

Biological and Immunological Properties of Recombinant Human, Rat, and Chicken Nerve Growth Factors: A Comparative Study

Carlos F. Ibáñez, Finn Hallböök, *Stine Söderström, *Ted Ebendal, and Håkan Persson

Department of Medical Chemistry II, Laboratory of Molecular Neurobiology, Karolinska Institute, Stockholm;
and *Department of Developmental Biology, Biomedical Center, Uppsala, Sweden

Abstract: Biological and immunological properties of recombinant human, rat, and chicken nerve growth factors (NGFs) were studied and compared. Recombinant NGF proteins were produced in a transient expression system using COS cells and levels of secreted NGF protein were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of conditioned media from *in vivo* [³⁵S]cysteine-labeled cell cultures. Antigenic differences among the three NGFs were studied by immunoblotting and immunoprecipitation of secreted cell products using a rabbit polyclonal antiserum against purified mouse NGF, and by a two-site enzyme immunoassay (EIA) with a monoclonal antibody against mouse NGF. Although all three NGFs were recognized equally well in the immunoblotting, only one-third of the chicken NGF protein could be detected by immunoprecipitation or by the EIA as compared to the rat and human NGFs. Thus, changes in the three-dimensional structure of the NGF molecule are most likely responsible for the antigenic differences between avian and mammalian NGFs. The three NGF proteins were also compared in their ability to displace ¹²⁵I-mouse NGF

from low-affinity NGF receptors on rat pheochromocytoma PC12 cells. Similar displacement curves and values were obtained for each NGF protein, indicating that structural differences among these molecules do not affect low-affinity binding to NGF receptors. Biological activities were studied by the ability of the conditioned media to promote neurite outgrowth from explants of E9 chick sympathetic ganglia and from PC12 cells. Although the rat system showed a slight preference for the homologous molecule, the morphological changes, dose-response curves, and maximal stimulation values obtained with the different NGFs were practically indistinguishable in the chicken bioassay. The observed differences and similarities among the three NGF proteins are discussed in the context of their evolutionary relationship and their potential therapeutic applications. **Key Words:** Binding—Biological activity—Immunoblotting—Immunoprecipitation—PC12 cells—Sympathetic ganglia. **Ibáñez C. F. et al.** Biological and immunological properties of recombinant human, rat, and chicken nerve growth factors: A comparative study. *J. Neurochem.* 57, 1033–1041 (1991).

Nerve growth factor (NGF) is a 118-amino-acid polypeptide essential for the development and survival of sympathetic and neural crest-derived sensory neurons in the PNS (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980). In recent years, NGF has also been found in the brain, where it exerts a trophic function for cholinergic neurons of the basal forebrain (Korsching et al., 1985; Large et al., 1986; Whitemore and Seiger, 1987; Thoenen et al., 1987).

The richest source of NGF is the adult male mouse submandibular gland, from where the protein has been originally purified and sequenced (Cohen, 1960; Angeletti and Bradshaw, 1971; Angeletti et al., 1973). The

NGF molecule consists of two noncovalently linked identical polypeptide chains, each containing three disulfide bonds. Other sources rich in NGF have also been identified such as the venom of a variety of snakes (Cohen, 1959; Bailey et al., 1975; Oda et al., 1989); the prostate glands of guinea pig (Harper et al., 1979), rabbit, and bull (Harper and Thoenen, 1980); bovine seminal plasma (Harper et al., 1982); and the submandibular gland of the house musk shrew (Ueyama et al., 1986).

Comparative studies among NGFs from different species have so far been restricted to those cases where the biochemical purification of the NGF protein is fea-

Received November 7, 1990; revised manuscript received January 14, 1991; accepted February 18, 1991.

Address correspondence and reprint requests to Dr. C. F. Ibáñez at Department of Medical Chemistry II, Laboratory of Molecular Neurobiology, Karolinska Institute, Box 60400, Stockholm S-104 01, Sweden.

Abbreviations used: BU, biological unit; DMEM, Dulbecco's modified Eagle's medium; EIA, enzyme immunoassay; FCS, fetal calf serum; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; SDAT, senile dementia of the Alzheimer type; SDS, sodium dodecyl sulfate; SSC, saline-sodium citrate.

sible. Snake NGF has been found to promote outgrowth of nerve fibers at concentrations similar to those of the mammalian NGFs, although the fiber outgrowth was significantly weaker (Angeletti et al., 1976; Nakata et al., 1988). The specific activities of rabbit and guinea pig NGFs have been estimated using crude tissue preparations in a semiquantitative biological assay, and were found to be similar to that of mouse NGF (Harper and Thoenen, 1980). The biological activity of bovine NGF purified from seminal plasma has also been compared in detail with that of mouse NGF (Harper et al., 1983) and found to be identical. In spite of the similarities in their biological properties, significant immunological differences have been observed among the different NGFs tested so far. As expected, antisera raised against different NGFs react preferentially with the homologous antigen (Bailey et al., 1976; Harper et al., 1983; Ueyama et al., 1986; Nakata et al., 1988). However, nonreciprocal cross-reactivity has also been observed among NGFs from some species and their corresponding antisera (i.e., the antiserum against NGF from species A is able to recognize NGF from species B whereas the antiserum against NGF from species B is not able to recognize NGF from species A), suggesting that different NGFs contain a variable number of immunodominant epitopes that can be partially or completely shared by NGF proteins from different species.

As an alternative to the biochemical purification approach, methods of molecular genetics have also proved to be useful for the production and characterization of NGFs from different species. Following the initial cloning and sequencing of a mouse NGF cDNA (Scott et al., 1983), several NGF genes have been isolated from a number of species including human (Ullrich et al., 1983), bovine (Meier et al., 1986), chicken (Ebendal et al., 1986; Meier et al., 1986; Wion et al., 1986), cobra (Selby et al., 1987), rat (Whittemore et al., 1988), and guinea pig (Schwarz et al., 1989). Nucleotide sequence analysis of these clones has shown that the mature NGF protein is evolutionarily highly conserved, and that the amino acid changes among the different NGFs are clustered. The finding that the less conserved domains are located predominantly in hydrophilic regions of the NGF molecule provides an explanation for the limited immunological cross-reactivity observed among different NGFs (Meier et al., 1986). Cloned NGF genes have been used to produce recombinant NGF both in transient and stable mammalian expression systems (Hallböök et al., 1988; Rosenberg et al., 1988; Edwards et al., 1988; Ernfors et al., 1989; Bruce and Heinrich, 1989). The production of biologically and immunologically active recombinant human NGF has led to the establishment of a two-site enzyme immunoassay (EIA) for human NGF (Heinrich and Meyer, 1988; Söderström et al., 1990), opening up the possibility to evaluate the levels of NGF protein in human autopsy-derived samples. This is of particular interest in the case of samples derived from patients with senile dementia of the Alzheimer type (SDAT), because

