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Cellular localization of nerve growth factor synthesis by *in situ* hybridization

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A very sensitive and specific method for *in situ* hybridization has been developed. This method detects low copy numbers of mRNA^{NGF} transcripts in both tissue sections and cultured cells using ^{35}S-labelled cRNA and oligonucleotide probes. In order to reduce the high nonspecific background occurring with ^{35}S-labelled probes, prehybridization in the presence of non-labelled thioαUTP at pH 5.5 proved to be essential, together with a series of additional changes in the standard procedures for *in situ* hybridization. With this improved method it was possible to demonstrate that in tissues densely innervated by sensory (whisker pad) or both sympathetic and sensory (iris) fibers, NGF is synthesized not only by Schwann cells ensheathing these fibers, but also — and even to a much larger extent — by the target cells of the sensory and sympathetic neurons, i.e. epithelial cells, smooth muscle cells and fibroblasts. Moreover, in the sciatic nerve of newborn rats (where the mRNA^{NGF} levels are 15× higher than in adults) it was demonstrated that all Schwann cells have the capacity to express mRNA^{NGF}, not just those ensheathing the axons of NGF-responsive neurons.

Key words: in situ hybridization/mRNA^{NGF}/[^{35}S]cRNA probes

Introduction

The embryonic development and maintenance of specific properties of neurons are influenced by continuous retrograde signals from their target tissues (see Oppenheim, 1981). The molecular basis for such an interaction has been established for Nerve Growth Factor (NGF), a well-characterized protein which acts on peripheral sympathetic and neural crest-derived sensory neurons (see Levi-Montalcini and Angeletti, 1968; Greene and Shooter, 1980; Thoenen and Barde, 1980) and, as more recently demonstrated, also on a subpopulation of central cholinergic neurons (Gnahn *et al.*, 1983; Mobley *et al.*, 1985). The evidence that NGF acts as a retrograde messenger between target tissues and innervating neurons was initially deduced indirectly; the impairment of retrograde transport had the same effect as the neutralization of endogenous NGF by anti-NGF antibodies (see Hendry, 1980; Thoenen and Barde, 1980; Schwab and Thoenen, 1983). This indirect evidence was more recently complemented by the observation that a positive correlation exists between the density of sympathetic innervation of target tissues and their levels of NGF (Korsching and Thoenen, 1983) and mRNA^{NGF} (Heumann *et al.*, 1984; Shelton and Reichardt, 1984). Moreover the relatively high levels of NGF in sympathetic and sensory ganglia result from NGF accumulation by retrograde axonal transport rather than by local synthesis (Heumann *et al.*, 1984; Korsching and Thoenen, 1985). An essential open question in the understanding of the physiological action of NGF is the cellular localization of its synthesis, i.e. whether a single cell type is responsible for the supply of NGF to the target neurons, or whether several cell types are involved in NGF synthesis. We chose to approach this question by using *in situ* hybridization. However, it was first necessary to improve available methods. A particular problem was the serious background which occurred when ^{35}S-labelled cRNA and oligonucleotide probes were used in an attempt to improve the resolution over that obtained using ^{32}P-labelled probes. The necessity for improving the signal to noise ratio arises from the fact that mRNA^{NGF} is present in extremely low copy numbers (10^{-6} to 10^{-7} molecules of polyA$^+$–RNA) even in the most densely innervated tissues, and that this low copy number of mRNA^{NGF} is not concentrated in a small number of cells. Based on the well-documented biochemical data referring to levels of mRNA^{NGF} we have selected various representative tissues of mouse and rat to localize mRNA^{NGF} by *in situ* hybridization.

Results and Discussion

Specificity and sensitivity

In initial experiments, using ^{32}P-labelled cRNA^{NGF} probes, we obtained specific signals over sections and cultured cells (data not shown). However, the resolution of ^{32}P-labelled probes was unsatisfactory due to the high energy and consequent long path length (200 μm) of the β-emission through the emulsion (see Rogers, 1979). We therefore used ^{35}S-labelled probes which have a much shorter path length (2–5 μm) but achieve, in contrast to tritiated probes, higher specific activities resulting in acceptable exposure times (2–3 weeks compared with months when using ^{3}H). Unexpectedly, we observed a marked non-specific labelling over sections and cultured cells using ^{35}S-labelled cRNA or synthetic oligonucleotide probes (Figure 1).

Two observations suggested that the non-specific binding of ^{35}S-labelled probes was due to the replacement of an O by an S in the phosphate group of the nucleotides. First, the nonspecific labelling was independent of the size and sequence of the cRNA probes. Moreover, synthetic oligonucleotides, and even [^{35}S]αUTPs, showed the same nonspecific labelling pattern. Second, this nonspecific binding could be reduced neither by more stringent washing conditions nor by an intense RNase digestion (100 μg/ml RNase A and 10 μg/ml ribonuclease T1). We therefore concluded that the substitution of an O by an S in the phosphate groups of the nucleotides led to a change in their chemical reactivity probably resulting in a covalent binding to non-RNA macromolecules. The specificity of hybridization was increased by: (i) prehybridization of the sections and cultured cells with non-labelled thioαUTP. The use of appropriate concentrations was critical, since at high concentrations not only the nonspecific binding was reduced but the specific signal as well. The best signal to noise ratio was obtained at a concentration of 500 nmol non-labelled thioαUTP/ml; (ii) replacing dithiothreitol (DTT) with β-mercaptoethanol which has a better

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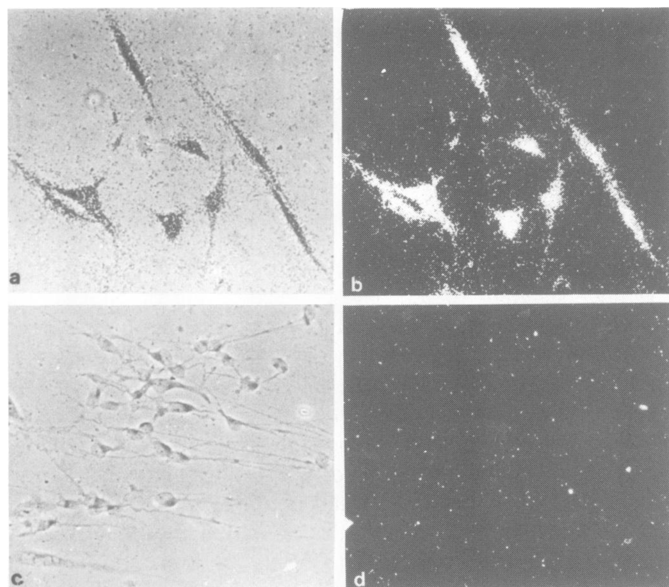

Fig 1. Phase contrast and corresponding darkfield photomicrographs showing unspecific binding of the $[^{35}S]cRNA^{NGF-}$ (control) probe on sciatic nerve cells of newborn rats cultured for 24 h, before (**a** and **b**) and after (**c** and **d**) pre-incubation with non-labelled thioαUTP (500 nmol/ml, β-mercaptoethanol (10 mM/ml) at pH 5.5. Exposure time was 10 days.

stability at higher temperatures (see CRC-Handbook). Since we hybridized at 50–55°C for at least 24 h, the instability of DTT at higher temperatures made an inactivation likely; (iii) decreasing the pH of the hybridization buffer to pH 5.5–6. According to Wetmur *et al.*, 1968 this decrease in the pH should not affect the hybridization kinetics (Figure 1).

