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Detection of nerve growth factor mRNA in the developing chicken embryo

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Summary

Nerve growth factor (βNGF) is a protein supporting sympathetic and sensory innervation in the peripheral tissues as well as cholinergic innervation in the brain. A DNA probe derived from a genomic clone coding for chicken NGF was used to study NGF mRNA levels during development. NGF mRNA was detected in the chicken embryo as early as day 3·5 of incubation. The level of NGF mRNA in total embryo increased four-fold until day 8, remained high until day 12, and subsequently decreased. No corresponding peak in NGF mRNA expression was found in heart and brain measured separately. Instead these organs showed increased NGF mRNA levels after hatching. The highest levels of NGF mRNA in the day-8 embryo were found in skin and eye (in particular cornea, but also iris, sclera–choroid and neural retina) suggesting a correlation between sensory innervation and this early peak of NGF expression.

Key words: nerve growth factor, chicken embryo, messenger RNA, developmental regulation

Introduction

Nerve growth factor supports sympathetic and sensory innervation of peripheral tissues (Levi-Montalcini & Angeletti, 1968; Thoenen & Barde, 1980). Recently NGF has also been found in the mammalian brain (Korsching *et al.* 1985; Whittemore *et al.* 1986; Shelton & Reichardt, 1986; Goedert *et al.* 1986) and attributed a trophic function for cholinergic neurones situated in the basal forebrain (Gnahn *et al.* 1983; Korsching *et al.* 1985; Large *et al.* 1986). The recent isolation of the gene for chicken βNGF (Ebendal *et al.* 1986; Meier *et al.* 1986; Wion *et al.* 1986) has made it possible to document the presence of NGF mRNA also in the avian brain (Ebendal *et al.* 1986; Wion *et al.* 1986; Goedert, 1986). However, the developmental appearance and function of NGF in avian embryos are still largely obscure, despite the fact that the classical biological assay for NGF (Cohen *et al.* 1954) is based on stimulation of neurite formation in embryonic chicken ganglia. In this report, we have used a DNA probe for chicken NGF to address the question of when and where NGF mRNA is synthesized in the chicken embryo.

Materials and methods

RNA blots

Total RNA was prepared from entire embryos (embryonic day (E) 3·5–18), and organs from embryos (E8–18), or posthatch chicks (day P1 and P7), as previously described (Ebendal *et al.* 1986). Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography (Aviv & Leder, 1972) and used for RNA blots.

10 μg of poly(A)$^+$ RNA from each sample were electrophoresed in 1 % agarose gels containing 0·7 % formaldehyde, transferred to nitrocellulose filters and hybridized to a 900 bp *PstI* fragment encoding the mature chicken NGF protein (Ebendal *et al.* 1986). The probe was labelled by nick translation using α-^{32}P-dCTP to a specific activity of around 5×10^8 cts min^{-1} μg^{-1}. Filters were washed at high stringency (15 mM-NaCl, 1·5 mM-sodium citrate, pH 7·0, 0·1 % SDS, 54°C) and exposed to Kodak XAR-5 films at −80°C using Du Pont intensifying screens. Filters were then boiled for 10 min in 1 % glycerol and rehybridized with

