyIleGlnGlyAsnAsnMetAspGlnArgSerLeuProGluAspSerLeuAsnSerLeuIleIleLysLeuIleGln
GCTTATCTCCGTGGCATCCAAGGTAACAACATGGATCAAAGGAGTTTGCCAGAAGACTCGCTCAATTCCCTCATTATTAAGCTGATCCAG  163

                    -90                 -80                 -70
AlaAspIleLeuLysAsnLysLeuSerLysGlnMetValAspValLysGluAsnTyrGlnSerThrLeuProLysAlaGluAlaProArg
GCAGATATTTTGAAAAACAAGCTCTCCAAGCAGATGGTGGACGTTAAGGAAAATTACCAGAGCACCCTGCCCAAAGCTGAGGCTCCCCGA  253

                    -60                 -50                 -40
GluProGluArgGlyGlyProAlaLysSerAlaPheGlnProValIleAlaMetAspThrGluLeuLeuArgGlnGlnArgArgTyrAsn
GAGCCGGAGCGGGGAGGGCCCGCCAAGTCAGCATTCCAGCCAGTGATTGCAATGGACACCGAACTGCTGCGACAACAGAGACGCTACAAC  343

                    -30                 -20                 -10
SerProArgValLeuLeuSerAspSerThrProLeuGluProProProLeuTyrLeuMetGluAspTyrValGlySerProValValAla
TCACCGCGGGTCCTGCTGAGCGACAGCACCCCCTTGGAGCCCCCGCCCTTGTATCTCATGGAGGATTACGTGGGCAGCCCCGTGGTGGCG  433

●          -2   +1                  +10                 +20
AsnArgThrSerArgArgLysArgTyrAlaGluHisLysSerHisArgGlyGluTyrSerValCysAspSerGluSerLeuTrpValThr
AACAGAACATCACGGCGGAAACGGTACGCGGAGCATAAGAGTCACCGAGGGGAGTACTCGGTATGTGACAGTGAGAGTCTGTGGGTGACC  523

                    +30                 +40                 +50
AspLysSerSerAlaIleAspIleArgGlyHisGlnValThrValLeuGlyGluIleLysThrGlyAsnSerProValLysGlnTyrPhe
GACAAGTCATCGGCCATCGACATTCGGGGACACCAGGTCACGGTGCTGGGGGAGATCAAAACGGGCAACTCTCCCGTCAAACAATATTTT  613

                    +60                 +70                 +80
TyrGluThrArgCysLysGluAlaArgProValLysAsnGlyCysArgGlyIleAspAspLysHisTrpAsnSerGlnCysLysThrSer
TATGAAACGCGATGTAAGGAAGCCAGGCCGGTCAAAAACGGTTGCAGGGGTATTGATGATAAACACTGGAACTCTCAGTGCAAAACATCC  703

                    +90                 +100                +110
GlnThrTyrValArgAlaLeuThrSerGluAsnAsnLysLeuValGlyTrpArgTrpIleArgIleAspThrSerCysValCysAlaLeu
CAAACCTACGTCCGAGCACTGACTTCAGAGAACAATAAACTCGTGGGCTGGCGGTGGATACGGATAGACACGTCCTGTGTGTGTGCCTTG  793

                    +119
SerArgLysIleGlyArgThr
TCGAGAAAAATCGGAAGAACATGAATTGGCATCTCTCCCCATATATAAATTATTACTTTAAATTATATGATATGCATGTAGCATATAAAT  883
```

```
c
                    Signal              Pro
human   MSILFYVIFLAYLRGIQGNNMDQRSLPEDSLNSLIIKLIQADILKNKLSKQMVDVKENYQSTLPKAEAPREPE
rat     MSILFYVIFLAYLRGIQGNNMDQRSLPEDSLNSLIIKLIQADILKNKLSKQMVDVKENYQSTLPKAEAPREPE