it has been shown that the NGF-sensitive magnocellular cholinergic neurons of the nucleus basalis of Meynert undergo a profound degeneration in SDAT patients. This results in cholinergic deficits in the target areas of these neurons (Kubanis and Zornetzer, 1981; Bartus et al., 1982; Whitehouse et al., 1982; Collerton, 1986), and a strong correlation exists between the reduction of cholinergic activity and the severity of the dementia (Perry et al., 1978; Wilcock et al., 1982). The known function of NGF in promoting the survival of basal forebrain cholinergic neurons has been used to argue that in SDAT a continuous administration of NGF into the brain may prevent degeneration of basal forebrain cholinergic neurons. However, NGF is probably not causally linked to the degeneration of basal forebrain cholinergic neurons in SDAT because the level of NGF mRNA is not significantly changed in hippocampal neurons of SDAT compared to age-matched control brains (Goedert et al., 1986; Ernfors et al., 1990a). Interestingly, a threefold higher level of NGF receptor mRNA was found in the surviving magnocellular neurons of nucleus basalis in SDAT compared to age-matched controls, arguing that exogenous treatment with NGF may be beneficial for these neurons in SDAT (Ernfors et al., 1990a).

Although NGFs from several species have recently been produced using recombinant DNA techniques, no further work has been carried out on the biological and immunological relationships among these molecules. Knowledge of the comparative biological and immunological behavior of some of these molecules, particularly human NGF and NGFs from the preferred animals for NGF research, namely chicken and rat, could be of importance, particularly for future evaluations of human NGF samples using bioassay systems based on chicken or rat cells and ganglia. This information could also provide insight into the various biochemical features of the NGF molecule with regard to its biological and immunological properties. Here we report the results of a comparative study on the biological and immunological properties of recombinant human, rat, and chicken NGFs, produced in a transient expression system using COS cells.

MATERIALS AND METHODS

Bacterial strains, cells, and plasmids

The *E. coli* K12 strain MC1061 (Huynh et al., 1985) was used to propagate and maintain all plasmid DNAs. The *E. coli* strain NM538 was used to grow λ EMBL3 bacteriophages. COS cells (Gluzman, 1981) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Rat pheochromocytoma PC12 cells (Greene and Tischler, 1976) were grown in DMEM supplemented with 5% FCS and 10% horse serum. pXM (Yang et al., 1986) was used as expression vector for all NGF genes. pXRN, pXCN, and pXHN are pXM derivatives containing fragments including the preproNGF coding sequences from the rat (Whittemore et al., 1988), chicken (Ebendal et al., 1986), and human (see below) NGF genes, respectively. pCH110 (Phar-

macia, Uppsala, Sweden), an eukaryotic expression plasmid carrying a β -galactosidase gene, was used to control for the transfection efficiency.

Isolation of the 3' exon of the human NGF gene

A human genomic library was prepared by partial *Mbo*I digestion of leukocyte genomic DNA and cloning into the *Bam*HI site of 1 EMBL-3. Eight hundred thousand clones were plated and screened with a 1-kb mouse NGF cDNA insert (Scott et al., 1983) labeled with [α - 32 P]dCTP by nick-translation to a specific activity of approximately 5×10^8 cpm/ μ g. Hybridization was carried out in $4 \times$ SSC ($1 \times$ SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 40% formamide, $1 \times$ Denharts' solution, and 10% dextran sulfate at 42°C. The filters were washed at 45°C in $0.5 \times$ SSC, 0.1% sodium dodecyl sulfate (SDS) and exposed to Kodak XAR-5 films at -70°C using an intensifying screen. Three positive clones were isolated and phage DNA was analyzed with restriction enzymes. The hybridizing region was contained in a 1.8-kb *Bgl*II fragment that was subcloned in the *Bam*HI site of plasmid pBS-KS (Stratagene, La Jolla, CA, U.S.A.). The identity of this fragment with human NGF (Ullrich et al., 1983) was confirmed by dideoxy chain termination sequencing (Sanger et al., 1977). The fragment was digested further with *Sma*I and *Sac*I, generating a 1.5-kb insert which was then subcloned in the expression vector pXM.

Production of recombinant NGF

COS cells grown to about 70% confluency were transfected with 25 μ g of plasmid DNA per 100-mm dish using the DEAE dextran/chloroquine protocol (Luthman and Magnusson, 1983). A plasmid expressing a β -galactosidase gene (pCH110) was transfected in parallel dishes, and β -galactosidase activity was measured in cytoplasmic extracts as a control of transfection efficiency. Transfected cells were grown for 16 h in complete medium and then for 3 days in medium with 0.5% FCS. The medium was then collected and concentrated 50 times by ultrafiltration (Amicon, MA, U.S.A.). Thirty-five-millimeter dishes transfected in parallel were grown over the third night after transfection in the presence of 200 μ Ci/ml of [35 S]cysteine (Amersham, U.K.). Aliquots of 10–20 μ l of the *in vivo* labeled conditioned media were analyzed by SDS-polyacrylamide electrophoresis (SDS-PAGE). The gels were treated with EnHance (NEN, Boston, MA, U.S.A.), dried, and exposed to Kodak XAR5 films with intensifying screens for 24–48 h at -80°C. Autoradiograms were scanned in a Shimadzu densitometer and the results were expressed as the area corresponding to each NGF protein relative to that obtained with the rat NGF.

RNA preparation and Northern blots

Total RNA was extracted as previously described by Hallböök et al. (1988). One microgram of total RNA was electrophoresed in a 1% agarose gel containing 0.7% formaldehyde and transferred to nitrocellulose membranes. The membranes were then hybridized as described above with 2×10^7 cpm of each nick-translated 32 P-radiolabeled rat, human, and chicken NGF gene fragments. The filters were then washed at high stringency and exposed to Kodak XAR5 films for 24 h at room temperature. Autoradiograms were scanned as described above.