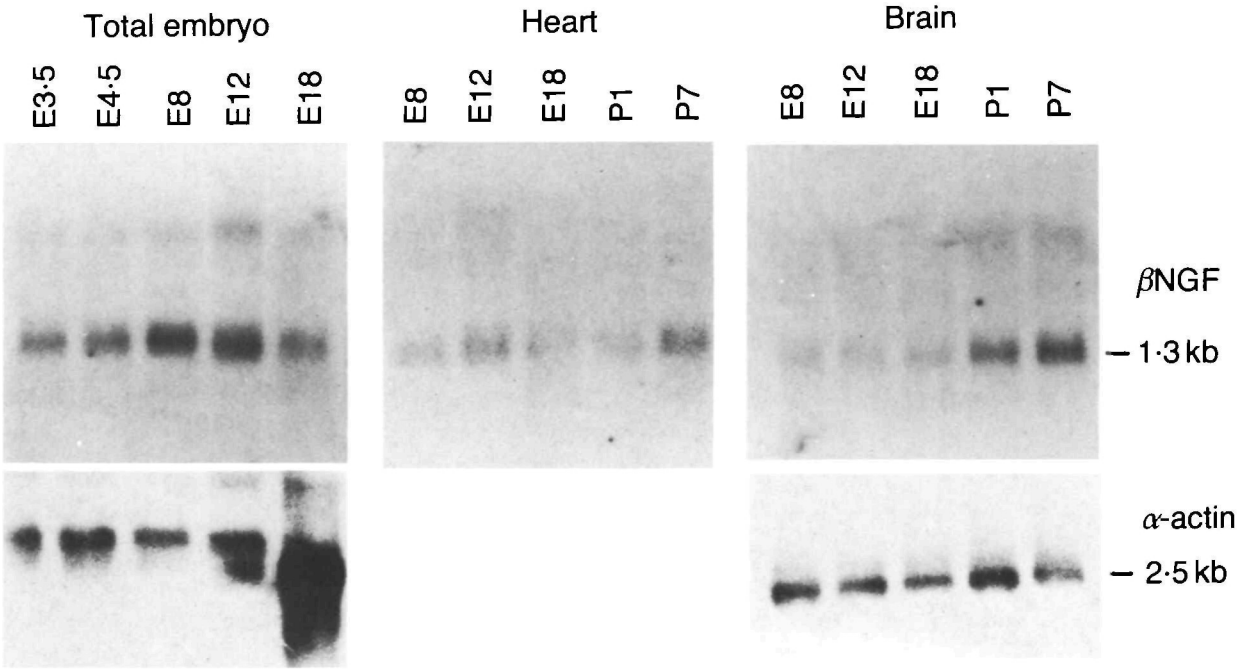

Fig. 1. NGF mRNA during chick development as revealed by RNA blots. 10 μg poly(A)$^+$ RNA in each lane were elctrophoresed in formaldehyde-containing agarose gels. The gels were blotted to nitrocellulose filters for hybridization with a 900 bp ^{32}P-labelled *Pst*I fragment encoding the mature chicken NGF. Filters were washed at high stringency followed by autoradiography.

a probe for mouse α-actin as a control for the amount of RNA in each lane.

Autoradiograms were scanned using a Shimadzu CS-390 densitometer and the linearity was checked by scanning of serial dilutions of RNA obtained from COS cells transfected with a eukaryotic expression vector containing the chicken NGF gene (Ebendal & Persson, 1987; Hallböök *et al.* 1987).

Results

Developmental changes in NGF mRNA levels

A DNA fragment from a genomic chicken NGF clone (Ebendal *et al.* 1986) was used as a probe to measure the levels of NGF mRNA in the chicken embryo. Blots of RNA prepared from total embryos at different ages showed the presence of NGF mRNA already at E3·5 (stage 20; Hamburger & Hamilton, 1951), the earliest stage examined (Fig. 1). The size of the embryonic NGF transcript was the same as in the adult (1·3 kb; Ebendal *et al.* 1986). In addition, weak hybridization was seen to a 5 kb RNA species that may represent a NGF RNA precursor or cross-hybridization of the probe to ribosomal RNA (Fig. 1). The level of NGF mRNA in total embryo increased slightly at E4·5 (stage 25), showed peak levels at E8 and E12, and was markedly lower during late embryonic development (E18) (Figs 1, 2) .

For comparison, levels of NGF mRNA were studied also in heart and brain from E8 until one week after hatch. These two organs were previously found to contain higher NGF mRNA levels than most organs in the adult chicken (Ebendal *et al.* 1986). Low levels of NGF mRNA were detected in the embryonic heart (E8, E12 and E18), whereas increasing levels were found after hatch (Fig. 2). Similarly, brain showed low levels of NGF mRNA throughout the