                                                                    Mature
human   RGGPAKSAFQPVIAMDTELLRQQRRYNSPRVLLSDSTPLEPPPLYLMEDYVGSPVVANRTS-RRKRYAEHKSH
rat     QGEATRSEFQPMIATDTELLRQQRRYNSPRVLLSDSTPLEPPPLYLMEDYVGNPVVTNRTSPRRKRYAEHKSH
        * **** *   *  *                                          *   *   *

human   RGEYSVCDSESLWVTDKSSAIDIRGHQVTVLGEIKTGNSPVKQYFYETRCKEARPVKNGCRGIDDKHWNSQCK
rat     RGEYSVCDSESLWVTDKSSAIDIRGHQVTVLGEIKTGNSPVKQYFYETRCKEARPVKNGCRGIDDKHWNSQCK

human   TSQTYVRALTSENNKLVGWRWIRIDTSCVCALSRKIGRT
rat     TSQTYVRALTSENNKLVGWRWIRIDTSCVCALSRKIGRT
```

Fig. 1. (a) Schematic diagram of the cloned human NGF-2 cDNA showing the restriction endonuclease cleavage sites. The cDNA inserts of λβGN1321 and λHNT31 are aligned below the diagram with the coding sequence boxed. The stippled and dashed boxes indicate the putative signal and pro-sequences, respectively. The closed box shows the putative mature region. Restriction sites: N, *Nco*I; Sa, *Sac*II; Sc, *Sca*I; A, *Aha*III. (b) Nucleotide sequence and deduced amino acid sequence of the cloned human NGF-2 cDNA. The additional potential initiation codon is underscored with a double line. The putative signal sequence is underlined. The putative mature region is boxed. The Lys-Arg dipeptide which precedes the proteolytic cleavage site is underscored with a bold line. The one potential N-glycosylation site in the pro-region is indicated by a large dot. (c) Alignment of amino acid sequences of human prepro-NGF-2 and rat prepro-NGF-2. Asterisks indicate different amino acids. Hyphens are introduced for optimal alignment.

(Hs683) cDNA library under low stringent hybridization conditions using a 0.38 kb synthetic DNA fragment encoding human NGF as a probe. One positive clone, λβGN1321, was isolated from 5×10^5 plaques. The clone had an insert of 0.72 kb (Fig. 1a). Clones containing additional 5′ sequences were also isolated from the same library using the insert in λβGN1321 as a probe. One of the clones, λHNT31, had an insert of 1.1 kb (Fig. 1a).

The nucleotide sequence of the cDNA cloned in λHNT31 and the deduced amino acid sequence are shown in Fig. 1b. The cDNA encodes a polypeptide of 257 amino acids containing a region structurally related to human NGF. We named this polypeptide NGF-2. Comparison with the prepro-NGF suggests that the polypeptide contains a putative signal sequence of 18 amino acid residues (residues −138 through −121) and a putative pro-sequence of 120 amino acid residues (residues −120 through −1) preceding the putative mature region (residues +1 through +119). The amino acid sequence of human NGF-2 (the putative mature region) exhibits 58% similarity with that of human NGF, and the positions of the 6 cysteine residues are conserved.