Immunoblotting and immunoprecipitation of recombinant NGF

Aliquots of 10–50 μ l of $50 \times$ concentrated conditioned medium were analyzed by SDS-PAGE and immunoblotting

(Pettmann et al., 1988). The primary antibody used at a 1:300 dilution was a rabbit antiserum against NGF purified from the male mouse submandibular gland (Ebendal et al., 1989). Biotinylated goat anti-rabbit immunoglobulin G (Vector) was used as the secondary antibody at a concentration of 7.5 μ g/ml. Immunoreactive proteins were detected using 125 I-streptavidin (Amersham, U.K.) at about 2.5×10^4 cpm/ml. Dried blots were exposed to Kodak XAR5 films with intensifying screens for 24–48 h at -80°C. Autoradiograms were scanned as described above and the amount of NGF protein was determined by comparing the area in the autoradiogram corresponding to the processed monomer form of each protein with a standard curve generated with purified mouse NGF which was run in the same gel. Yields of recombinant NGF in the culture medium ranged from 100 to 300 ng/ml. Media from transfected COS cells, grown over the third night after transfection in the presence of [35 S]cysteine (Amersham, U.K.), were immunoprecipitated with a rabbit antiserum against purified mouse NGF for 16 h at 4°C. Immunocomplexes were collected using streptococcal cells (Calbiochem, San Diego, CA, U.S.A.), washed five to six times, boiled in SDS-PAGE sample buffer, and electrophoresed in 13% polyacrylamide gels. The gels were then treated and exposed as described above. Results were expressed as the area in the autoradiogram corresponding to each NGF protein relative to that obtained with the rat recombinant NGF.

EIA of recombinant NGF

A two-site EIA for NGF was essentially performed as described previously by Lärkfors et al. (1987) and Söderström et al. (1990). Monoclonal anti-mouse-NGF antibody 27/21 (Korsching and Thoenen, 1983) and antibody 27/21- β -galactosidase conjugate were obtained from Boehringer Mannheim Biochemica, Germany.

Binding assay of recombinant NGF to PC12 cells

Mouse NGF was labeled with 125 I by the chloramine-T method to an average activity of 2×10^7 cpm/ μ g. Steady-state binding of each NGF protein was measured in a competition assay against 125 I-NGF as previously described by Ibáñez et al. (1990). Control experiments using medium from mock transfected COS cells showed that other proteins present in the conditioned medium had no effect on the binding of purified mouse NGF to PC12 cells. Nonspecific binding was measured in a parallel incubation to which at least a 1,000-fold excess of unlabeled NGF was added. All results were corrected for this nonspecific binding, which was always <10% of total binding. Percent of binding was calculated as the cpm obtained with each competitor divided by the cpm obtained when concentrated medium from mock transfected cells was used as competitor times 100. The concentration of each NGF protein that gave 50% binding (IC_{50}) was determined and used to calculate relative binding compared to that obtained with rat recombinant NGF.

Biological assays of recombinant NGF

The biological activity of recombinant NGF was measured by the ability of conditioned medium from transfected COS cells to stimulate neurite outgrowth from explanted sympathetic ganglia from 9-day-old chicken embryos (Ebendal, 1984, 1989) and from rat PC12 cells (Greene and Tischler, 1976). In the ganglion bioassay, the fiber outgrowth was scored on a semiquantitative scale in biological units (BUs) by comparison to standards obtained with purified mouse NGF, for

which 1 BU is equivalent to approximately 5 ng/ml (200 pM). Serial dilutions of concentrated conditioned medium were assayed and the concentration of each NGF protein that gave 0.5 BU in this scale was determined, and used to calculate relative activity compared to that obtained with rat recombinant NGF. For the PC12 cell assay, 18,000 cells were plated in 15-mm wells that had previously been coated with poly-D-lysine. After 3 days of incubation with serial dilutions of concentrated conditioned medium, the percentage of cells bearing fibers longer than one cell diameter was determined microscopically and used to calculate relative activity compared to that obtained with rat recombinant NGF.

RESULTS

Production and quantitation of recombinant NGF

Genomic fragments containing the preproNGF coding sequences from the human (see Materials and Methods), rat (Whittemore et al., 1988), and chicken (Ebendal et al., 1986) NGF genes were subcloned in the expression plasmid vector pXM (Yang et al., 1986) and transiently expressed in COS cells. Conditioned medium from transfected cells was then concentrated by ultrafiltration and the recombinant NGF protein present in this medium was tested for a number of immunological and biological properties. To correct for differences in the amount of recombinant NGF protein produced by each construct, parallel dishes of transfected COS cells were labeled *in vivo* with [³⁵S]cysteine and aliquots of conditioned media analyzed by SDS-PAGE. A specific band with a molecular mass of approximately 13 kDa corresponding to the mature NGF monomer polypeptide could be detected in the media from cells that had been transfected with the different NGF gene constructs (Fig. 1A). No band of this size was detected in media from cells that had been transfected with a control plasmid. The level of secreted NGF protein was about three times higher in the medium from cells transfected with the rat or human NGF genes compared to the media from cells that were transfected with the chicken NGF construct (Fig. 1B). To test if these variations were due to differences in the efficiency of transcription of the different NGF gene constructs, the steady-state levels of NGF mRNA produced by the transfected cells were examined by Northern blot analysis (Fig. 1C). Transfections with the rat NGF gene construct resulted in a major RNA transcript of 1.9 kb. As previously reported by Hallböök et al. (1988), this mRNA species includes the 770-bp rat NGF insert plus 1,150 bp of vector sequences preceding the SV40 polyadenylation site. The plasmid construct containing the chicken NGF gene produced a major RNA transcript of 1.2 kb, due to the use of an endogenous polyadenylation site included in the chicken NGF insert. A less abundant RNA of 2.1 kb was also detected, which probably arose from the alternative use of the SV40 polyadenylation site included in the vector. A similar situation was observed for the human NGF gene construct, which gave a major 1.4-kb transcript and a larger, although less abundant,

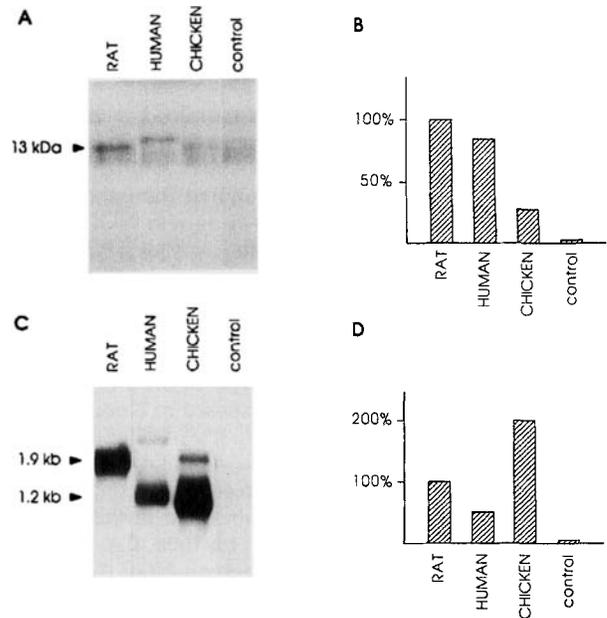


FIG. 1. Levels of recombinant NGF protein and mRNA after transient expression of transfected NGF genes in COS cells. **A:** SDS-PAGE of conditioned media from *in vivo* [³⁵S]cysteine-labeled cultures (2×10^5 cpm loaded in each well) of cells transfected with the rat NGF, human NGF, chicken NGF, or control plasmid constructs, respectively. **B:** Densitometric analysis of the autoradiogram shown in A. The value for rat NGF is arbitrarily set to 100%. Background protein contribution was compensated by densitometric scanning of the control lane. Analysis of duplicate gels produced similar results to those shown here. **C:** Northern blot of total RNA from COS cells transfected with the rat NGF, human NGF, chicken NGF, or control plasmid constructs, respectively. One microgram of total RNA from each sample was electrophoresed and blotted onto a nitrocellulose membrane. The membrane was probed with equal amounts of cpm of ³²P-radiolabeled rat, human, and chicken NGF gene fragments labeled to the same specific activity. The filter was then washed at high stringency and exposed to an x-ray film. **D:** Densitometric analysis of a shorter exposure of the autoradiogram shown in C. The value for rat NGF is arbitrarily set to 100%.