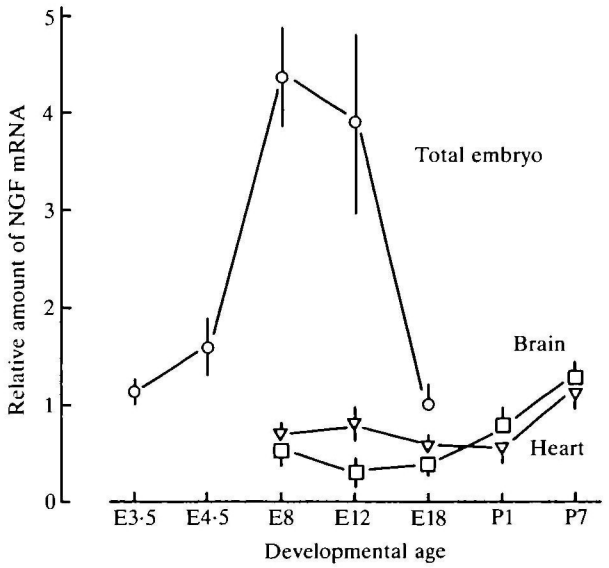

Fig. 2. Results from densitometric scanning of autoradiograms from three independent experiments as shown in Fig. 1. The results presented are mean values with error bars included.

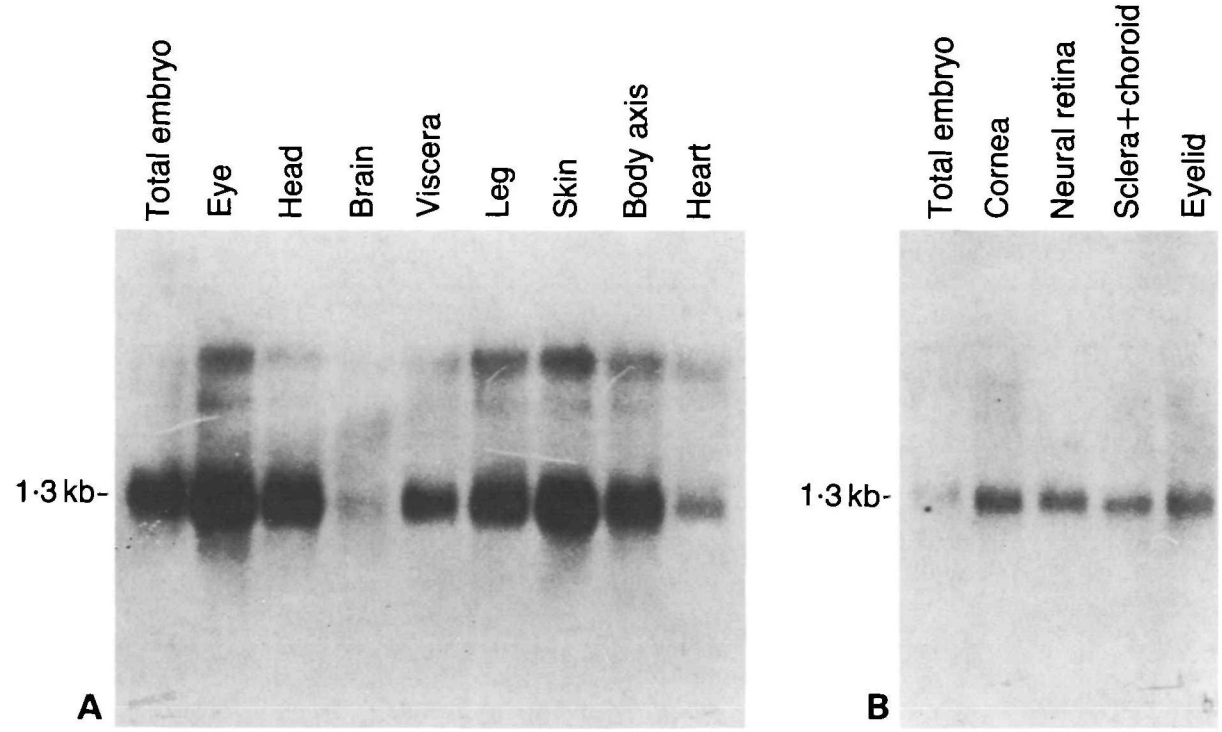

Fig. 3. (A) RNA blots probed for chicken NGF. Poly(A)$^+$ RNA was prepared from the indicated organs of E8 embryos. (B) RNA-blots with poly(A)$^+$ RNA from different parts of the eye of E8 chick embryos.

embryonic period. At E8, the level of NGF mRNA in the entire embryo was around ten times higher than the levels found at the same developmental age in the brain and heart (Fig. 2). As a control for the developmental changes in NGF mRNA, and to ensure similar amounts of mRNA in each lane, the same blots were reprobed with an α-actin probe. Both the entire embryo and the brain contained similar levels of α-actin mRNA at all stages analysed (Fig. 1). The heart, on the other hand, showed a more complex developmental change in α-actin mRNA levels, with two mRNA species represented at different relative levels at different stages (not shown).

