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Synthesis of Chimeric Mouse Nerve Growth Factor Precursor and Human β-Nerve Growth Factor in *Escherichia coli:* Immunological Properties

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The complete mouse prepro-nerve growth factor (NGF) DNA was fused to the carboxyl terminus of the β-galactosidase (lac-z) gene of *Escherichia coli*. Similarly, a genomic fragment encoding the human NGF comprising codons 11 to 106 (from a total of 118) was fused to the fifth codon of the amino terminus of β-galactosidase. Both bacterial vectors produce high amounts of the chimeric proteins. After cell lysis most of the chimeric mouse preproNGF protein is insoluble and appears in the pellet, whereas the majority of the chimeric human β-NGF remains in the supernatant.

Purification of the fusion proteins from the soluble fraction was achieved by affinity chromatography to p-aminophenyl β-D-thio-galactoside Sepharose. Yields of the purified chimeric proteins were increased threefold to fourfold by the addition of protease inhibitors in the lysis and chromatography buffers. Their antigenic similarity to the preproNGF and mouse β-NGF was examined by their interaction to sera raised against synthetic peptides which reproduce sequences of the precursor protein and to sera directed against native and denatured mouse β-NGF using enzyme-linked immunoabsorbent assay (ELISA) techniques. Antibodies to the peptide N_2 (−163 to −139) interacted with high affinity with the chimeric mouse preproNGF protein. Antisera to native and denatured mouse β-NGF interacted with both chimeric proteins but with a variable degree of affinity. These results provide direct evidence that certain antisera to mouse β-NGF can cross-react with the human β-NGF molecule.

Key words: β-galactosidase fusions, NGF precursor, human β-NGF, ELISA

INTRODUCTION

The rapid progress in the field of the β-nerve growth factor (NGF) is due essentially to the discovery of high levels of this factor in the male mouse submandibular gland [Cohen, 1960]. Purification of the factor from this gland allowed determination of its amino acid sequence [Angeletti and Bradshaw, 1971], the production of specific antibodies, and, more recently, the isolation of a complementary deoxyribonucleic acid (cDNA) encoding the mouse NGF precursor [Scott et al., 1983; Ullrich et al., 1983]. Using the mouse clone as a probe, the human β-NGF gene was isolated by Ullrich et al. [1983]. The nucleotide sequence of the cDNA predicts that β-NGF is synthesized from a larger precursor of a molecular mass of about 35,000 Da (preproNGF), which may be cleaved at dibasic residues (Arg/Arg, Arg/Lys) to generate three additional peptides besides the β-NGF protein, which is situated at the carboxyl terminus.

Antibodies to the mature β-NGF failed to detect the precursor protein synthesized in vitro [Dicou and Brachet, 1984; Wion et al., 1984] or in vivo [Dicou et al., 1986]. Using an antiserum to a synthetic peptide that reproduces an epitope of 25 amino acids (−163 to −139) [Ullrich et al., 1983] of the precursor molecule, a polypeptide of about 30,000–32,000 Da, the putative NGF precursor protein, was detected in submandibular gland and thyroid gland extracts by immunoprecipitation [Dicou et al., 1986]. Immunohistochemical studies with affinity-purified antipeptide sera localized the proNGF in the basal parts of the granular convoluted tubules of the submandibular gland [Dicou et al., 1988] and in parafollicular cells of the thyroid gland [Dicou et al., 1986].

Comparison of mouse β-NGF nucleotide sequences with corresponding human sequences revealed 90% identity. In order to study their immunological and biological properties, we fused the mouse preproNGF cDNA and a human β-NGF genomic fragment to β-galactosidase gene

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so that fusion proteins could be expressed in *Escherichia coli*. We report here the construction of these fused genes and the antigenic properties of the chimeric proteins to sera raised against synthetic peptides that reproduce epitopes of the precursor protein and to various mouse anti-β-NGF sera.

MATERIALS AND METHODS

Bacterial Strains

E. coli strain BMH 71-18 (lac pro) Δ thi$^-$SupE/F′ lac I^qz^- ΔM15 Y^+ pro^+) was used for transformation [Maniatis et al., 1982]. Lac-z^+ colonies were identified on indicator plates containing 5-bromo 4-chloro 3-indolyl β-D galactoside (X-gal) and ampicillin. A blue-colored colony indicates the presence of β-galactosidase activity.