Southern blotting analysis of human chromosomal DNA showed that NGF-2 gene exists as a single gene per haploid genome (data not shown). The NGF-2 cDNA was also isolated from a human placental cDNA library.

3.2. *Expression of human NGF-2 in mammalian cells*

The 1.1. kb *Eco*RI fragment of human NGF-2 cDNA prepared from λHNT31 was inserted downstream of the LTR region of Abelson murine leukemia virus and the SV40 promoter in the expression plasmid [14] to give pNTL145. The conditioned medium of COS-7 cells transfected with the plasmid pNTL145 supported the survival of sensory neurons of chicken E8 dorsal root ganglia (Fig. 2), and stimulated neurite outgrowth from rat pheochromocytoma PC12 cells (data not shown). In contrast, the conditioned medium of COS-7 cells transfected with the control plasmid pTB389 having no NGF-2 cDNA showed only a weak biological activity; this may be due to NGF endogenously produced by COS-7 cells (Fig. 2).

3.3. *Northern blotting analyses of NGF-2 mRNA*

Northern blotting analysis with human NGF-2 cDNA as a probe showed the presence of a 1.5 kb NGF-2 mRNA in human glioma Hs683 cells (Fig. 3a; left lane). In addition, a 1.3 kb NGF mRNA was also detected in the cells using human NGF gene as a probe (Fig. 3a; right lane).

Using rat NGF-2 gene as a probe, the synthesis of NGF-2 mRNA was examined in several tissues of the young adult rat (Fig. 3b). A large amount of NGF-2 mRNA was detected in all the tissues examined except muscle and testis. The highest level of mRNA synthesis was observed in kidney. The developmental time course

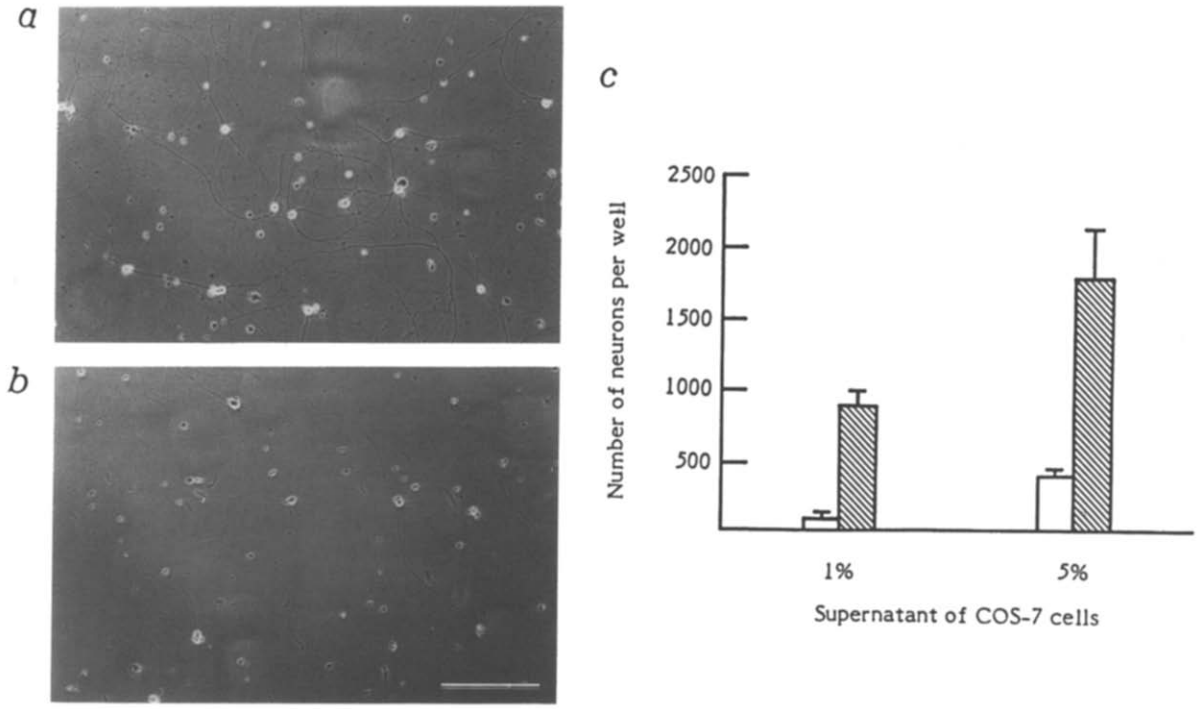


Fig. 2. The biological activity of conditioned medium of transfected COS-7 cells. Sensory neurons of chicken E8 dorsal root ganglia were grown for 5 days in the medium containing 5% of the conditioned medium of COS-7 cells transfected with human NGF-2 expression plasmid pNTL145 (a) and with the control plasmid pTB389 (b); bar = 250 μm. Neurons were plated at 5000 cells per well and grown for 3 days, and the numbers of phase-bright neurons with neurites were counted (c). Dashed bars indicate the conditioned medium of COS-7 cells transfected with pNTL145, and blank bars indicate the conditioned medium of COS-7 cells transfected with pTB389. The values are the means of duplicate experiments.

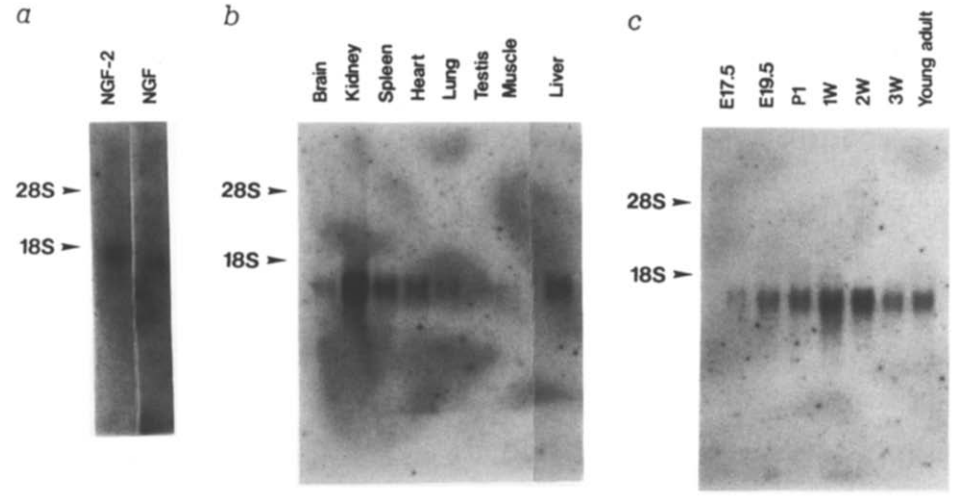


Fig. 3. (a) Analysis of NGF-2 mRNA in human glioma cells. Poly $(A)^+$ RNA (4.5 μg) was analyzed. Human NGF-2 cDNA (left lane) and human NGF gene (right lane) labelled were used as probes. Positions of ribosomal RNA markers are shown on the left. (b) Tissue distribution of NGF-2 mRNA. Poly $(A)^+$ RNAs (about 6 μg) from the indicated tissues of the young adult rat were analyzed. Rat NGF-2 gene labelled was used as a probe. (c) Developmental expression pattern of NGF-2 mRNA in rat brain. Poly $(A)^+$ RNAs (about 10 μg) from rat brain were analyzed as above. E17.5 and E19.5 indicate embryonic days 17.5 and 19.5, respectively; P1, 1W, 2W, and 3W indicate 1 day, 1 week, 2 weeks, and 3 weeks postnatally, respectively.