Distribution of NGF mRNA at E8

In order to determine the origin of the high levels of NGF mRNA at E8, a number of organs from embryos of this age was dissected for RNA blot hybridization (Fig. 3). The result clearly showed that NGF mRNA is expressed in many regions of the embryo at E8 (Fig. 3A). Particularly high levels of expression were found in skin (collected mainly from the back of the embryo). The level of expression in skin at E8 exceeds that found in the heart of the adult chicken (Fig. 4). High levels were also found in eye, head (skin, eyes and brain removed) and hind limb (skin removed) (Figs 3, 4). Thigh muscle was collected separately to test if the developing muscles of the limb could account for the high NGF mRNA level found in hind limb. This analysis showed a somewhat

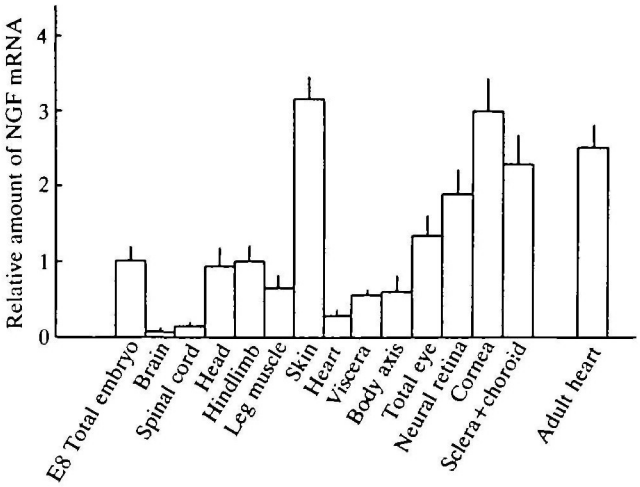

Fig. 4. Schematic presentation of NGF mRNA levels in the E8 chicken embryo. The results shown are mean values (error bars indicated) for two independent experiments as shown in Fig. 3. The level of NGF mRNA in the chicken heart is included for comparison.

lower level of NGF mRNA in the muscle mass than in the entire limb (Fig. 4). The P7 pectoralis muscle contained very low levels of NGF mRNA (not shown).

Brain and spinal cord (Fig. 4), together with spinal ganglia, contained the lowest levels of NGF mRNA of the E8 organs analysed. Viscera (excluding heart but including liver, gizzard, intestine, cloaca, lungs

and kidneys) showed a somewhat higher level of NGF mRNA than heart. The yolk sac contained a low but detectable level of NGF mRNA (data not shown). The remaining axial structures of the embryo (excluding skin and limbs but including axial muscles, vertebrae, connective tissue and major nerves and blood vessels) also contained intermediate levels of NGF mRNA (Figs 3, 4).

The eye was further subdivided to determine which parts of the eye are expressing NGF mRNA at E8. The highest levels of NGF mRNA were found in cornea and eyelids (Figs 3B, 4). The sclera, including the choroid and pigment epithelium, also showed high levels of NGF mRNA as did neural retina.

Discussion

The present finding of NGF mRNA as early as E3·5 (stage 20) suggests a function for NGF in early chick embryo development. However, evidence is lacking for the presence of the NGF protein until late in development (day 18; Belew & Ebendal, 1986). Unequivocal demonstration of NGF protein at earlier stages of development would require a sensitive enzyme immunoassay for chicken NGF like that available for rat and mouse NGF (Korsching *et al.* 1985; Whittemore *et al.* 1986; Lärkfors & Ebendal, 1987; Auburger *et al.* 1987).

The pattern of NGF mRNA expression in the chicken embryo reveals several interesting features. One obvious characteristic is the presence of NGF mRNA already at a time when the first formation of peripheral ganglia is occurring. The early appearance of NGF mRNA in the chicken embryo at day 3 was recently also observed by Goedert (1986).