Construction of Plasmids

Restriction enzyme digests, ligation, transformation, and plasmid DNA preparations were performed as described by Maniatis et al., [1982].

Purification of β-Galactosidase Chimeric Proteins

To purify chimeric β-galactosidases we used affinity chromatography on p-aminophenyl-β-D-thio-galactoside (APTG) Sepharose as described by Steers et al. [1971] and modified by Ullmann [1984]. Briefly, bacteria were grown in the presence of the appropriate antibiotic to OD_{600} of 1.2. Then isopropyl -1-thio-β-D-galactoside (IPTG) was added at 1mM for 3–4 hr, and cells were collected and resuspended in buffer A containing 20 mM Tris pH 7.4, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol and sonicated three times for 1 min in an ice bath. Cell debris was spun down at 12,000 × g for 40 min, and the supernatant was adjusted to 1.6M NaCl and applied to the column, which also was equilibrated in buffer A containing 1.6M NaCl. To remove nonspecifically bound proteins, the column was washed with the same buffer until the OD_{280} of the flow through was less than 0.05. Adsorbed β-galactosidase chimeric proteins were eluted with 100 mM sodium borate, pH 10, and neutralized with 1M NaH_2PO_4. Fractions containing the activity were pooled, sterilized by filtration through Schleicher and Schüell membranes (0.45 μm), and kept at 4°C for several weeks without any significant change of the activity or adjusted to 50% glycerol and stored at −20°C.

Enzyme Assay

β-galactosidase activity was assayed as described by Miller [1972] by the hydrolysis of 2-nitrophenyl β-D-galactopyranoside (ONPG) at 28°C.

Preparation of Bacterial Extracts

Bacterial suspensions were lysed with lysozyme/deoxycholate and then treated with DNase I as described by Marston et al. [1984]. Aliquots of such extracts were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [1970].

ELISA

Enzyme-linked immunosorbent assays (ELISA) were performed essentially by the method of Engvall and Perlmann [1972]. Immunoplates were coated with β-galactosidase or β-galactosidase chimeric protein at 2 μg/ml enzymatic activity (about 20–100 μg/ml of protein) in 0.1 M carbonate buffer, pH 9.2, or with purified mouse β-NGF at 5 μg/ml in the same buffer at 37°C for 2 hr. Appropriate dilutions of the various sera in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 0.5% gelatine were added to the wells and incubated at 4°C for 16 hr. Bound immunoglobulins were measured using goat antirabbit IgGs coupled to peroxidase and o-phenylene diamine as a substrate. Reactions were stopped with HCl at a final concentration of 1 N, and the adsorbance was measured at 492 nm with a Titertek Multiskan plate reader.

Sera

Serum N_{24} is a polyclonal rabbit serum raised against a 25 amino acid synthetic peptide (N_2) that reproduces the sequence (−163 to −139) of the preproNGF [Ullrich et al., 1983]. N_3 is raised against a 10 amino acid synthetic peptide reproducing the sequence (−49 to −40), and N_{4G} is directed against a 26 amino acid synthetic peptide (N_4) reproducing the sequence (−71 to −46) of the preproNGF. Sera β_2, β_3, L_{6-9}, V_1, and V_2 were raised against a mixture of β and 2.5S NGF purified according to Mobley et al. [1976]. Sera L_1 and L_2 were raised against denatured β/2.5S NGF by treatment with iodoacetic acid as described by Berger and Shooter [1977].

RESULTS

Construction of the Vector Containing the Mouse preproNGF cDNA Fragment

A 960 bp SmaI/PstI cDNA fragment containing the complete coding sequence of the mouse preproNGF protein (a gift of M. Selby) was introduced into the polylinker region of pTRBO. For this purpose, the plasmid DNA was cut with BamHI, in a polylinker region at the end of the lac-z gene, made blunt-ended with the Klenow fragment of DNA polymerase, and then cut with PstI and ligated to the SmaI/PstI cDNA fragment (Fig. 1). pTRBO differs from pUR290 [Rüther and Müller-Hill, 1983] in that it contains a transcription stop signal downstream from the lac-z gene. This prevents read through and contributes to an increased recovery of fusion protein.