The specificity of the hybridization signals observed under these experimental conditions was deduced from the following observations: (i) RNase pretreatment of the tissue sections and cultured cells before hybridization (100 μg/ml of RNaseA and 5 μg/ml of ribonuclease T1), as a control for nonspecific binding of the probes to molecules other than mRNA, prevented hybridization; (ii) the disappearance of signals observed over sections and single

Table I. Selection of tissues for *in situ* hybridization

Tissues		pg $mRNA^{NGF}$/ mg wet weight ± SEM[a]
Rat iris	Native	0.354 ± 0.05
	Cultured	12.4 ± 0.31
Rat sciatic nerve	Newborn	0.052 ± 5
	Adult	0.004 ± 0.001
Mouse whisker pad E13		1.1 ± 0.01

[a]SEM is derived from at least three triplicate determinations.

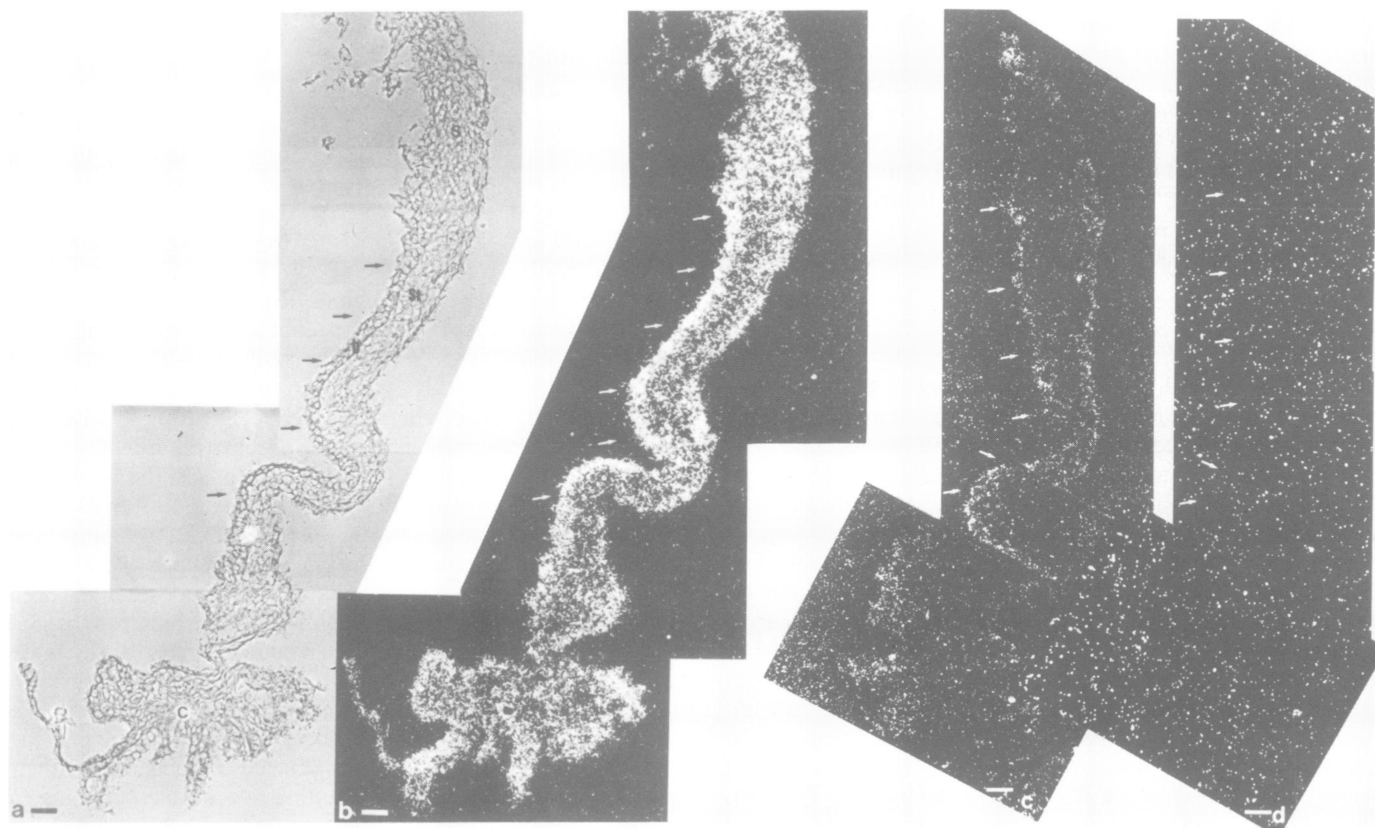

Fig. 2. Localization of $mRNA^{NGF}$ on cryostat sections of irides from adult rats, hybridized with a $[^{35}S]cRNA^{NGF+}$ probe. Exposure time was 21 days. Spacebar: 100 μm. **(a)** Phase contrast photomicrograph illustrating the two smooth muscle regions: the mainly sympathetically innervated dilator (D), a one-cell layer structure covered at the posterior side with a cuboidal epithelial cell layer (arrows), and the parasympathetically innervated sphincter (S). C = ciliary body, St = anterior stroma. **(b)** Darkfield photomicrograph of an 'induced' (12 h in culture) iris. All cells are labelled; the highest signal is over the posterior border layer (arrows); homogeneous distribution of grains over the anterior connective tissue, the sphincter and the ciliary body. **(c)** Darkfield photomicrograph of a cross section of a native iris. A specific signal is seen over the posterior and anterior border layers. **(d)** Darkfield photomicrograph illustrating the background density found over a native iris cross section hybridized with a probe of opposite polarity ($[^{35}S]cRNA^{NGF-}$).

cells was accomplished by RNaseA digest at high concentrations (50 μg/ml) after hybridization; (iii) both ^{35}S-labelled probes (cRNA^{NGF+} as well as the synthetic oligonucleotide NGF1) resulted in the same labelling-pattern whereas the corresponding control probes (cRNA^{NGF-}, NGF1A) showed no signal above background.

In agreement with previous observations (Lawrence and Singer, 1985; Griffin and Morrison, 1985), in the present experiments the choice of fixative and the time of fixation proved to be of foremost importance for the reproducible localization of mRNA molecules present at low copy numbers, as is mRNA^{NGF}. It is necessary to compromise between a good preservation of tissue morphology and an optimal retention of cellular RNA on the one hand, and the accessibility of the target RNA sequences for the corresponding hybridization probes on the other hand. We have successfully used AFE-fixation (5% acetic acid, 4% formaldehyde, 85% EtOH) for paraffin and cryostat tissue sections as well as cultured cells. In agreement with previous observations of Haase *et al.*, 1985 fixation times longer than 30 min (cultured cells and tissues) markedly reduced the hybridization signals. In cultured cells the reduction was much larger than in sections (data not shown).