of NGF-2 gene expression in rat brain indicated that NGF-2 mRNA can be detected at embryonic day 17.5 and that the level of NGF-2 mRNA synthesis reached a maximum 1–2 weeks postnatally (Fig. 3c). In contrast, the level of actin mRNA synthesis was found almost equal in all above experiments (data not shown).

4. DISCUSSION

Cloning from a human glioma cDNA library using a synthetic human NGF gene as a probe under low stringent hybridization conditions resulted in the isolation of a cDNA structurally related to NGF. Transfection experiments suggested that the cDNA encodes a novel neurotrophic factor (NGF-2). Comparison with the primary structure of prepro-NGF suggests that NGF-2 has a prepro-sequence preceding the mature region. In fact, Western blotting using rabbit anti-human NGF-2 peptide antisera showed that COS-7 cells transfected with the expression plasmid pNTL145 produced in the medium an immunoreactive protein which migrated to the position corresponding to 15 kDa (data not shown). This suggests that proteolytic cleavage occurs between Arg(−1) and Tyr(+1) in Fig. 1b.

A comparison of the amino acid sequences of human NGF-2, human NGF, and pig BDNF showed that human NGF-2 exhibits 58% and 55% similarity with human NGF and pig BDNF, respectively (Fig. 4). Recently Hohn et al. and Maisonpierre et al. reported the isolation of the genes for named neurotrophin-3 (NT-3) from mouse and rat genomic DNA libraries, respectively [15,16]. The amino acid sequence of human NGF-2 was identical to those of the mouse and rat NT-3, although the sequences in the pro-sequence were significantly different between human NGF-2 and NT-3. This indicates that there are at least 3 NGF-related genes, NGF, NGF-2/NT-3, and BDNF. The high degree of structural conservation of NGF-2/NT-3

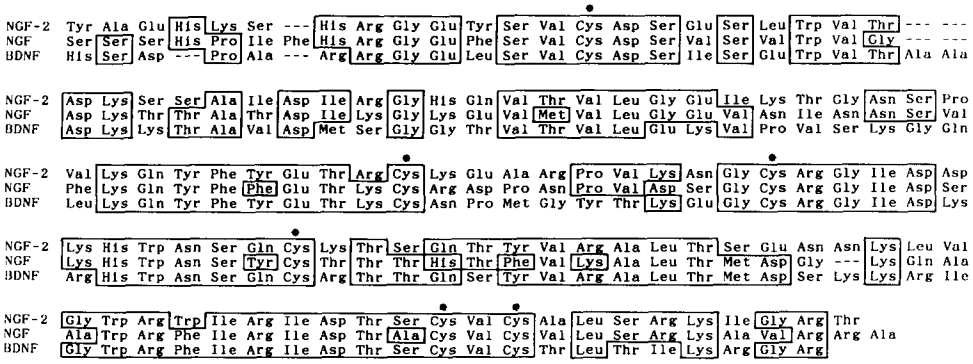


Fig. 4. Alignment of amino acid sequences of human NGF-2, human NGF, and pig BDNF. Residues commonly present among the polypeptides are boxed. Hyphens are introduced for optimal alignment. Cysteine residues are emphasized with asterisks.

among species indicates that NGF-2/NT-3 could have a primordial role in vivo.