2.3-kb mRNA. The human NGF gene construct was found to produce half the amount of NGF mRNA when compared to the rat, whereas the chicken NGF plasmid produced about two times as much (Fig. 1D). Thus, the level of the chicken NGF mRNA does not correlate with the decreased level of the secreted chicken NGF protein (compare Fig. 1B and D), indicating that the lower level of the secreted chicken NGF protein was not due to a lower transcription efficiency of the chicken NGF plasmid construct.

Immunological studies: immunoblotting, immunoprecipitation, and EIA

Antigenic differences among rat, chicken, and human NGFs were first studied by immunoblotting and immunoprecipitation using a polyclonal rabbit antiserum against purified mouse NGF (Ebendal et al., 1989). Equal amounts of concentrated conditioned media were analyzed by western blotting as described in Materials and Methods, and differences among the

different NGFs assessed by scanning of the corresponding autoradiograms (Fig. 2A and B). The differences observed in the immunoblotting paralleled those detected in the *in vivo* labeled products secreted by the transfected cells (compare Fig. 1B and 2B). Thus, the lower level of the secreted chicken NGF protein seen also after the immunoblotting was probably due to the lower level present in the conditioned media and not to an ability of the antiserum to discriminate among the primary sequences of the three NGF molecules. Western immunoblotting was therefore used to quantitate the actual levels of NGF protein present in the concentrated conditioned media by comparison to a dilution curve generated using known amounts of purified mouse NGF which were run in the same gel (not shown). The concentration of NGF protein in the 50 times concentrated conditioned media was estimated to be 7, 6.5, and 2.5 $\mu\text{g}/\text{ml}$ for the rat, human, and chicken samples, respectively.

For the immunoprecipitation, data from Fig. 1B

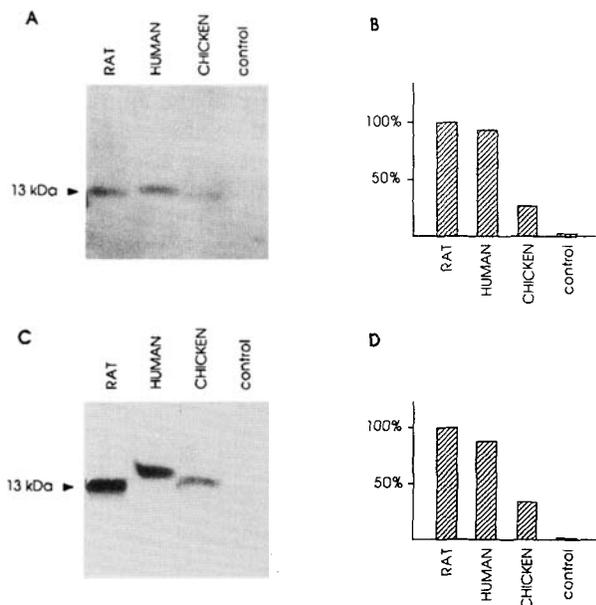


FIG. 2. Immunoblotting and immunoprecipitation of transfected COS cell conditioned media using a rabbit polyclonal antiserum against purified mouse NGF. **A:** Western blot of concentrated conditioned medium from COS cells transfected with rat NGF, human NGF, chicken NGF, and control plasmid constructs, respectively. Thirty microliters of 50 times concentrated medium was analyzed in each case. **B:** Densitometric analysis of the autoradiogram shown in A. The value for rat NGF is arbitrarily set to 100%. Analysis of duplicate gels produced similar results to the ones shown here. **C:** SDS-PAGE analysis of immunoprecipitated proteins from conditioned media of *in vivo* [^{35}S]cysteine-labeled cultures of cells transfected with the rat NGF, human NGF, chicken NGF, or control plasmid constructs, respectively. Prior to the immunoprecipitation, samples were compensated for differences in the amount of recombinant NGF protein produced by each NGF gene construct according to data from Fig. 1B. **D:** Densitometric analysis of the autoradiogram shown in C. The value for rat NGF is arbitrarily set to 100%. Analysis of duplicate gels produced similar results to the ones shown here.

were used to equilibrate aliquots of *in vivo* labeled conditioned media so that equal amounts of rat, human, and chicken ^{35}S -labeled NGF protein were used for immunoprecipitation with the anti-mouse NGF antiserum. Unlike immunoblotting, immunoprecipitation did reveal differences in antigenicity among the three NGF molecules (Fig. 2C and D). Although rat and human NGF were similarly immunoprecipitated, only one-third of the chicken NGF protein could be immunoprecipitated by the anti-mouse NGF antiserum (Fig. 2D).

The immunological properties of recombinant human, rat, and chicken NGFs were also studied in a two-site EIA using a monoclonal antibody to purified mouse NGF (Korsching and Thoenen, 1983) (Fig. 3). Comparison of EIA values at a given concentration of NGF showed that both human and rat NGFs were recognized by this antibody with equal efficiency, whereas the EIA value obtained with chicken NGF was only one-third of that obtained with rat NGF. This result indicates that the interaction of this antibody with the chicken NGF protein is less efficient, probably due to a lower affinity as suggested by the smaller slope of the chicken NGF EIA curve compared to those of rat or human NGFs.

Biological studies: receptor binding and biological activities

Rat, human, and chicken NGFs were also compared for their ability to displace ^{125}I -mouse NGF from NGF receptors on PC12 cells. Competitive binding assays were performed using concentrations of ^{125}I -NGF ($\sim 1.5 \text{ nM}$) at which about 80% of the bound ^{125}I -NGF is bound to the low-affinity site in the absence of competitor (Sutter et al., 1979; Cohen et al., 1980). All three NGFs were able to displace ^{125}I -mouse NGF from PC12 cells and showed similar IC_{50} values (concentration of competitor that gave 50% displacement) (Fig. 4).

