Molecular cloning of the avian β-nerve growth factor gene: transcription in brain

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A chicken gene cross-hybridizing with a murine β-nerve growth factor (βNGF) cDNA probe was identified by Southern blot analysis and isolated from a genomic DNA library. The DNA sequence coding for the putative mature βNGF protein was determined, providing direct evidence for the existence in birds of a neurotrophic factor sharing a high degree of sequence homology with mammalian βNGF. In addition this gene is shown to be transcriptionally active in adult avian brain as demonstrated by Northern blot analysis.

(Brain) Gene Nerve growth factor Nucleotide sequence

1. INTRODUCTION

Nerve growth factor (βNGF) is a neurotrophic factor which exerts its effects on several cell types derived from the neural crest. This includes sympathetic and sensory neurons, and chromaffin cells from the adrenal medulla [1,2]. βNGF is synthesised in exceptionally large amounts in a few exocrine glands of some vertebrate species [3], such as the male mouse submaxillary gland (MSG) [4]. The physiological role of βNGF in rodents was demonstrated by eradicating endogenous factor from embryos or newborn animals with antisera raised against the MSG βNGF protein. This treatment resulted in an atrophy of sympathetic and sensory ganglia, and the adrenal medulla [5]. More recently, the use of cDNA probes encoding the sequence of MSG βNGF mRNA and enzyme immunoassays have provided evidence for the presence of the factor in numerous peripheral organs in mammals [6,7]. Furthermore, levels of expression of the βNGF gene were shown to correlate closely with the density of sympathetic innervation of effector organs. NGF is apparently not confined to peripheral tissues since the presence of βNGF mRNA and mature protein were reported in the central nervous system of the rat [8].

The neurotrophic activity of purified MSG βNGF in avian embryo sensory and sympathetic neurons has been largely demonstrated, both in vivo and in vitro [9,10]. Despite the presence in chick embryo extracts of substances eliciting an NGF-like activity on the expected target neurons, and of NGF receptors in the developing chicken embryo [11], the existence of an avian βNGF protein homologous to the mouse βNGF has never been clearly established. This is mainly due to the fact that antibodies directed against the MSG βNGF show little or no cross-reactivity with the chicken neurotrophic molecules [12]. Here, we provide direct evidence that a chicken gene which is transcribed in adult avian brain encodes a molecule with high sequence homology with mammalian βNGF.

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2. MATERIALS AND METHODS

2.1. *Screening of chicken genomic library*

A λCharon 4A genomic library [13] was screened with a ^{32}P-labelled *Ava*II-*Pst*I probe derived from the mouse βNGF cDNA cloned by Scott et al. [14]. Screening of the library was carried out as described by Benton and Davis [15]. Nitrocellulose filters were prewashed for 2 h at 42°C in 50 mM Tris (pH 8.0), 1 M NaCl, 1 mM EDTA, 0.1% SDS, prehybridized for 4 h at 42°C in 50% formamide, 5 × Denhardt, 5 × SSPE, 0.1% SDS, 1 μg/ml poly(A), 1 μg/ml poly(C), 100 μg/ml denatured salmon sperm DNA. The nick-translated probe (10^8 cpm/μg) was then added to the same buffer and hybridization carried out overnight at 42°C. The filters were washed 4 times for 10 min at room temperature, twice for 1 h at 45°C, and twice for 1 h at 55°C in 2 × SSC, 0.1% SDS. Positive clones were purified by rescreening under the same hybridization conditions.

2.2. *DNA preparation and characterization*

DNA from positive clones was isolated [16]. A partial restriction map was established by single and double digestions using various restriction enzymes followed by electrophoresis in 1% agarose gels and Southern blot analysis [17] using the mouse cDNA probe.

2.3. *Subcloning in pUC19*

A 0.91 kb *Pst*I fragment was subcloned in the *Pst*I site of pUC19. *E. coli* JM 83 was transformed with the ligated DNA, and positive clones were identified by in situ colony hybridization with the *Ava*II-*Pst*I mouse βNGF probe.