Selection of tissues for in situ hybridization

Based on the available biochemical data for mRNA^{NGF} levels in peripheral target organs we have chosen the rat iris, the mouse whisker pad and the rat sciatic nerve as representative tissues for localization of mRNA^{NGF} by *in situ* hybridization (Table I).

The rat iris

The native iris. The rat iris is an organ with a dense sympathetic, sensory and parasympathetic innervation (Hedlund *et al.*, 1984) which also shows a regional difference in the distribution of NGF (Barth *et al.*, 1984) and mRNA^{NGF} levels (Shelton and Reichardt, 1986). The dilator region, which is mainly sympathetically innervated, has a 3-fold higher level of endogenous NGF than the sphincter muscle which has a predominantly cholinergic and a relatively sparse adrenergic innervation (Barth *et al.*, 1984). The differences in the NGF levels between sphincter and dilator regions are also reflected by corresponding differences in the mRNA^{NGF} levels.

In agreement with these biochemical data consistent labelling of [^{35}S]cRNA^{NGF+} was observed over the ciliary body and along the posterior and anterior border layers (Figure 2). Ultrastructural studies (Hedlund *et al.*, 1984) have demonstrated a very thin epithelial lining covering the anterior surface and a thicker, cuboidal epithelial cell layer covering the posterior side of the iris. The hybridization labelling with the [^{35}S]cRNA^{NGF+} probe is mainly restricted to these areas, indicating that the epithelial cells do synthesize mRNA^{NGF}. However, the resolution of our method was insufficient to decide whether, at the posterior side of the sections, only the epithelial cell layer is labelled or also the underlying dilator muscle (Figure 2). No detectable signal above background was seen over the predominantly parasympathetically innervated sphincter. These results are in good agreement with the biochemical data discussed above (Barth *et al.*, 1984; Heumann and Thoenen, 1986; Shelton and Reichardt, 1986). With the help of these data we calculated that there were 1−2 copies of mRNA^{NGF}/iris cell. Considering the unequal distribution of the mRNA^{NGF} it seems reasonable to assume that we can detect 10−20 copies/cell by our hybridization procedure.

The cultured iris. The endogenous NGF content of an intact iris increases up to 200-fold upon culturing (Ebendal *et al.*, 1980; Barth *et al.*, 1984), with a corresponding increase in the levels

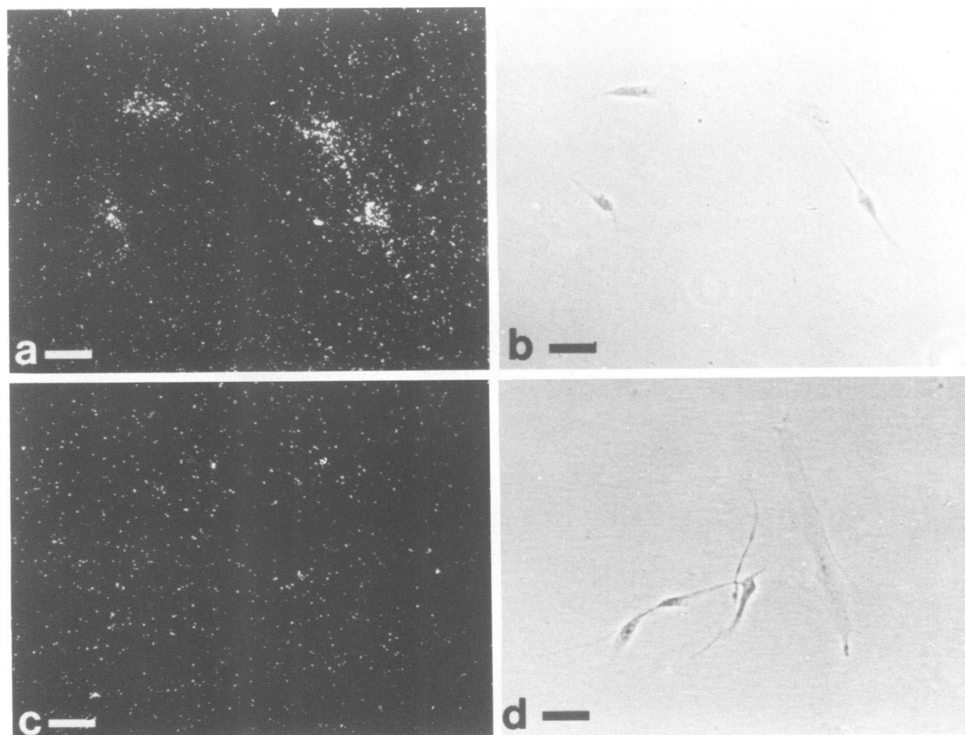

Fig. 3. Primary cultures of adult rat irides, 24 h *in vitro*. (**a** and **b**) Phase contrast and darkfield photomicrographs showing specific signals with different intensities over all cells; hybridized with the [^{35}S]cRNA^{NGF+} probe; (**c** and **d**) Phase contrast and darkfield photomicrographs of cells hybridized with the control strand [^{35}S]cRNA^{NGF-}. Exposure time was 16 days. Spacebar: 40 μm.

of $mRNA^{NGF}$ by a factor of up to 35. (Heumann and Thoenen, 1986; Shelton and Reichardt, 1986).

The increased levels of $mRNA^{NGF}$ were also reflected in the present *in situ* hybridization experiments. A very intense signal was seen all over the sections, indicating that all major cell types produce $mRNA^{NGF}$ after culturing the iris *in vitro* (Figure 2). Again the grain density was higher over the posterior layer, comprised of the cuboidal epithelial cell layer and the dilator, than over the loose anterior stroma. Moreover, the relative labelling over the sphincter was also lower, reflecting recent biochemical data demonstrating that the relative $mRNA^{NGF}$ content of the two muscle systems remained unchanged after culturing (Shelton and Reichardt, 1986).

Table II. Characterization of dissociated rat iris cells by immunohistochemistry

Specific marker	Cell type	% of total cells
Desmin	Smooth muscle cells	23
Keratin	Epithelial cells	11
ThyI	Fibroblasts	63
O_4	Schwann cells	5

Dissociated rat iris cells. Since our method did not allow us to decide the precise site of NGF synthesis within a single cell of a section, we studied $mRNA^{NGF}$ in primary cultures of rat irides. Virtually all cells were labelled by hybridization with the $[^{35}S]cRNA^{NGF+}$ probe, but with different signal intensities (Figure 3). However, it cannot be decided whether these variations of signal intensities over individual cells reflect a specific variation of $mRNA^{NGF}$ expression or result from variations in the accessibility of $mRNA^{NGF}$ sequences for the cRNA probes. Characterization of the various cell types with different antibodies (Table II) showed that 23% of the cells stained positive for desmin, a specific marker for smooth muscle cells (Debus *et al.*, 1984) but with different intensities. Up to 10% of the positive cells were heavily stained while the remaining cells showed a more moderate or even weak staining. Using ThyI, a selective marker for fibroblasts in this system (Mason and Williams, 1980), an average of 63% of the cells reacted positively. O_4, a specific marker for Schwann cells (Sommer and Schachner, 1981), stained an average of 5% of the cells, whereas keratin, a specific marker for epithelial cells (Sun and Green, 1978), stained 11% of the