Biological activities of the various recombinant NGFs were studied in two different systems: stimulation of neurite outgrowth from E9 chick sympathetic ganglion explants and from rat pheochromocytoma PC12 cells. All three NGFs (tested in serial dilutions ranging from 7.5 to 240 pM in all cases) produced the same morphological responses in the chick assay, with similar dose-response curves and maximal stimulation values (Fig. 5A and B). In the rat bioassay, the homologous NGF protein was somewhat more efficient in eliciting differentiation and neurite outgrowth from PC12 cells (Fig. 5C and D). The human and chicken NGFs showed similar dose-response curves, although the human protein appeared to be more effective at higher concentrations (Fig. 5D).

DISCUSSION

In this study, the immunological and biological properties of human, rat, and chicken NGFs were compared using recombinant NGF proteins produced

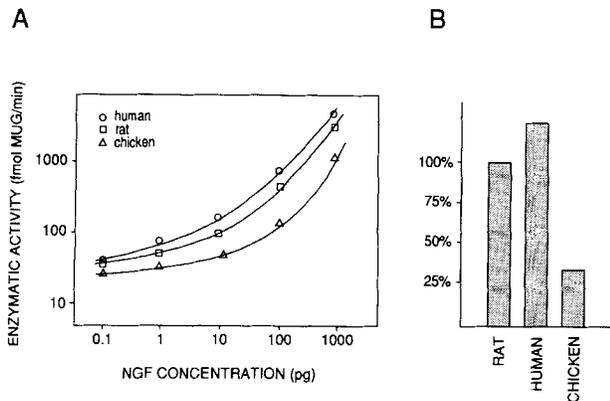


FIG. 3. Two-site EIA of recombinant NGFs using a monoclonal antibody against purified mouse NGF. **A:** Dose-response curves for enzymatic activity obtained with recombinant rat (\square), human (\circ), and chicken (\triangle) NGFs. **B:** Comparison of EIA values at 100 pg of recombinant NGF protein. The value for rat NGF is arbitrarily set to 100%. Data from two experiments varied by $\pm 20\%$ of the average values reported here.

in a transient expression system in COS cells. Levels of recombinant NGF protein in conditioned media from transfected cells were found to vary over a three-fold range as assessed by SDS-PAGE of *in vivo* labeled proteins. The differences in NGF production were not due to differences in the transcription yield of the different NGF gene constructs. Instead, these variations most likely reflect differences in protein synthesis, stability, or secretion among the human, rat, and chicken NGF proteins in COS cells. Interestingly, the human NGF protein showed a higher apparent molecular weight in SDS-PAGE compared to both rat and chicken NGFs. This variation is probably not due to differences in the polypeptide chain length because the mature NGF proteins from all these species are 118 amino acids long. Nor is it likely that this shift in mobility is due to differences in glycosylation among the human, rat, or chicken NGFs because all three proteins contain only one site for *N*-glycosylation and other types of glycosylation would cause a larger shift in mobility than the one observed. For proteins with a molecular weight of $<15,000$, the binding of SDS does not always completely mask the inherent charge and conformation of the protein (Weber and Osborn, 1975). Therefore, the apparently higher molecular weight of human NGF in SDS-PAGE could be due to a difference in the isoelectric point of this protein. This possibility is to some extent supported by the fact that bovine NGF, which shares with human NGF almost all the amino acid differences with respect to the rat NGF molecule (Fig. 6), also shows a higher apparent molecular weight in SDS-PAGE when compared to that of mouse NGF, probably due to the higher isoelectric point of the bovine protein (Harper et al., 1982).

The immunological properties of the recombinant NGF molecules were compared by immunoblotting, immunoprecipitation, and EIA. In the immunoblotting

the molecules are detected in a denatured state and the fact that the results of these blots paralleled those obtained with SDS-PAGE of crude [^{35}S]cysteine-labeled conditioned media indicates that the polyclonal anti-mouse NGF antiserum does not recognize the amino acid differences in the primary structure of the three molecules (see Fig. 6). However, the rat, human, and chicken NGF proteins were differently recognized by the same antiserum when used for immunoprecipitation. In this case, the chicken NGF protein was only one-third as efficiently recognized compared to rat and human NGFs. This suggests that the amino acid differences between chicken and rat NGFs generate conformational changes affecting the three-dimensional structure of the major immunogenic epitope(s) recognized by the mouse NGF antiserum. In this context, it is interesting to note that the human NGF protein, which compared to chicken NGF has only half the number of amino acid differences with respect to rat NGF (Fig. 6), was as efficiently recognized as the rat NGF protein when either a polyclonal antiserum or a monoclonal antibody was used. This result indicates that the amino acid differences between these two molecules, which include the nonconservative replacements Arg for Met⁹, Asp for Ala⁶⁰, Met for Thr⁹², and Gly for Asp⁹⁴, do not affect any of the epitopes recognized by the antibodies used in this study. All of these nonconservative differences are also present in chicken NGF, suggesting that only the "chicken-specific" changes are responsible for the observed immunological differences. Among these, nonconservative changes such as Asn for Ser⁴⁷, Arg for Asn⁶², and Ser for Glu⁶⁵, all contained in hydrophilic regions of the molecule, might be residues affecting the epitope(s) recognized by the anti-mouse NGF antiserum. In fact,

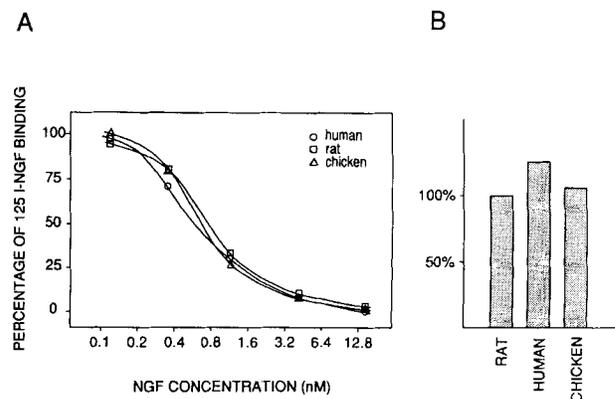


FIG. 4. Displacement of ^{125}I -mouse NGF from low-affinity NGF receptors on PC12 cells by rat, human, and chicken recombinant NGFs. **A:** Serial dilutions of rat (\square), human (\circ), and chicken (\triangle) NGF protein were assayed for their ability to displace ^{125}I -mouse NGF from NGF receptors on PC12 cells. **B:** Comparison of relative binding calculated from the IC_{50} values obtained with each NGF protein. The value for rat NGF is arbitrarily set to 100%. Data from three experiments varied by $\pm 20\%$ of the average values reported here.