2.4. *ExoIII-S1 deletion of the chicken βNGF* Pst*I insert, and DNA sequence determination*

Ordered deletions of the 0.91 kb *Pst*I fragment were generated using the ExoIII-S1 deletion protocol of Henikoff [18], using the *Bam*HI site to start deletion, and the *Kpn*I or *Sac*I sites in the pUC19 polylinker to generate blocked termini. ExoIII reactions were stopped every 3 min. After ligation and transformation, plasmid DNA was prepared for rapid analysis by the minilysate procedure [19]. The deleted plasmids were labelled at the *Eco*RI or *Hin*dIII site in the pUC19 polylinker using [^{32}P]dATP and the Klenow fragment of *E. coli* DNA polymerase I. After recutting with *Hin*dIII or *Eco*RI respectively, the labelled fragments were isolated by electroelution from agarose gels, purified by DEAE-cellulose chromatography, and sequenced by the chemical degradation procedure of Maxam and Gilbert [20]. The sequencing strategy is shown in fig.2.

2.5. *DNA and RNA blotting*

High-M_r chicken DNA was isolated from whole chicken embryos (7 days old) by the method of Blin and Stafford [21]. Brain RNA was prepared from adult quail using the LiCl/urea method [22]. Poly(A)$^+$ RNA was purified by two passages on oligo(dT)-cellulose [23]. Southern and Northern blots were performed as described [24].

2.6. *Computer-assisted sequence analysis*

Nucleotide sequences were compiled using the COMPSEQ package (Genofit). Alignments were performed using the program BESTFIT [25] with parameters of 3.0 (gap weight) and 0.5 (gap length) using the BISANCE facility (CITI2, Paris).

3. RESULTS

3.1. *Isolation of a chicken gene hybridizing with a murine βNGF probe*

A murine βNGF cDNA probe hybridizes with a 0.91 kb *Pst*I DNA fragment of the chicken genome as demonstrated by Southern blot analysis (fig.1). After screening 8 × 10^5 phage plaques from a chicken genomic DNA library, we isolated 8 clones hybridizing with the probe. Two clones, λCβNGF-2 and λCβNGF-6, were analysed in detail. They had an identical restriction map in their region of overlap containing the 0.91 kb *Pst*I fragment (not shown).

3.2. *Nucleotide sequence of the chicken βNGF gene*

After subcloning the *Pst*I fragment from λCβNGF-2 into pUC19, ordered deletions were generated in both directions, and the DNA coding for the so far undetermined avian mature βNGF was sequenced. The nucleotide sequence determined as shown in fig.2 is presented in fig.3 together with its comparison with the murine cDNA sequence and their conceptual translation into amino acids. The position of the gaps which

Fig.1. Characterization of chicken βNGF gene. Southern blot analysis was performed as described in section 2. Lanes: (1) 100 ng of the pUC19 plasmid containing the *Pst*I chicken βNGF insert was cleaved by *Pst*I and hybridized with mouse βNGF cDNA (exposure time 16 h). (2) 20 μg chicken genomic DNA was cleaved by *Pst*I and hybridized with chicken βNGF *Pst*I probe (exposure time 2 days). (3) 20 μg chicken genomic DNA was cleaved by *Pst*I and hybridized with mouse βNGF cDNA (exposure time 10 days).

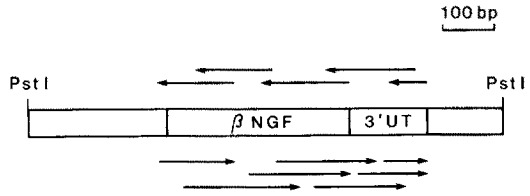

Fig.2. Sequencing strategy. A partial sequence of the 0.91 kb *Pst*I fragment hybridizing with the murine βNGF probe was determined as described in section 2. Arrows indicate the extent of sequence reading for each fragment. βNGF, coding portion for the mature form; 3'UT, 3'-untranslated region.

have been introduced in order to align the two sequences indicates that insertions and deletions which have accumulated with time in these homologous sequences have preserved a long open reading frame encoding a polypeptide chain sharing a high degree of sequence homology with murine βNGF (fig.4). The open reading frame terminates at the same position in chicken and mouse, and is followed by a 163 bp 3'-untranslated sequence. Like in mammals the polyadenylation site is ATTAAA, slightly different from the canonical AATAAA.