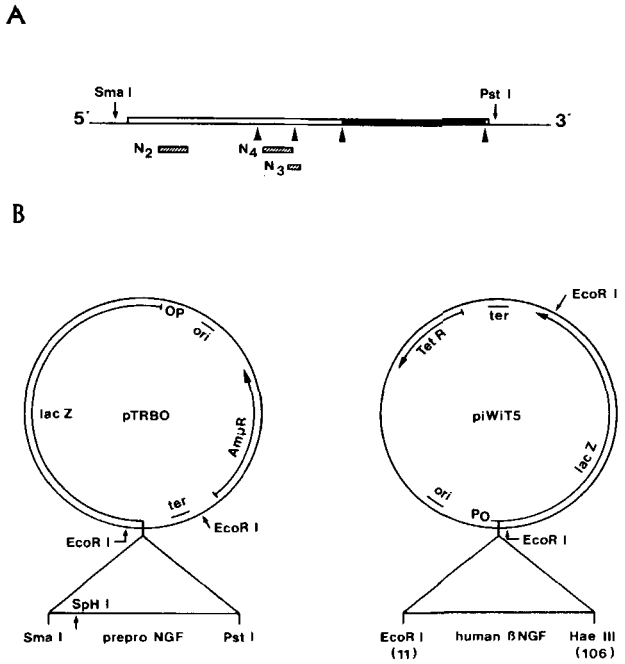

Fig. 1. Schematic representation of preproNGF and human β-NGF fusions to β-galactosidase gene. **A:** The 960 bp mouse preproNGF cDNA fragment was obtained after digestion with SmaI and PstI. The box indicates the sequence encoding preproNGF and the dark area that of the β-NGF protein. Arrows correspond to possible proteolytic sites. Shaded boxes represent sequences reproduced by synthetic peptides that served to raise antisera. **B:** pTRBO plasmid was cut at the carboxy-terminus of the β-galactosidase gene with BamHI, filled in, then cut with PstI and ligated to the SmaI/PstI preproNGF cDNA fragment. The EcoRI/HaeIII genomic fragment containing the sequence between codons 11 to 106 of the human β-NGF was fused to the fifth codon of the β-galactosidase gene of the plasmid piWiT5, as described in the text.

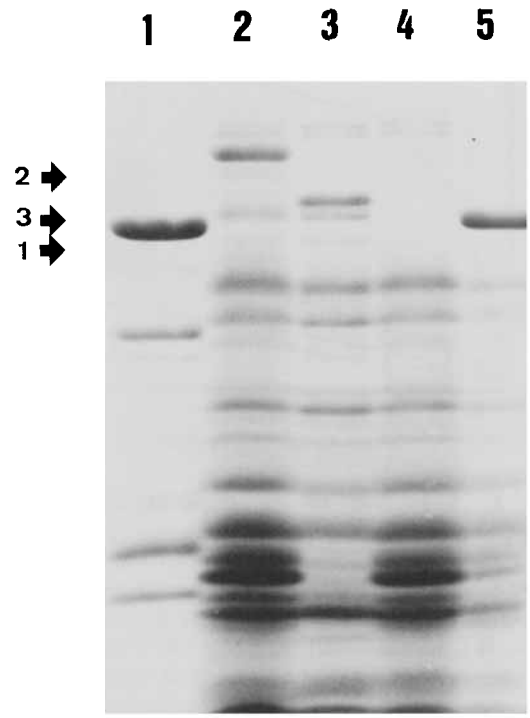

Fig. 2. Analysis of the chimeric proteins by SDS-polyacrylamide gel electrophoresis. **Lane 1:** Purified β-galactosidase (shown by arrow); **Lane 2:** Bacterial extract of the clone expressing the β-galactosidase/preproNGF chimeric protein (shown by arrow); **Lane 3:** Bacterial extract of the clone expressing the β-galactosidase/human β-NGF chimeric protein (shown by arrow); **Lane 4:** bacterial extract of the same clone grown without IPTG; **Lane 5:** Bacterial extract of the pTRBO plasmid before fusion. The protein aliquot is one-fifth that in the other slots. Electrophoresis was performed in 7.5% polyacrylamide slab gels. Proteins were stained with Coomassie blue.

After transformation of *E. coli* and selection on X-gal/ampicillin plates, blue colonies were screened for the presence of a 131 bp EcoRI/SpHI fragment of the preproNGF sequence. Protein extracts were prepared from clones containing the EcoRI/SpHI fragment and analysed for the presence of the chimeric protein in SDS/polyacrylamide gels. The gene fusion of the preproNGF cDNA fragment to the β-galactosidase would give rise to a polypeptide of about 150,000 Da. The fused protein was easily detectable in the protein extracts of the recombinant clones (Fig. 2, lane 2) as compared with the β-galactosidase (Fig. 2, lane 1).