The highest level of NGF mRNA expression in early development was found in the skin, an area of sensory innervation, whereas organs densely innervated by adrenergic fibres such as the heart, showed low levels of NGF mRNA before hatch. A peak in the embryonic level of NGF mRNA was recently demonstrated also in skin of the E12·5 mouse embryo by Davies *et al.* (1987). The distribution of NGF mRNA in the E8 chick embryo, with high levels in the skin and eye, suggest a role for NGF also in exteroceptive sensory innervation during chicken development.

Binding of radiolabelled NGF to receptors has been observed in sections of the E4 chick embryo (Raivich *et al.* 1985), while premigratory and early migratory neural crest cells during E2 and E3 lack the NGF receptor (Bernd, 1985). The first survival-promoting effects of NGF injected into the chick embryo at days 3 and 4 were seen at E4·5 (stage 25) in the cervical primary sympathetic ganglion (Oppenheim *et al.* 1982) and thoracic level spinal ganglia (Hamburger *et al.* 1981).

The peak of NGF mRNA levels (E8–12) coincides with the maximum number of NGF receptors on the spinal chick ganglia (Herrup & Shooter, 1975) and with maximal fibre outgrowth from explanted sympathetic, trigeminal and spinal ganglia responding to mouse NGF (Ebendal & Hedlund, 1975; Ebendal, 1979; Davies & Lindsay, 1984). A similar fibre outgrowth response has recently been demonstrated using a biologically active recombinant chicken NGF protein (Ebendal & Persson, 1987). At around E8, maximal neuronal death in spinal ganglia has also been observed (Hamburger *et al.* 1981) which has been suggested to be the result of competition between neurones for a limited supply of a trophic factor.

Interestingly, the developing hindlimb contained a high level of NGF mRNA. This high level was not accounted for by the skin (which was removed before preparation of RNA) nor by the developing muscle mass of the thigh. Goedert (1986) also observed substantial amounts of NGF mRNA in the embryonic leg and suggested a functional correlation to the fibre projections from the dorsal root ganglia.

The cellular origin of the NGF mRNA in the skin and leg is not known. Production of NGF may be widespread with several cell types contributing. Moreover, the proportion of cells expressing NGF mRNA at any developmental stage may vary. This issue needs to be resolved by the use of *in situ* hybridization. Indeed, Davies *et al.* (1987) recently presented *in situ* hybridization data from the mouse embryo suggesting synthesis of NGF mRNA in both epithelial and mesenchymal layers of the skin.

The pattern of NGF mRNA expression in the central nervous system is of particular interest. The brain and spinal cord contain relatively high levels of NGF mRNA in the adult chicken (Ebendal *et al.* 1986; Goedert, 1986). Developmentally, expression of NGF mRNA in these organs increases significantly after hatch with only low levels of NGF mRNA during the embryonic period. A similar time course has been found in the mammalian brain, where NGF mRNA appears postnatally in the rat brain with a peak level three weeks after birth (Whittemore *et al.* 1986; Large *et al.* 1986; Auburger *et al.* 1987). Despite the low levels of NGF mRNA prenatally, the NGF protein was found in the fetal rat brain at levels 25–50% of those found in the adult brain (Whittemore *et al.* 1986; Large *et al.* 1986; Auburger *et al.* 1987). It is not known whether this is also true for the chicken embryo brain. An early function for NGF in brain development is suggested by the presence of the NGF receptor in fetal rat brain (Yan & Johnson, 1987). NGF binding in the chicken brain has been reported at E8 by Frazier *et al.* (1974) and more

recently by Raivich *et al.* (1987) demonstrating transient, widespread receptor binding of NGF in several areas of the chick brain at E4–12.

A function for NGF in the visual system is also suggested by the finding of NGF mRNA in the embryonic neural retina. Interestingly, optic tectum is the part of adult chicken brain with the highest level of NGF mRNA (Ebendal *et al.* 1986). Similar observations were made by Goedert (1986) who also showed that the retina maintains a high level of NGF mRNA in adult hens.

The mechanisms regulating the tissue- and stage-specific expression of NGF mRNA revealed by the present study remains to be elucidated.

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