3.3. *The βNGF gene is transcribed in avian brain*

When the *Pst*I fragment from the chicken gene was used as probe, it was possible to detect the murine βNGF transcript in MSG mRNA (fig.5). The intensity of the signal was in the range of 50-fold lower than that obtained with the murine probe. This is explained by the fact that the murine and chicken sequences have at most 80% sequence homology in the segment coding for mature βNGF. When quail brain mRNA was analysed under similar conditions, the chicken probe hybridized to mRNA in the range of 1.3 kb (fig.5). Again, the intensity of the signal was much lower than that obtained with the same probe in mouse SMG mRNA. Altogether these results indicate that the avian βNGF is transcribed at low levels in adult avian brain.

4. DISCUSSION

We have isolated chicken genomic clones containing the 0.91 kb *Pst*I fragment hybridizing with a murine βNGF probe on Southern blots. As in mammals, it appears that there is a single gene in the chicken genome with the potential of encoding a neurotrophic factor similar to mouse and human βNGF. As in mouse and human βNGF [26], the cysteine residues involved in maintaining the tertiary structure are conserved [27]. Tryptophan residue 21 which is critical for receptor binding is also maintained [28], and amino acid sequence homologies reach 82.5–87.5%. Only one of the possible *N*-glycosylation sites present in mouse and man is conserved in chicken, that located at position −8, suggesting a possible involvement in the maturation step of pro-βNGF involving cleavage at the dibasic peptide located just in front of the mature βNGF.