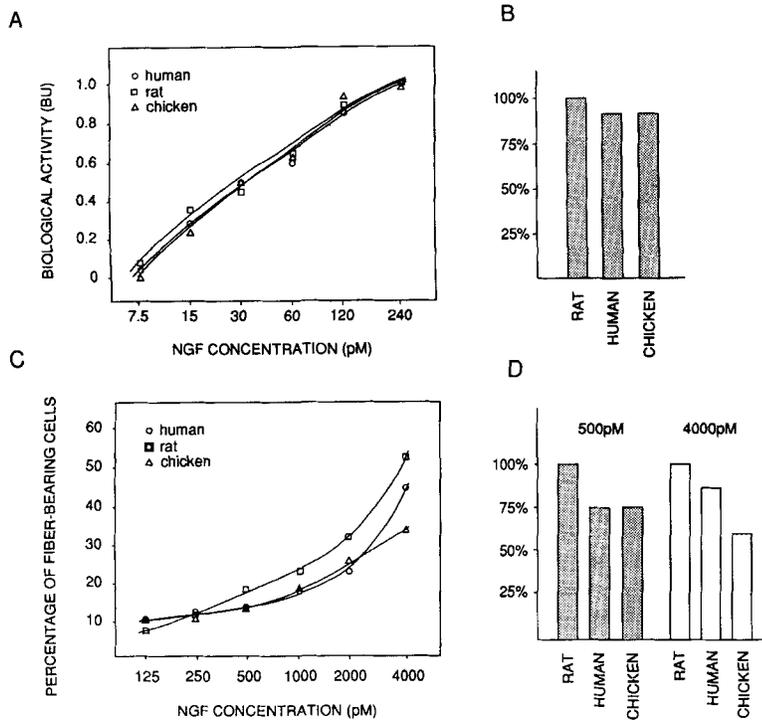


FIG. 5. Biological activities of recombinant rat, human and chicken NGFs. **A:** Dose-response curves of neurite outgrowth stimulation from E9 chick sympathetic ganglion explants obtained with recombinant rat (□), human (○), and chicken (△) NGFs. The fiber outgrowth was scored on a semi-quantitative scale in BUs by comparison to standards obtained with purified mouse NGF, for which 1 BU is equivalent to approximately 5 ng of protein per milliliter (200 pM). **B:** Comparison of relative biological activity in the sympathetic ganglion bioassay calculated from the concentration of NGF protein that gave 0.5 BU. The value for rat NGF is arbitrarily set to 100%. Data from three experiments varied by ±20% of the average values reported here. **C:** Same as A but using PC12 cells. Fiber outgrowth is scored as the percentage of cells bearing fibers longer than one cell diameter per microscope field. **D:** Comparison of percentages of fiber-bearing PC12 cells at 500 (□) and 4,000 (○) pM of recombinant NGF protein. The value for rat NGF is arbitrarily set to 100%. Standard deviation from three determinations was at or generally below ±20% of the average values reported here.

the last two amino acids are contained in a postulated loop of the molecule (positions 57–67) which concentrates most of the interspecies amino acid variation observed in the NGF protein. Based on this, it can be argued that these residues are important for the folding and tertiary structure of the chicken NGF molecule. A recent study on the functional importance of highly conserved amino acid residues in the chicken NGF protein using site-directed mutagenesis has identified two conserved amino acids (Ser¹⁸ and Trp⁹⁸) which also appear to be important for the recognition of the chicken NGF protein by the same polyclonal anti-NGF antiserum (Ibáñez et al., 1990).

Unlike the immunological studies, low-affinity binding to NGF receptors on PC12 cells did not show any differences among the three NGF proteins. The nonidentical amino acids among these molecules are therefore not likely to be involved in low-affinity binding to the NGF receptor. In agreement with this observation, it has been recently shown that low-affinity NGF receptors cannot discriminate between either

NGF and brain-derived neurotrophic factor (Rodríguez-Tébar et al., 1990). Moreover, the third member of the NGF family (hippocampus-derived neurotrophic factor/neurotrophin-3) can displace the binding of ¹²⁵I-NGF from low-affinity NGF receptors on PC12 cells (Ernfors et al., 1990b). Taken together, these results suggest that the amino acids involved in low-affinity binding to the NGF receptor are most likely conserved among all members of the NGF family.

The biological activity of the recombinant NGF proteins was assayed by the ability of the concentrated conditioned media to promote neurite outgrowth from explants of E9 chick sympathetic ganglia and from rat pheochromocytoma PC12 cells. In the chicken bioassay, all three NGF proteins showed indistinguishable dose-response curves and maximal stimulation values, indicating that the nonconservative amino acid differences among these molecules do not affect the efficiency whereby the NGF protein elicits its biological response. In agreement with this, it has recently been shown by site-directed mutagenesis (Ibáñez et al., 1990) that the

FIG. 6. Alignments of the amino acid sequences of rat (Whittemore et al., 1988), human (Ullrich et al., 1983), and chicken (Ebendal et al., 1986) mature NGF proteins. Positions are numbered from the NH₂ terminal residue of the mature NGF protein. The dots indicate amino acids identical to those found in rat NGF. The arrow shows postulated proteolytic sites at the COOH ends of rat and human NGFs.

rat	1	SSTHPVFHMGFEFSVCDSSVSVWVGDKTTATDIKKGKVTVLGEVNNNSVFK
human	1	..S..I..R.....M.....
chicken	1	TA...L.R.....M.....N...
	51	QYFFETKCRAPNEVESGCRGIDSKHWNSYCTTHTFVKALTTDDKQAAWR
	51D...D.....M.G.....
	50D.R..S...A.....MEG.....
	101	FIRIDTACVCLSRKAARRG
	101V..A
	100SG.P

biological activity of the NGF protein is not changed as the result of many single nonconservative amino acid replacements, even in positions that are highly conserved among different species. Human and chicken NGF elicited the same response in the PC12 cell bioassay, whereas the rat molecule appeared to be somewhat more effective. These results might reflect differences in the binding to the high-affinity NGF receptor and suggest that coevolution of both ligand and receptor may in some cases result in a more efficient interaction between the two molecules.

The results presented in this report constitute the first detailed study of the immunological and biological properties of human NGF and their comparison to those of rat and chicken NGF. Information on the biochemical characteristics of these molecules is of importance in view of the potential therapeutic applications of recombinant human NGF. Our data indicate that human, rat, and chicken NGFs have very similar biological activities, but that the avian molecule differs immunologically from the mammalian NGFs. The variations observed in the yield of production of the different recombinant NGFs suggest that the chicken molecule may have a lower stability compared to those of the rat or human NGF proteins. These results are in agreement with the hypothesis that the NGF molecules contain a highly conserved active site responsible for the biological properties of the protein, whereas the remaining parts of the molecule are under less evolutionary constraint, and have therefore diverged more extensively.