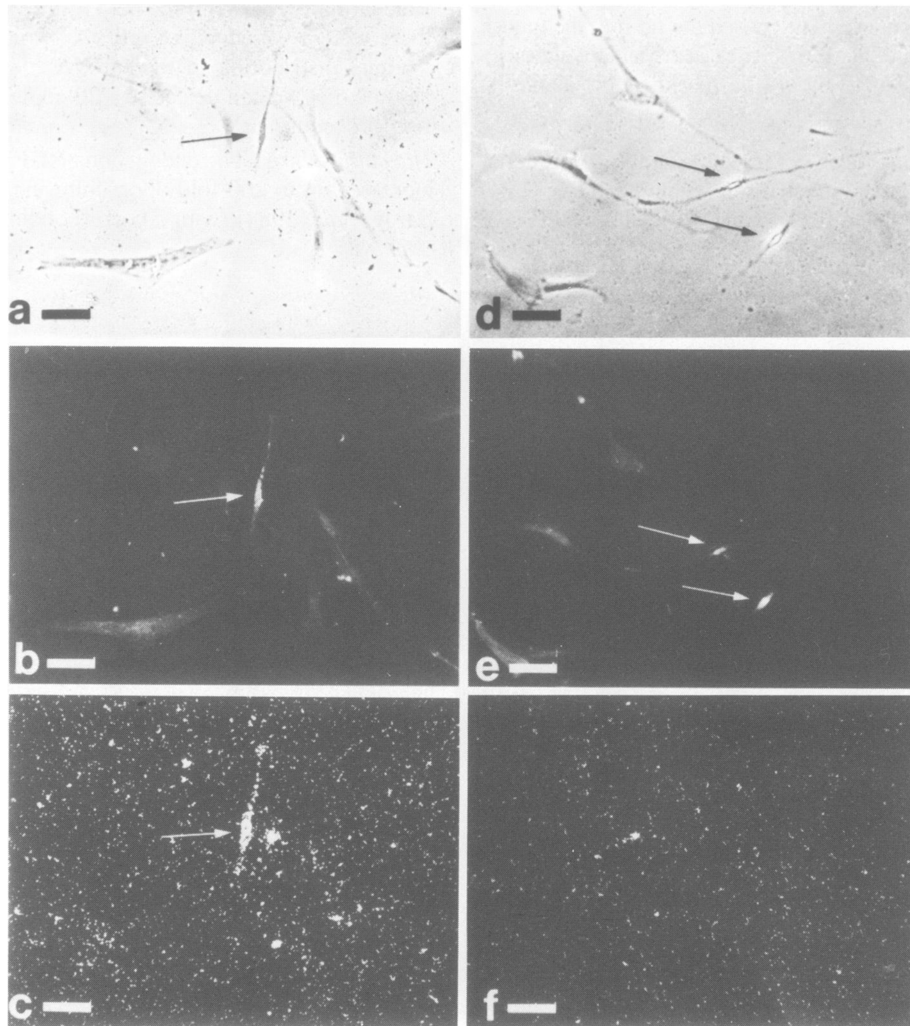

Fig. 4. Combination of autoradiography of iodinated NGF and immunohistochemical identification of dissociated rat iris cells after 48 h in culture. (**a**) Phase contrast; (**b**) corresponding indirect immunofluorescence picture of an O_4-positive cell (arrow) which is labelled by ^{125}I-NGF (arrow) as seen in the darkfield photograph (**c**). (**d–f**) Control pictures of cells incubated with an excess of non-labelled NGF. Exposure time was 5 weeks. Spacebar: 40 μm.

total cell number. Since the used antibodies require different fixation procedures for optimal staining (O_4 and ThyI on living cells, desmin and keratin on permeabilized cells) a double labelling was not possible. Nevertheless, the hybridization signals showed that all cells were labelled.

Recent results by Rush and collaborators (Rush, 1984; Finn *et al.*, 1986) demonstrated that an affinity-purified polyclonal antibody directed against mouse NGF stained cells with Schwann cell-like morphology in organ-cultured iris and 10-day-denervated iris, but not in the intact iris. Since no other cells were stained these results were interpreted as indicating NGF production predominantly or even exclusively by Schwann cells. The recent finding (Taniuchi *et al.*, 1986) that adult Schwann cells reexpress NGF receptors after denervation [in early stages of development all Schwann cells express NGF receptors (Zimmermann and Sutter, 1983; Rohrer, 1985)] suggests an alternative explanation for this observation. NGF, produced by both Schwann cells and other cells, could be bound by the Schwann cell NGF receptors (or even be accumulated by internalization), thereby resulting in detectable staining with anti-NGF antibodies of Schwann cells only. Our observation that binding of radioiodinated NGF ($[^{125}I]$NGF) to dissociated rat iris cells occurs exclusively on Schwann cells (O_4 positive) (Figure 4) supports this hypothesis.

Identification and localization of mRNA^{NGF} in the mouse whisker pad

The developing mouse whisker pad was used as a characteristic example of an organ with a dense and exclusively sensory innervation. Emerging fibers from the trigeminal ganglion reach their maximal density of target-field contact at E13 (Davies and Lumsden, 1984). Recent findings suggest that the commencement of target-field innervation is correlated with the onset of mRNA^{NGF} synthesis (Davies *et al.*, 1987). Therefore the whisker pad seemed to be an appropriate tissue in order to investigate the cellular localization of mRNA^{NGF}. Hybridizing sections with $[^{35}S]$cRNA^{NGF+} showed a higher labelling of the epithelium and its adjacent mesenchyme than of the central mesenchyme. The silver grain density was equally distributed over both the surface and follicular epithelium with their immediately adjacent mesenchyme (Figure 5). This observation further supports the concept that the synthesis of NGF takes place predominantly in the target tissues of NGF-responsive neurons and certainly not exclusively by Schwann cells ensheathing them (see below).