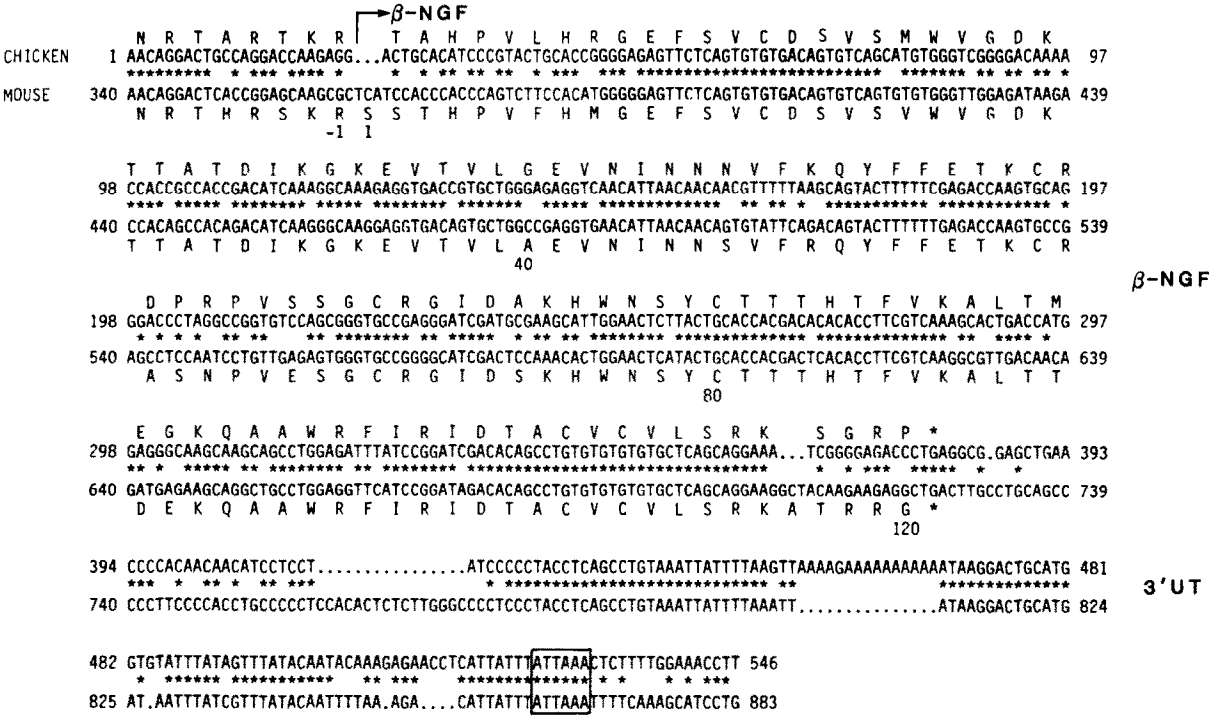

Fig.3. Sequence of the βNGF gene. The sequence determined as described in fig.2 was aligned with the murine sequence [14] using the program BESTFIT [25]. Matching nucleotides are indicated by stars, and gaps by dots at missing positions. The coding portion is translated using the one-letter code for amino acids. Numbering of nucleotides starts at the initiation codon (amino acid −121) in the murine sequence. By convention amino acids are numbered below the murine sequence starting at the beginning of mature βNGF (+1) and accordingly negative numbers are assigned to the pro moiety. The non-canonical poly(A) addition site ATTAAA is boxed.

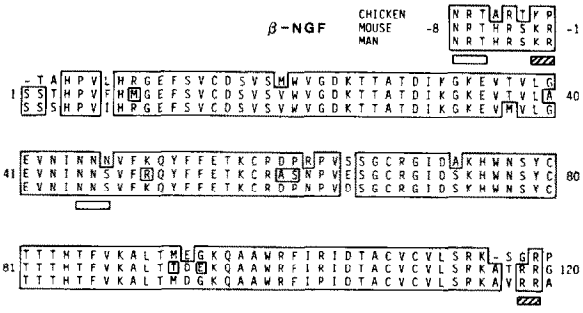

Fig.4. Comparison of βNGF amino acid sequences. The chicken sequence was derived from the nucleotide sequence as shown in fig.3, and aligned with the mouse [14] and human [26] sequences. Gaps indicated by dashes were introduced to maximize homology. Homologous positions in at least two sequences are boxed. The dibasic peptides are indicated by hatched bars and potential glycosylation sites by open bars. Numbering of amino acids is as indicated in legend to fig.3.

In addition this gene appears to be transcribed in adult brain, a situation similar to that found recently in mammals [29]. Thus the chicken probe will help in clarifying the expression of βNGF especially during development in birds.

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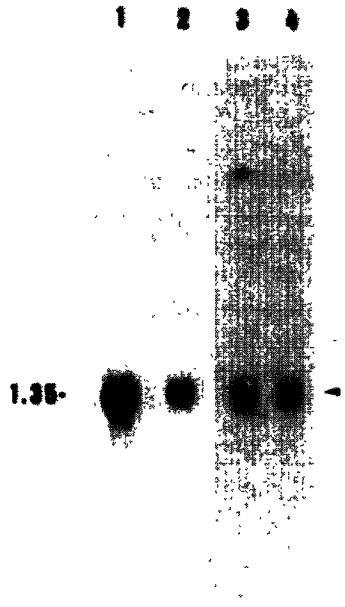

Fig.5. Transcription of the βNGF gene in quail brain. Northern blot analysis was performed as described in section 2. Lanes: (1) Mouse submaxillary gland total RNA (15 μg) probed with the murine cDNA (exposure time 12 h). (2) As lane 1 with the chicken βNGF 0.91 kb *PstI* fragment as probe (exposure time 10 days). (3,4) Poly(A)$^+$ RNA from adult quail brain, 12 μg (lane 3) and 8 μg (lane 4), probed with the 0.91 kb *PstI* fragment (exposure time 15 days). Size of hybridizing transcripts is indicated in kilobases.

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