Construction of the Vector Containing a Fragment of the Human NGF Gene

A pBR322-subcloned EcoRI fragment of about 2kb containing the human β-NGF genomic sequence starting 10 amino acids down from the amino terminus of the β-NGF protein (a gift of A. Ullrich) was digested with EcoRI/HaeIII to generate a 286bp fragment from position 1847 to 2185 of the sequence shown in Figure 2 of Ullrich et al. [1983]. This fragment comprises the human β-NGF sequence between codons 11–106 (over a total of 118 for the human β-NGF protein). A fragment containing the lac promotor and up to the first five codons of the lac-z gene was taken from a plasmid (piWiT5) that contains an EcoRI site in the amino terminus of the lac-z gene in the same frame as the EcoRI site within the β-NGF gene at position 1847. The HaeIII blunt end was ligated to the sixth codon of the lac-z gene (Fig. 1).

Lac-positive transformants were selected as blue colonies on X-gal/tetracycline plates, and recombinant clones were identified by the presence of a 320 bp EcoRI fragment. The human β-NGF fragment codes for a polypeptide of about 11,000 Da; after gene fusion, it would give rise to a β-galactosidase chimeric protein of about

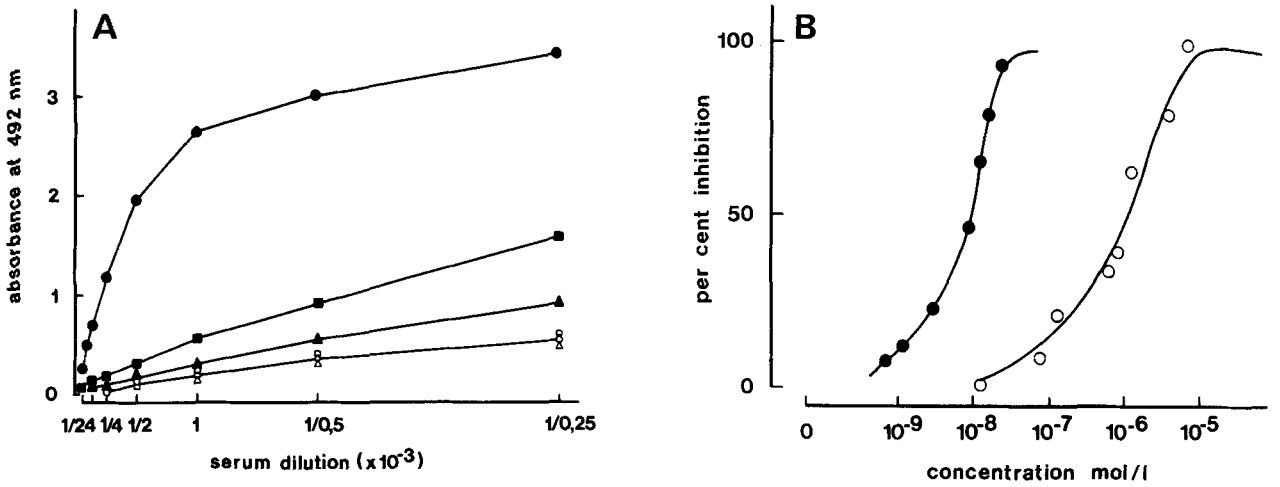

Fig. 3. **A:** Interaction of the chimeric β-galactosidase/preproNGF protein with antipeptide sera. Eluted fractions from APTG-Sepharose column containing the β-galactosidase/preproNGF protein were used to coat microtiter plates and then incubated with varying dilutions of the antipeptide sera N_{24}(●----●), N_3 (■----■), and N_{4G} (▲----▲). Bound immunoglobulins were assayed as described in Methods. Open symbols correspond to controls using purified β-galactosidase as substrate for coating. **B:** Inhibition of the binding of N_{24} antibodies to N_2 peptide by the chimeric protein and by the N_2 peptide. Plates were coated with N_2 peptide at about 2 μg/ml concentration. N_{24} antibodies (dilution 1:3,000) were preincubated with varying concentrations of the β-galactosidase/preproNGF protein eluted from APTG-Sepharose (●----●) or with peptide N_2 (○----○).