Identification and localization of NGF-specific mRNA in the sciatic nerve of newborn rats

The rat sciatic nerve contains fibers of both NGF responsive

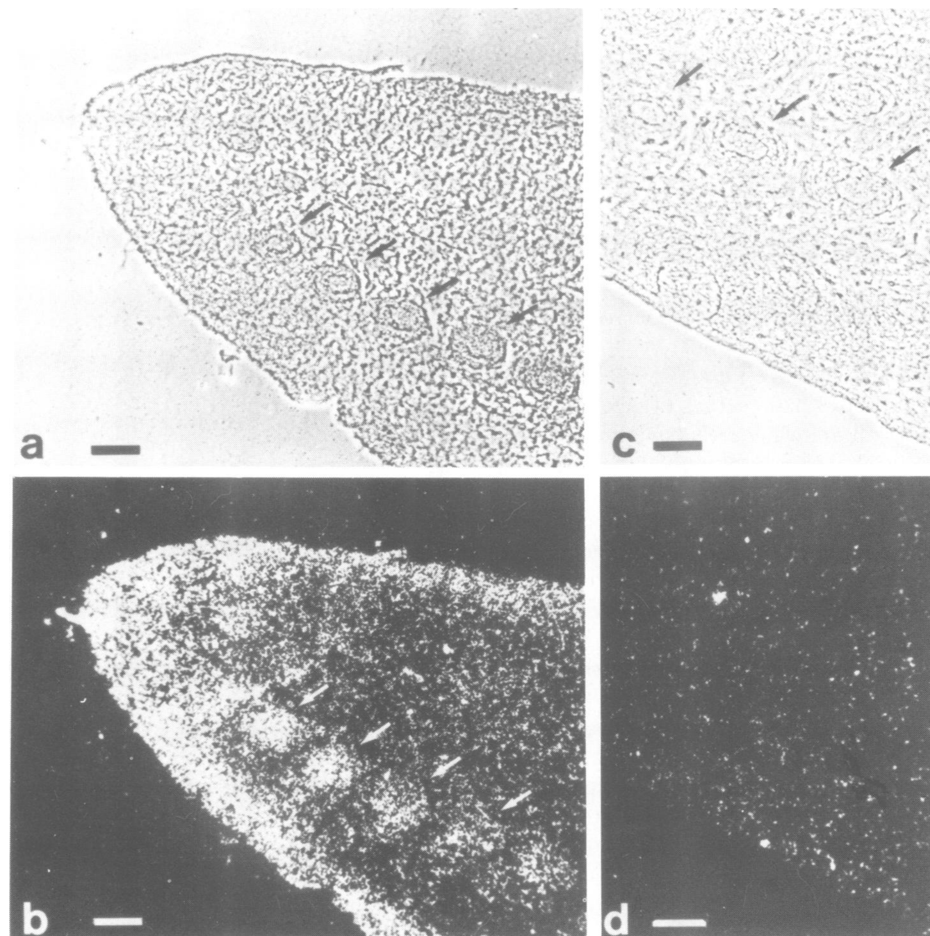

Fig. 5. Localization of mRNA^{NGF} over a paraffin section through a mouse whisker pad at E13. Spacebar: 100 μm. (**a** and **b**) Phase contrast and darkfield photomicrographs of a section passing a row of whisker follicles (arrows); hybridized with the $[^{35}S]$cRNA^{NGF+} probe. Both the follicular and the surface epithelium with their adjacent mesenchyme are densely labelled, whereas the central mesenchyme shows moderate labelling throughout. (**c** and **d**) Corresponding control sections. Exposure time was 20 days.

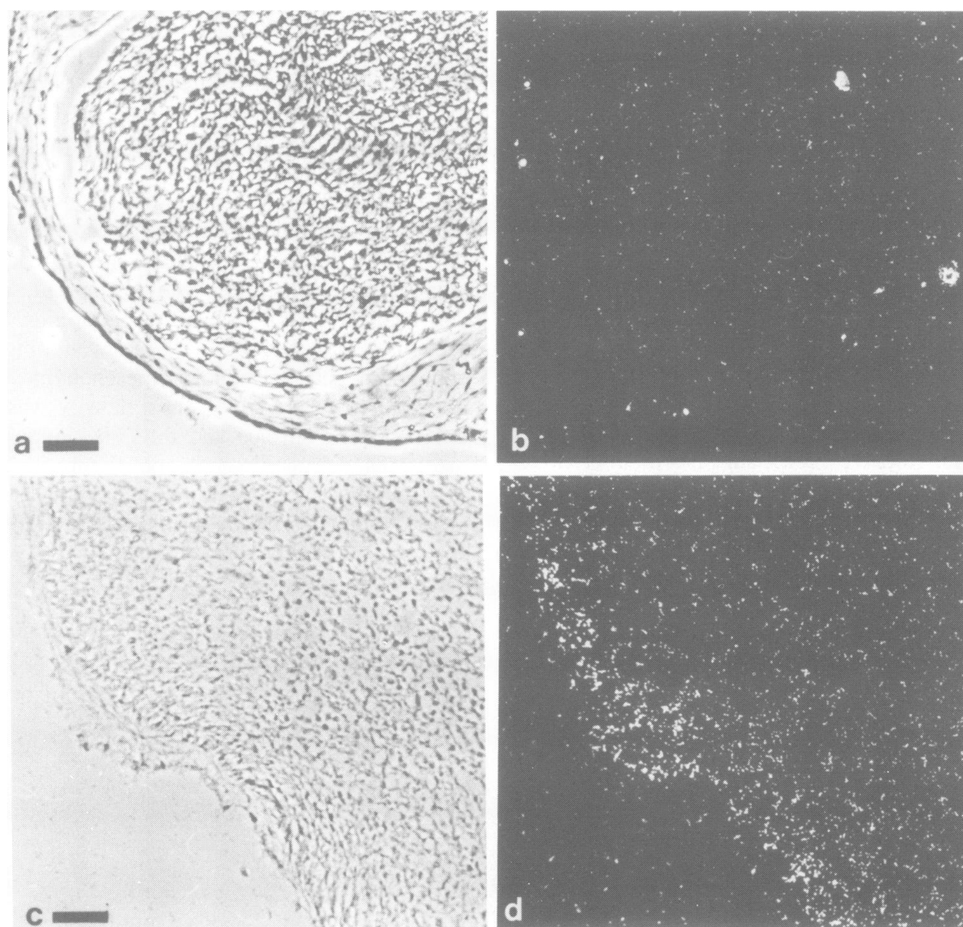

Fig. 6. Phase contrast and darkfield photomicrographs of *in situ* hybridization of $mRNA^{NGF}$ in a cross section of sciatic nerve of adult rats (**a** and **b**) showing no labelling above background with the $[^{35}S]cRNA^{NGF+}$ probe and sciatic nerve of newborn animals (**c** and **d**) showing uniform labelling throughout the section with a denser labelling over the epineurium. Exposure time was 18 days. Spacebar: 100 μm.

(sympathetic and sensory) and NGF non-responsive (motor) neurons. In newborn animals the levels of $mRNA^{NGF}$ are about 15× higher than in adult animals. We therefore analysed sections as well as cultured cells of sciatic nerves of newborn rats, in order to decide whether all Schwann cells have the capacity to synthesize NGF or only those ensheathing sympathetic and sensory fibers.

In contrast to sections of adult animals, intense signals were detectable over sections of newborn animals, hybridized under the same conditions; the highest signal intensity occurring over the epineurium (Figure 6). Since the labelling over the sections did not show any patch-pattern we concluded that all Schwann cells, not only those ensheathing sympathetic and sensory fibers (representing 20–40% of total fibers) produce $mRNA^{NGF}$.