Acknowledgment: Technical assistance was given by Mrs. Mona Gullmert. Support was obtained from the Swedish Natural Science Research Council, The Swedish Board for Technical Development, Petrus och Augusta Hedlunds Stiftelse, Magnus Bergvalls Stiftelse, and funds from the Karolinska Institute. C.F.I. was supported by a fellowship from the Karolinska Institute.

REFERENCES

- Angeletti R. H. and Bradshaw R. A. (1971) Nerve growth factor from mouse submaxillary gland: amino acid sequence. *Proc. Natl. Acad. Sci. USA* **68**, 2417–2420.
- Angeletti R. H., Hermodson M. A., and Bradshaw R. A. (1973) Amino acid sequences of 2.5S nerve growth factor. II Isolation and characterization of the thermolytic and peptic peptides and the complete covalent structure. *Biochemistry* **12**, 100–115.
- Angeletti R., Frazier W., Jacobs J., Nial H., and Bradshaw R. (1976) Purification, characterization and partial amino acid sequence of nerve growth factor from cobra venom. *Biochemistry* **15**, 26–34.
- Bailey G. S., Banks B. E., Pearce F. L., and Shipolini R. A. (1975) A comparative study of nerve growth factors from snake venoms. *Comp. Biochem. Physiol. B* **51**, 429–438.
- Bailey G. S., Banks B. E., Carstairs J. R., Edward D. C., Pearce F. L., and Vernon C. A. (1976) Immunological properties of nerve growth factors. *Biochim. Biophys. Acta* **437**, 259–263.
- Bartus R. T., Dean R., Beer B., and Lippa A. S. (1982) The cholinergic hypothesis of geriatric memory dysfunction. *Science* **217**, 408–414.
- Bruce G. and Heinrich G. (1989) Production and characterization of biologically active recombinant human nerve growth factor. *Neurobiol. Aging* **10**, 89–94.
- Cohen P., Sutter A., Landreth G., Zimmermann A., and Shooter E. M. (1980) Oxidation of tryptophan-21 alters the biological activity and receptor binding characteristics of mouse nerve growth factor. *J. Biol. Chem.* **255**, 2949–2954.
- Cohen S. (1959) Purification and metabolic effects of a nerve growth-promoting protein from snake venom. *J. Biol. Chem.* **234**, 1129–1137.
- Cohen S. (1960) Purification of a nerve-growth promoting protein from the mouse salivary gland and its neuro-cytotoxic antiserum. *Proc. Natl. Acad. Sci. USA* **46**, 302–311.
- Collerton D. (1986) Cholinergic function and intellectual decline in Alzheimer's disease. *Neuroscience* **19**, 1–28.
- Ebendal T. (1984) Nerve growth-promoting activities in embryonic and adult tissues, in *Organizing Principles of Neural Development* (Sharma S., ed), pp. 93–107. Plenum Press, New York.
- Ebendal T. (1989) Use of collagen gels to bioassay nerve growth factor activity, in *Nerve Growth Factors* (Rush R. A., ed), pp. 81–93. John Wiley & Sons, Chichester.
- Ebendal T., Larhammar D., and Persson H. (1986) Structure and expression of the chicken β nerve growth factor. *EMBO J.* **5**, 1483–1487.
- Ebendal T., Persson H., Larhammar D., Lundströmer K., and Olson L. (1989) Characterization of antibodies to synthetic nerve growth factor (NGF) and proNGF peptides. *J. Neurosci. Res.* **22**, 223–240.
- Edwards R. H., Selby M. J., Mobley W. C., Weinrich S. L., Hruby D. E., and Rutter W. J. (1988) Processing and secretion of nerve growth factor: expression in mammalian cells with a vaccinia virus vector. *Mol. Cell. Biol.* **8**, 2456–2464.
- Ernfors P., Ebendal T., Olson L., Mouton P., Strömberg I., and Persson H. (1989) A cell line producing recombinant nerve growth factor evokes growth responses in intrinsic and grafted central cholinergic neurons. *Proc. Natl. Acad. Sci. USA* **86**, 4756–4760.
- Ernfors P., Lindfors N., Chan-Palay V., and Persson H. (1990a) Cholinergic neurons of nucleus basalis express elevated levels of nerve growth factor receptor mRNA in senile dementia of the Alzheimer type. *Dementia* **1**, 138–145.
- Ernfors P., Ibáñez C. F., Ebendal T., Olson L., and Persson H. (1990b) Molecular cloning and neurotrophic activities of a protein with structural similarities to β -nerve growth factor: developmental and topographical expression in the brain. *Proc. Natl. Acad. Sci. USA* **87**, 5454–5458.
- Gluzman Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23**, 175–182.
- Goedert M., Fine A., Hunt S., and Ullrich A. (1986) Nerve growth factor mRNA in peripheral and central rat tissue and in the human central nervous system: lesion effects in the rat brain and levels in Alzheimer's disease. *Mol. Brain Res.* **1**, 85–92.
- Greene L. A. and Tischler A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* **73**, 2424–2428.
- Hallböök F., Ebendal T., and Persson H. (1988) Production and expression of biologically active recombinant beta nerve growth factor. *Mol. Cell. Biol.* **8**, 452–456.
- Harper G. P. and Thoenen H. (1980) The distribution of nerve growth factor in the male sex organs of mammals. *J. Neurochem.* **34**, 893–903.
- Harper G., Barde Y.-A., Burnstock G., Carstairs J., Dennison M., K. S., and Vernon C. A. V. (1979) Guinea pig prostate is a rich source of nerve growth factor. *Nature* **279**, 160–162.
- Harper G. P., Glanville R. W., and Thoenen H. (1982) The purification of nerve growth factor from bovine seminal plasma. Biochemical characterization and partial amino acid sequence. *J. Biol. Chem.* **257**, 8541–8548.
- Harper G. P., Barde Y. A., Edgar D., Ganten D., Hefti F., Heumann R., Naujoks K. W., Rohrer H., Turner J. E., and Thoenen H. (1983) Biological and immunological properties of the nerve growth factor from bovine seminal plasma: comparison with the properties of mouse nerve growth factor. *Neuroscience* **8**, 375–387.
- Heinrich G. and Meyer T. E. (1988) Nerve growth factor (NGF) is