126,000 Da. A polypeptide with the corresponding molecular mass was readily detected in the protein extracts of such clones after electrophoresis in SDS/polyacrylamide gels (Fig. 2, lane 3). The reading frames of the fusion junctions of both constructions were verified by sequence analysis according to the method of Maxam and Gilbert [1983].

Purification of the Chimeric Proteins

Bacterial suspensions of the recombinant clones were disrupted by sonication, and β-galactosidase activity was assayed before and after centrifugation at 12,000 × g for 40 min at 4°C. Only 15–20% of the β-galactosidase/preproNGF protein was found in the supernatant, indicating that the majority of this protein was insoluble. In contrast, about 70–80% of the β-galactosidase/human β-NGF protein was present in the soluble fraction.

Purification of the chimeric proteins from the bacterial extract was performed by affinity chromatography on APTG-Sepharose. Addition of protease inhibitors (1 mM phenylmethyl sulfonyl fluoride PMSF, 5 mM N-α-tosyl-L-lysine chloromethyl ketone, and 25 μg/ml aprotinin) in the sonication buffer and performing the affinity chromatography at 4°C rather than at room temperature in the presence of 1 mM PMSF in the chromatography buffer resulted in a threefold to fourfold increase in the recovery of the chimeric proteins. From 1 liter of the appropriate bacterial culture, about 60–200 μg of purified β-galactosidase/preproNGF protein and 280–600 μg of β-galactosidase/human β-NGF protein were routinely obtained.

Immunological Analysis of the Chimeric Proteins

To determine if the chimeric proteins are antigenically related to preproNGF and to the mouse β-NGF, we employed ELISA techniques to monitor their interaction to sera against the native and denatured mouse β-NGF and to sera raised against synthetic peptides that reproduce epitopes of the preproNGF molecule.

The antipeptide serum N_{24} interacted with high affinity with the β-galactosidase/preproNGF protein, whereas sera N_3 and N_{4G} interacted weakly (Fig. 3A). Controls using β-galactosidase as a substrate for the ELISA were performed in parallel. The difference in the inhibitory capacities of the synthetic peptide N_2 and of the chimeric protein (mol/liter of the peptide or chimeric protein necessary for 50% inhibition) reflects the difference in the affinities of the N_{24} antibodies for these two antigens (Fig. 3B). N_{24} serum exhibits about a 100-fold higher apparent affinity for the chimeric protein as compared with the synthetic peptide.

The polyclonal rabbit serum L_1, which is raised against the denatured form of the mouse β-NGF, interacted with both chimeric proteins (Fig. 4A,B). Sera β_2 and β_3 are directed against the native mouse NGF. However, β_2 showed a strong affinity for both chimeric pro-