This interpretation is in agreement with observations in dissociated cells of the newborn sciatic nerve. Primary cultures of sciatic nerves were seeded on laminin-coated dishes. On this substrate Schwann cells attach and differentiate within 1 h after plating (McGarvey, 1984). Therefore after 1 h the medium was changed to remove any non-attached cells thereby enriching for Schwann cells. The cultures consisted of 95% Schwann cells (0_4-positive) and 5% fibroblasts (ThyI-positive). We looked after 4, 12 and 24 h in culture for the presence of $mRNA^{NGF}$. A specific signal after hybridization with $cRNA^{NGF+}$ was observed at all time points over both Schwann cells and

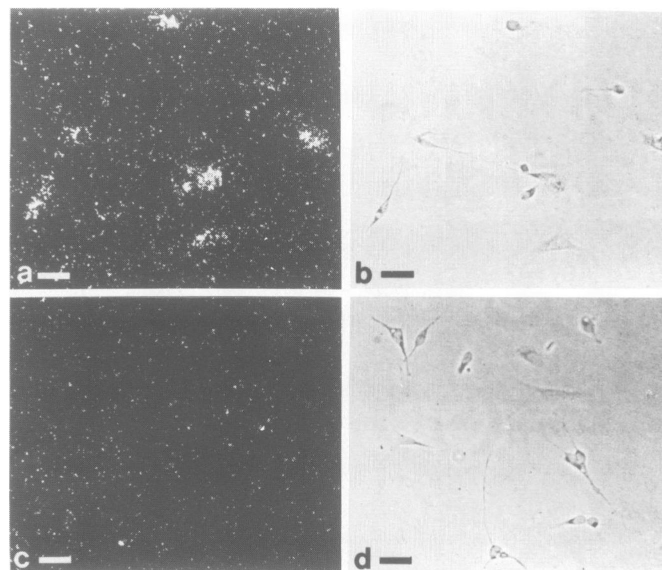

Fig. 7. Phase contrast and corresponding darkfield photomicrographs illustrating the localization of $mRNA^{NGF}$ in primary cultures of sciatic nerve cells of newborn rats after 12 h *in vitro*, hybridized with the $[^{35}S]cRNA^{NGF+}$ probe (**a** and **b**) and the control probe $[^{35}S]cRNA^{NGF-}$ (**c** and **d**). Exposure time was 18 days. Spacebar: 40μm.

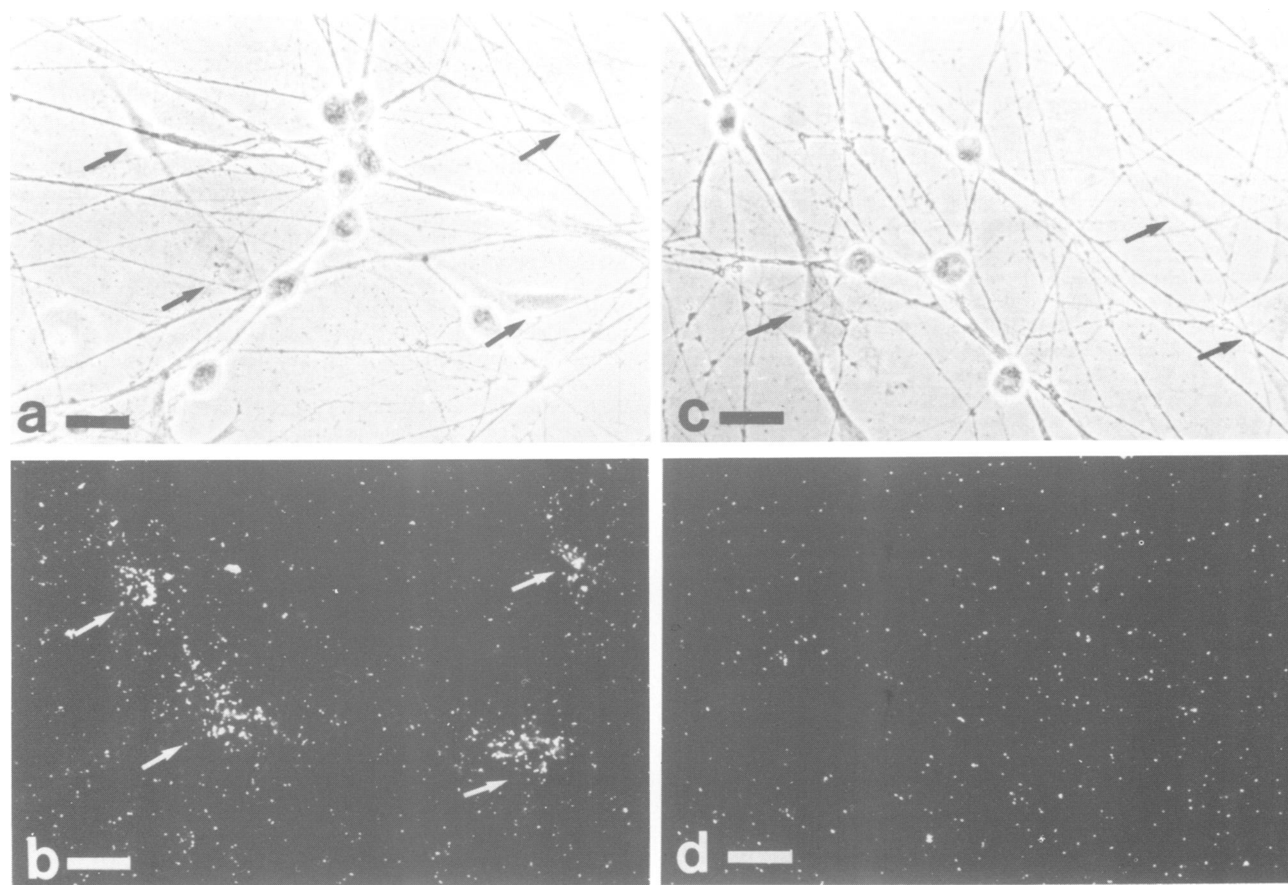

Fig. 8. Phase contrast and darkfield photomicrographs of primary cultures of rat SCG. Notice that only the non-neuronal cells are labelled (arrows) (**a** and **b**); no labelling of cells with the control strand (**c** and **d**).. Exposure time 18 days. Spacebar: 40 μm.

fibroblasts, with the highest signal intensity over all cells after 12 h in culture (Figure 7), indicating that both Schwann cells and fibroblasts express $mRNA^{NGF}$ even after short periods in culture. These observations suggest that dissociated Schwann cells, even those ensheathing motor-fibers, synthesize $mRNA^{NGF}$ in sciatic nerves of newborn animals but decrease the NGF expression when they turn into a quiescent state. As discussed above the expression of $mRNA^{NGF}$ in sciatic nerves of adult rats was below the detection limits of our method. However, as recently shown, a very rapid increase of $mRNA^{NGF}$ occurs after axotomy (Korsching *et al.*, 1986).

Primary cultures of neuronal and non-neuronal cells of rat superior cervical ganglia

In contrast to the primary cultures of non-neuronal cells, superior cervical ganglia (SCG) of newborn rats contain predominantly NGF-responsive sympathetic neurons, as well as Schwann cells and fibroblasts. These neurons were not labelled by hybridization with the $[^{35}S]cRNA^{NGF+}$ probe whereas all other non-neuronal cells showed a specific signal (Figure 8). These results not only showed that sympathetic neurons only respond to NGF and do not synthesize $mRNA^{NGF}$, they also support recent findings that the very high levels of NGF in SCGs result from retrograde axonal transport (Heumann *et al.*, 1984; Korsching and Thoenen, 1985). Moreover, they proved the specificity of the method.