- present in human placenta and semen, but undetectable in normal and Paget's disease blood: measurements with an anti-mouse-NGF enzyme immunoassay using a recombinant human NGF reference. *Biochem. Biophys. Res. Commun.* **155**, 482-486.
- Huynh T. V., Young R. A., and Davis R. W. (1985) Constructing and screening cDNA libraries in λ gt10 and λ gt11, in *DNA Cloning—A Practical Approach* (Glover D. M., ed), pp. 56-110. IRL Press, Oxford.
- Ibáñez C. F., Hallböök F., Ebendal T., and Persson H. (1990) Structure-function studies of nerve growth factor: functional importance of highly conserved amino acid residues. *EMBO J.* **9**, 1477-1483.
- Korsching S. and Thoenen H. (1983) Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation. *Proc. Natl. Acad. Sci. USA* **80**, 3513-3516.
- Korsching S., Auburger G., Heumann R., Scott J., and Thoenen H. (1985) Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. *EMBO J.* **4**, 1389-1393.
- Kubanis P. and Zornetzer S. F. (1981) Age-related behavioral and neurobiological changes: a review with an emphasis on memory. *Behav. Neurol. Biol.* **31**, 115-172.
- Large T. H., Bodary S. C., Clegg D. O., Weskamp G., Otten U., and Reichardt L. F. (1986) Nerve growth factor gene expression in the developing rat brain. *Science* **234**, 352-355.
- Lärkfors L., Ebendal T., Whittemore S. R., Persson H., Hoffer B., and Olson L. (1987) Decreased level of nerve growth factor (NGF) and its messenger RNA in the aged brain. *Mol. Brain Res.* **3**, 55-60.
- Levi-Montalcini R. and Angeletti P. (1968) Nerve growth factor. *Physiol. Rev.* **48**, 534-569.
- Luthman H. and Magnusson G. (1983) High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucleic Acids Res.* **11**, 1295-1305.
- Meier R., Becker-André M., Götz R., Heumann R., Shaw A., and Thoenen H. (1986) Molecular cloning of bovine and chick nerve growth factor (NGF): delineation of conserved and unconserved domains and their relationship to the biological activity and antigenicity of NGF. *EMBO J.* **5**, 1489-1493.
- Nakata Y., Nomoto H., Takahashi I., Furukawa Y., Furukawa S., and Hayashi K. (1988) Comparison of the biochemical and immunological properties of nerve growth factors from various animals. *J. Clin. Biochem. Nutr.* **4**, 73-86.
- Oda T., Ohta M., Inoue S., Ikeda K., Furukawa S., and Hayashi K. (1989) Amino acid sequence of nerve growth factor purified from the venom of the Formosan cobra *Naja naja atra*. *Biochem. Int.* **19**, 909-917.
- Perry E. K., Tomlinson B. E., Blessed G., Bergmann K., Gibson P. H., and Perry R. H. (1978) Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. *Br. Med. J.* **2**, 1457-1459.
- Pettmann B., Manthorpe M., Powell J. A., and Varon S. (1988) Biological activities of nerve growth factor bound to nitrocellulose paper by Western blotting. *J. Neurosci.* **8**, 3624-3632.
- Rodriguez-Tébar A., Dechant G., and Barde Y.-A. (1990) Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron* **4**, 487-492.
- Rosenberg M., Friedmann T., Robertson R. C., Tuszyński M., Wolf J. A., Breakefield X. O., and Gage F. H. (1988) Grafting genetically modified cells to the damaged brain: restorative effects of NGF expression. *Science* **242**, 1575-1578.
- Sanger F., Nicklen S., and Coulson A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.
- Schwarz M. A., Fisher D., Bradshaw R. A., and Isackson P. J. (1989) Isolation and sequence of a cDNA clone of beta-nerve growth factor from the guinea pig prostate gland. *J. Neurochem.* **52**, 1203-1209.
- Scott J., Selby M., Urdea M., Quiroga M., Bell G. I., and Rutter W. (1983) Isolation and nucleotide sequence of a cDNA encoding the precursor of mouse nerve growth factor. *Nature* **302**, 538-540.
- Selby M. J., Edwards R. H., and Rutter W. J. (1987) Cobra nerve growth factor: structure and evolutionary comparison. *J. Neurosci. Res.* **18**, 293-298.
- Söderström S., Hallböök F., Ibáñez C., Persson H., and Ebendal T. (1990) Recombinant human β -nerve growth factor (NGF): biological activity and properties in an enzyme immunoassay. *J. Neurosci. Res.* **27**, 665-677.
- Sutter A., Riopelle R. J., Harris-Warrick R. M., and Shooter E. M. (1979) Nerve growth factor receptors: characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. *J. Biol. Chem.* **254**, 5972-5982.
- Thoenen H. and Barde Y. A. (1980) Physiology of nerve growth factor. *Physiol. Rev.* **60**, 1284-1325.
- Thoenen H., Bandtlow C., and Heumann R. (1987) The physiological function of nerve growth factor in the central nervous system: comparison with the periphery. *Rev. Physiol. Biochem. Pharmacol.* **109**, 145-178.
- Ueyama T., Saito H., and Yohro T. (1986) Nerve growth factor and the submandibular gland of the house musk shrew *Suncus murinus*: a morphological and comparative immunological study. *Biomed. Res.* **7**, 379-387.
- Ullrich A., Gray A., Berman C., and Dull T. J. (1983) Human β -nerve growth factor gene highly homologous to that of mouse. *Nature* **303**, 821-825.
- Weber L. and Osborn M. (1975) *The Proteins* (Neurath H., Hill R., and Boeder C.-L., eds), pp. 179-223. Academic Press, New York.
- Whitehouse P. J., Price D. L., Struble R. G., Clark A. W., Coyle J. T., and Delon M. R. (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. *Science* **215**, 1237-1239.
- Whittemore S. R. and Seiger A. (1987) The expression, localization and functional significance of beta-nerve growth factor in the central nervous system. *Brain Res.* **434**, 439-464.
- Whittemore S. R., Friedman P. L., Larhammar D., Persson H., Gonzalez C. M., and Holets V. R. (1988) Rat beta-nerve growth factor sequence and site of synthesis in the adult hippocampus. *J. Neurosci. Res.* **20**, 403-410.
- Wilcock G. K., Esiri M. M., Bowen D. M., and Smith C. C. (1982) Alzheimer's disease. Correlation of cortical choline acetyltransferase activity with the severity of dementia and histological abnormalities. *J. Neurol. Sci.* **57**, 407-417.
- Wion D., Perret C., Frechin N., Keller A., Behar G., Brachet P., and Auffray C. (1986) Molecular cloning of the avian β -nerve growth factor gene: transcription in brain. *FEBS Lett.* **203**, 82-86.
- Yang Y. C., Ciarlette A. B., Temple P. A., Chung M. P., Kovacic S., Witek-Gianotti J. S., Leary A. C., Kritz R., Donahue R. E., Wong G. G., and Clark S. C. (1986) Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell* **47**, 3-10.