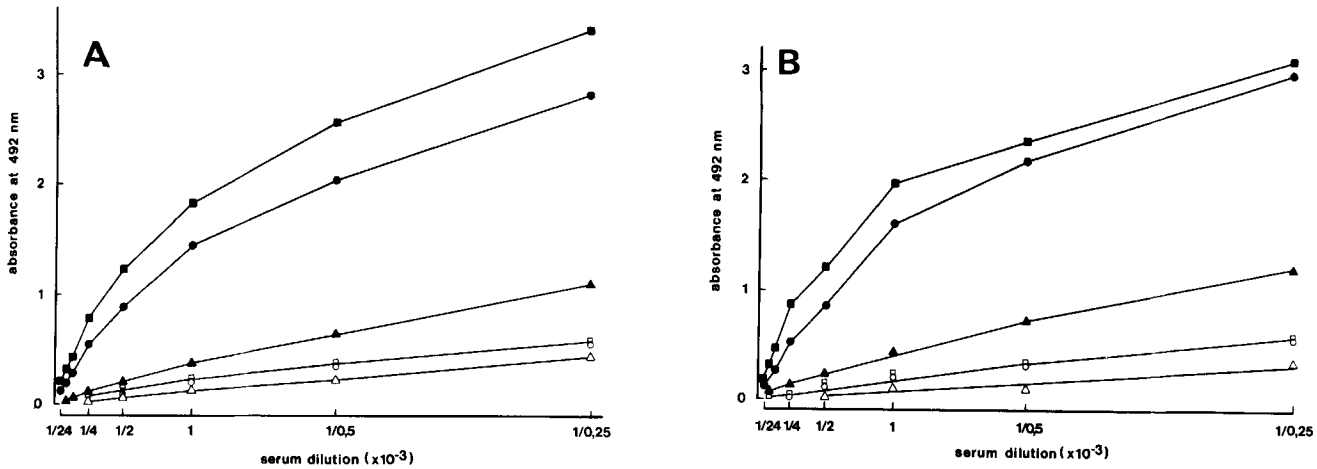

Fig. 4. **A:** Interaction of the β-galactosidase/preproNGF and **B:** β-galactosidase/human NGF chimeric proteins with antisera to mouse NGF. Eluted fractions from APTG-Sepharose columns containing the chimeric proteins were used to coat microtiter plates and were incubated with varying dilutions of the serum L_1 directed against denatured mouse β-NGF (■----■), and sera β_2 (●----●) and β_3 (▲----▲) directed against native mouse NGF. Open symbols correspond to controls using purified β-galactosidase as substrate.

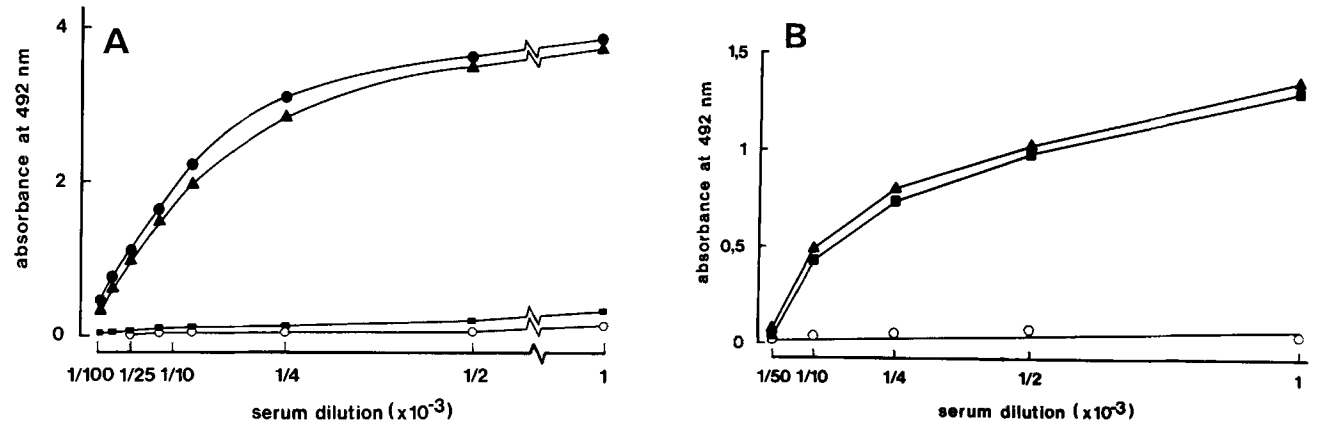

Fig. 5. Interaction of mouse NGF to anti-NGF sera. **A:** native. **B:** denatured. Plates were coated with purified native or denatured mouse NGF at a concentration of 5 μg/ml and incubated with varying dilutions of sera L_1 (■----■), β_2, (●----●), β_3 (▲----▲), and serum from unimmunized rabbit (○----○).

teins, whereas β_3 interacted weakly with both. For comparison, the interaction of the sera L_1, β_2, and β_3 with the native and denatured form of purified β-NGF is shown in Figure 5A,B.

We examined seven other anti-β-NGF sera, L_2, L_{6-9}, V_1, V_2 in a similar manner. L_2, which was raised against the denatured form of the mouse β-NGF, displayed an affinity for both chimeric proteins that was comparable to that shown by the serum L_1; sera L_7 and V_1, were comparable to β2 and β3, respectively. Sera L_{6-9}, L_8, V_1, and V_2 are directed against the native β-NGF protein and interacted with the chimeric proteins with a degree of affinity that varied between those observed with sera β_2 andβ_3 (results not shown).