Materials and methods

Animals and cell culture

Preparation of iris organ cultures. Organ cultures of adult rat iris were performed according to Barth *et al.* (1984).

Preparation of dissociated Iris cells. Wistar rats (150–200 g, both sexes) were killed by decapitation, and the irides were dissected and collected in PBS/glucose. 8–10 irides were incubated in 1 ml of 0.1% Collagenase (Worthington, 205 U/mg) in CMF–PBS (Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline) for 30 min at 37°C, followed by an additional 10 min in 0.1% collagenase/0.1% trypsin (Worthington, 245 U/mg). The enzymatic reaction was terminated by adding 100 μl/ml rat serum. Before trituration with a fire polished Pasteur pipette the tissue was washed in PBS. Triturated cells were passed through a 0.2 μm gauge mesh and plated at a density of 12.0 cells/cm^2 on a plastic Greiner 4-well dish. The cells were cultured in Dulbecco's modified Eagle's (DMEM)H21 medium with 10% rat serum and 1% penicillin/streptomycin at 37°C with 10% CO_2 in a water-saturated atmosphere.

Preparation of dissociated sciatic nerve cells. Newborn Wistar rats were killed by decapitation, the sciatic nerves were dissected and collected in PBS/glucose. The nerves were carefully freed from adhering connective tissue. Nerves of five animals were digested in the same way as the irides. The cells were plated on laminin-coated (5 μg/ml) 4-well Greiner dishes at a density of 50 000 cells/cm^2 and kept in culture for various time periods (4–72 h) in DMEM H21 with 10% fetal calf serum. (The laminin was a kind gift of R.Timpl, Max-Planck-Institute for Biochemistry, Martinsried.)

Preparation of primary cultures of superior cervical ganglia from newborn rats. Rat SCGs were dissected and processed as previously reported (Schwab and Thoenen, 1985). Dissociated cells were plated on laminin-coated (5 μg/ml) Greiner dishes at a density of 10 000 cells/cm^2. The cultures were grown in an enriched L15 medium (Mains and Patterson, 1973) with 10% FCS, penicillin (100 μg/ml)

and streptomycin (100 μg/ml), and 50 ng/ml of 2.5S NGF. The culture period varied from 2 to 3 days.

In situ hybridization

Pretreatment of glass slides. Treatment of the glass microscope slides (76 × 26 mm) with TESPA (3-aminopropyl-triethoxysilan, Fluka) followed by activation with paraformaldehyde (Fluka), according to Berger (1986), resulted in satisfactory adhesion of the sections, whereas gelatine or 3 × Denhardt's (1 × Denhardt's: 0.02% each of Ficoll, bovine serum albumin and polyvinylpyrrolidon) did not result in a sufficient attachment of the sections in the subsequent steps of the procedure.

Preparation of tissue sections. Adult (150–200 g) and newborn male Wistar rats were killed by decapitation. Irides and sciatic nerves were dissected and fixed by immersion in AFE (5% acetic acid, 4% formaldehyde, 85% ETOH) for 30–40 min before being placed in PBS containing 15% sucrose and stored overnight at 4°C. Whisker pads were dissected from late E13 mice embryos, fixed and processed under the same conditions. Although paraffin embedding results in a better preservation of the tissue morphology, it also causes tissue shrinkage, a major disadvantage when working with small and fragile tissues like the iris. We therefore embedded the iris in 11% gelatin/PBS and prepared 5–10 μm cryostat sections. All other fixed organs were dehydrated in ethanol and embedded in paraffin. 5–10 μm sections were collected on pretreated glass slides and processed through conventional xylene and ethanol steps before pretreatment for *in situ* hybridization.

Pretreatment of sections and cultured cells. To remove basic proteins which may bind nucleotides non-specifically, sections as well as cultured cells were treated with 0.2 N HCl for 20 min at room temperature. The slides were washed twice with diethylpyrocarbonate-treated H_20, then submerged in 2 × SSC buffer for 30 min at 70°C (1 × SSC: 150 mM NaCl, 15 mM Na-citrate, pH 3.5). Thereafter the slides were incubated in a freshly made Proteinase K (Boehringer, Mannheim) solution (1 μg/ml in 20 mM Tris–HCl pH 7.4/2 mM $CaCl_2$) for 15 min at 37°C. The Proteinase K reaction was terminated by rinsing the slides with an excess of PBS containing 2 mg/ml glycine, followed by a wash in PBS. At this point controls were treated with 100 μg/ml pancreatic and 5 μg/ml T1 ribonuclease (Bethesda Research Laboratory) in PBS for 1 h at 37°C. The slides were finally post-fixed in 4% paraformaldehyde/PBS for 15 min at room temperature, thereafter washed for 5 min each in 3 × PBS, then twice in PBS.

Prehybridization. Sections and cultured cells were prehybridized with a solution containing 50% deionized formamide, 0.9 M NaCl, 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 5 × Denhardt's, 20 mM β-mercaptoethanol, 250 μg/ml yeast tRNA (Sigma), 500 μg/ml salmon sperm DNA (Sigma), 30 μmol/ml non-labelled thioαUTP (New England Nuclear). In some cases the carrier DNA was substituted with 500 μg/ml heparin (Singh and Jones, 1984). The pH was adjusted to 5.5–6.

Labelling of cRNA probes. The *Pst*I-cut insert of the NGF cDNA (Scott *et al.*, 1983) was cloned into the *Pst*I site of the plasmid SP6 (Promega). Run-off transcripts of both template orientations (RNA^{NGF+}, RNA^{NGF-}) were synthesized using the SP6 polymerase (NEN) to achieve asymmetric RNA probes with a specific activity of $1-4 \times 10^8$ c.p.m./μg and a length of 160–180 bp. The reaction containing 0.04 mM [^{35}S]αUTP (650 Ci/mmol; Amersham) was done by a previously described protocol (Heumann, 1984).

Oligonucleotides

An oligodeoxyribonucleotide (18 mer, NGFl) was synthesized on an applied Biosysthems 380 A synthesizer and purified by reverse phase h.p.l.c. on a C18 column. The selected sequence was derived from the carboxyterminal sequence of the mouse NGF cDNA clone (Scott *et al.*, 1983) with a high homology to human, bovine and chick NGF (Meier *et al.*, 1986). As a negative control a synthetic oligonucleotide with complementary sequence (NGF 1A) was used. These oligonucleotides were 5'-end labelled using T4 polynucleotide kinase (Boehringer, Mannheim) and γ-[^{35}S]ATP (NEN) (Maniatis, 1982), and further purified by Sephadex G50 spin column chromatography to a specific activity of $1-2 \times 10^8$ c.p.m./μg.