NGF is a neurotrophic factor essential for the survival and development of sensory and sympathetic neurons and cholinergic neurons of the basal forebrain, and it is distributed in many peripheral tissues and in brain [17,18]. In rat brain NGF mRNA begins to accumulate after birth [19]. BDNF supports the survival of sensory neurons and is distributed mainly in brain [7]. NGF-2 also seems to act on sensory neurons and sympathetic neurons. It is distributed in peripheral tissues as well as in brain and, in rat brain, NGF-2 mRNA is synthesized from an early developmental stage. As these factors show specific tissue distribution and specific developmental expression, they may have distinct functions in vivo. The physiological role of NGF-2 is now under investigation.

Acknowledgements: The encouragement and support of Drs M. Nishikawa and A. Kakinuma are most gratefully acknowledged. We are greatly indebted to Drs M. Takeuchi and M. Satoh for biological assay, Drs M. Suno and M. Kakihana for their help in performing some of the experiments, and Dr T. Kurokawa for supplying rabbit antisera. Thanks are due to Drs K. Tsukamoto, K. Igarashi, R. Sasada, Y. Ono, and M. Iwane for helpful discussions and suggestions. We also thank Miss Y. Kitamura and Mrs A. Shintani for technical assistance.

REFERENCES

[1] Angeletti, R.H. and Bradshaw, R.A. (1971) Proc. Natl. Acad. Sci. USA 68, 2417–2420.
[2] Thoenen, H. and Barde, Y.-A. (1980) Physiol. Rev. 60, 1284–1335.
[3] Gnahn, H., Hefti, F., Heumann, R., Schwab, M.E. and Thoenen, H. (1983) Dev. Brain Res. 9, 45–52.
[4] Barde, Y.-A., Edgar, D. and Thoenen, H. (1982) EMBO J. 1, 549–553.
[5] Lindsay, R.M., Thoenen, H. and Barde, Y.-A. (1985) Dev. Biol. 112, 319–328.
[6] Davies, A.M., Thoenen, H. and Barde, Y.-A. (1986) J. Neurosci. 6, 1897–1904.
[7] Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H. and Barde, Y.-A. (1989) Nature 341, 149–152.
[8] Ullrich, A., Gray, A., Berman, C. and Dull, T.J. (1983) Nature 303, 821–825.
[9] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[10] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[11] Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408–1412.
[12] Graham, F.L. and Van der Eb, A.J. (1973) Virology 54, 536–539.
[13] Davies, A.M. and Lindsay, R.M. (1985) Dev. Biol. 111, 62–72.
[14] Ono, Y., Kikkawa, U., Ogita, K., Fujii, T. Kurokawa, T., Asaoka, Y., Sekiguchi, K., Ase, K., Igarashi, K. and Nishizuka, Y. (1987) Science 236, 1116–1120.
[15] Hohn, A., Leibrock, J., Bailey, K. and Barde, Y.-A. (1990) Nature 344, 339–341.
[16] Maisonpierre, P.C., Belluscio, L., Squinto, S., Ip, N.Y., Furth, M.E., Lindsay, R.M. and Yancopoulos, G.D. (1990) Science 247, 1446–1451.
[17] Shelton, D.L. and Reichardt, L.F. (1984) Proc. Natl. Acad. Sci. USA 81, 7951–7955.
[18] Heumann, R., Korsching, S., Scott, J. and Thoenen, H. (184) EMBO J. 3, 3183–3189.
[19] Whittemore, S.R., Ebendal, T., Lärkfors, L., Olson, L., Seiger, Å., Strömberg, I. and Persson, H. (1986) Proc. Natl. Acad. Sci. USA 83, 817–821.