DISCUSSION

The immunological detection of the preproNGF using anti-β-NGF sera has been a difficult task. In previous studies, antisera to the native and alkylated β-NGF failed to cross-react with in vitro synthesized translation products programmed with submandibular gland messenger RNAs (mRNA) [Dicou and Brachet, 1984; Wion et al., 1984] or with the in vivo precursor forms in homogenates of this gland [Dicou et al., 1986]. In contrast, several NGF precursor forms in submandibular gland extracts cross-reacted with antisera to β-NGF employed by others [Berger and Shooter, 1977; Darling et al., 1983; Saboori and Young, 1986]. Regarding the human β-NGF, there are contradictory reports on the cross-

reactivity of the human and mouse NGFs. A neurotrophic factor referred to as human NGF has been isolated from human placenta, but this molecule showed little or no cross-reaction to mouse βNGF by immunodiffusion [Perez-Polo et al., 1983a], radioimmunoassay (RIA) [Walker et al., 1980], or inhibition of the biological activity [Perez-Polo et al., 1983b] using rabbit polyclonal antibodies to mouse βNGF, whereas crossreaction was reported by others [Warren et al., 1980] by competition RIA using monoclonal antibodies to mouse β-NGF. An NGF-like activity secreted by human glial cells in culture was inhibited by antibodies to mouse β-NGF [Norrgren et al., 1980], whereas neurotrophic activity detected in human serum was inhibited only partially by antisera to mouse β-NGF [Banks et al., 1973; Riopelle et al., 1984; Stephani et al., 1987].

For this purpose, we designed plasmid constructions to obtain mouse preproNGF and partial human β-NGF chimeric proteins to study their cross-reactivity with anti-β-NGF sera. The β-galactosidase/human NGF protein is present primarily in the soluble fraction, unlike the mouse preproNGF fusion protein, which is found in the cell debris after lysis; this would indicate that the protein accumulates in an insoluble or aggregated form, which may be due to the presence of hydrophobic sites (−181 to −173 and −121 to −102, Fig. 3) [Ullrich et al., 1983] within the preproNGF sequence. Insoluble aggregates were observed for other bacterially synthesized proteins; this is thought to be due to intermolecular linking by disulfide bonds [Shoemaker et al., 1985].

The β-galactosidase/preproNGF protein exhibited preproNGF-like antigenicity, as shown by its interaction with the serum N_{24} directed against the synthetic peptide N_2; however, it interacted very weakly with the serum N_{4G}, which is directed against the synthetic peptide N_4. Peptide N_2 contains the sequence between amino acids 25 to 49 from the amino terminus of the 307 aa-long precursor. Peptide N_4, on the other hand, is situated between two potential cleavage sites by an arginine-specific esteropeptidase (Arg/Arg at −72 and −73 and Arg/Arg at −41 and −42) and contains the entire sequence of the generated cleavage product. It is interesting in this respect that another serum (N_{2G}) directed against the N_2 peptide immunoprecipitated a polypeptide of MW 30,000–32,000 in extracts of the submandibular and thyroid glands [Dicou et al., 1986], whereas serum N_{4G} failed to immunoprecipitate this protein [Dicou, unpublished results]. Some cross-reactivity was observed with serum N_3, raised against the hydrophilic peptide N_3, which fully overlaps a proteolytic cleavage site (Arg/Arg at −41 and −42).

These results suggest that epitopes shared by the N_2 and—to a certain degree—by the N_3 amino acid sequences, unlike the N_4 sequence, are "exposed" domains of the chimeric protein. However, they should be interpreted with caution, as the novel microenvironment created by gene fusion and by the probable aberrant formation of disulfide bonds may modify the tertiary structure of the precursor.

Data obtained with anti-β-NGF sera suggest that some may or may not cross-react with the mouse preproNGF protein, which agrees with our previous results and those of others.

Another conclusion of this study is that certain sera to mouse β-NGF recognize the fused human β-NGF fragment. The high degree of conservation of the DNA sequence of the murine and human NGF genes would argue in favor of antigenic similarities between the NGF proteins of these species. However, there are sera to mouse β-NGF that fail to cross-react with the chimeric human β-NGF molecule, although there is a 90% homology between the mouse and the human NGF proteins. In our constructions, the human β-NGF chimeric protein is missing the first 10 amino acids. A previous study indicated that the N-terminal nonapeptide is not an antigenic site [Butler and Revoltella, 1983].

Finally, sera raised against denatured mouse β-NGF interacted with either chimeric protein with higher affinity as compared to sera prepared against native protein. This may be due to misfolding of the hybrid proteins.

Our results provide direct evidence that certain antisera to mouse NGF may cross-react with the human NGF molecule and suggest that divergencies in previous reports regarding cross-reactivity between human and mouse NGF may arise from the variable affinity exhibited by the anti-NGF sera employed in the various studies.

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