Hybridization with single-stranded RNA probes

Following precipitation, the run-off transcripts were pelleted by centrifugation in a Hettich Eppendorf microfuge for 15 min and the pellet was washed 2× with 70% EtOH at room temperature. The dried pellets were resuspended in the above described prehybridization buffer without adding non-labelled thioαUTP to achieve a final probe concentration of 2 ng/ml. The labelled probes were boiled in the hybridization mixture for 1 min and then chilled on ice. 5–10 μl of the solution were applied to each slide and covered with a siliconized 20 × 20 mm coverslip (10 mm for cultures). To prevent evaporation of the hybridization solution the edges of the coverslip were sealed with rubber cement (Marabuwerke/Tamm, FRG). The slides and culture dishes were placed in a humid chamber and incubated at 55°C for 24–36 h. Thereafter, the rubber cement was removed and the coverslips were dislodged by floating in a solution containing 0.6 M NaCl/10 mM Tris pH 7.5/1 mM EDTA/10 mM β-mercaptoethanol. The slides were subsequently washed in the same buffer at 50°C for 2–4 h followed by a RNaseA digest (10 μg/ml in 0.5 M NaCl/10 mM Tris pH 7.5/1 mM EDTA) at 37°C for 15 min to achieve a better signal to noise ratio. As a final step the slides were washed in 0.03 M NaCl/10 mM Tris pH 7.5/1 mM EDTA/10 mM β-mercaptoethanol at 60°C for 4–6 h, followed by dehydration with increasing concentrations of EtOH. The slides were finally air dried.

Hybridization conditions for the synthetic oligonucleotides were the same with the following exceptions: hybridization was performed at 4°C overnight, followed by washing steps at 50°C in 0.3 M NaCl/10 mM Tris pH 7.5/1 mM EDTA/10 mM β-mercaptoethanol for 12 h and in 0.03 M NaCl/10 mM Tris pH 7.5/1 mM EDTA/10 mM β-mercaptoethanol for 2–4 h.

For autoradiography the slides were dipped in a 1:1 mixture of tap water and NTB-2 Kodak emulsion, and melted at 42°C. The emulsion-coated slides were dried and exposed at 4°C for 10–22 days. Development was done in Kodak D19 for 2 min. After rinsing in tap water the fixation was carried out in 24% sodium thiosulfate for 5 min followed by an extensive wash in tap water. After the slides were air dried they were embedded in Entellan (Merck, Darmstadt) under a glass coverslip, and examined under the light microscope.

Immunohistochemistry

Immunohistochemical analysis was performed on dissociated cells from adult rat irides. The antibodies used were as follows: (i) a mouse monoclonal antibody against Desmin (Boehringer, Mannheim), a specific marker for smooth muscle cells; (ii) a mouse monoclonal antibody against ThyI (Biochrom AG, Berlin), a selective marker for fibroblasts; (iii) a mouse monoclonal O_4 (generously provided by M.Schachner, Heidelberg) a specific marker for Schwann cells; (v) a mouse monoclonal antibody against keratin (Camon, Wiesbaden), a specific marker for epithelial cells. The antibodies O_4 and ThyI (both diluted 1:20) were applied on living cultures for 30 min at room temperature before fixation with AFE for 10 min. For all other antibodies (at various dilutions) the cultures had to be permeabilized by treatment with ice-cold methanol for 15 min at −20°C prior to antibody incubation. All cultures were incubated for 30 min at room temperature with fluorescein isothiocyanate-labelled sheep anti-mouse immunoglobulins (Gibco) (diluted 1:100) as a second antibody. The cultures were examined under a Zeiss fluorescence microscope.

NGF binding and autoradiography

Primary cultures of rat iris cells were processed according to Rohrer and Barde (1982) with minor modifications. Briefly, cells were washed with a modified Krebs–Ringer solution supplemented with 1% BSA (KRH/B), then incubated for 1 h at 37°C in KRH/B solution containing 10 ng/ml iodinated NGF and O_4-antibodies diluted 1:20. Control incubations also contained 10 μg/ml unlabelled NGF. After the incubation cells were washed in KRH/B solution, fixed for 1 h in 2.5% glutaraldehyde and incubated for a further 60 min at room temperature with fluorescine isothiocyanate-labelled sheep anti-mouse immunoglobulins (diluted 1:100) as a second antibody, before being processed for autoradiography.

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References

Barth,E.-M., Korsching,S. and Thoenen,H. (1984) *J. Cell Biol.*, **99**, 839–843.

Berger,C.N. (1986) *EMBO J.*, **5**, 85–93.

CRC, Handbook of Tables for Organic Compound Identification (1974) 3rd ed., CRC Press.

Davies,A.M and Lumsden,A.G.S. (1984) *J. Comp. Neurol.*, **223**, 124–137.

Davies,A.M., Bandtlow,C.E., Heumann,R., Korsching,S., Rohrer,H. and Thoenen,H. (1987) *Nature*, in press.

Debus,E., Weber,K. and Osborn,M. (1983) *EMBO J.*, **12**, 2305–2312.

Ebendal,T., Olson,L., Seiger,A. and Hedlund K. (1980) *Nature*, **286**, 25–28.

Finn,P.J., Ferguson,L.A., Renton,F.J. and Rush,R.A. (1986) *J. Neurocytol.*, **15**, 169–176.

Gnahn,H., Hefti,F., Heumann,R., Schwab,M.E. and Thoenen,H. (1983) *Dev. Brain. Res.*, **9**, 45–52.

Greene,L.A. and Shooter,E.M. (1980) *Annu. Rev. Neurosci.*, **3**, 353–402.

Griffin,W.S.T. and Morrison,M.R. (1985) *Peptides*, **6**, Suppl.2, 89–96.

Haase,A.T., Brahic,X., Strowing,L. and Blum,F. (1984) In Maramorosch,K. (ed.) *Methods in Virology*. Academic Press, Orlando, FL, Vol. 7, pp.189–226.

Hedlund,K.-O., Ayer-Lelievre,C., Brörklund,H., Hultgren,L. and Seiger,A. (1984) *J. Neurocytol.*, **13**, 703–725.

Hendry,I.A. (1980) In Bradshaw,R.A. and Schneider,D.M. (eds), *Proteins of the Nervous System*. Raven Press, New York, pp.183–211.

Heumann,R., Korsching,S., Scott,J. and Thoenen,H. (1984) *EMBO J.*, **3**, 3183–3189.
Heumann,R. and Thoenen,H. (1986) *J. Biol. Chem.*, **261**, 9246–9249.
Korsching,S. and Thoenen,H. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3513–3516.
Korsching,S. and Thoenen,H. (1985) *J. Neurosci.*, **5**, 1058–1061.
Korsching,S., Auburger,G., Heumann,R., Scott,J. and Thoenen,H. (1985) *EMBO J.*, **4**, 1389–1393.
Korsching,S., Davies,A., Heumann,R. and Thoenen,H. (1986) *Neurosci. Abstr.*, **12**, 1096.
Lawrence,J.B. and Singer,R.H. (1985) *Nucleic Acids Res.*, **13**, 1777–1799.
Levi-Montalcini,R. and Angeletti,P.U. (1968) *Physiol. Rev.*, **48**, 534–569.
Mains,R.E. and Patterson,P.H. (1973) *J. Cell Biol.*, **59**, 329–345.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, N.Y.
Mason,D.W. and Williams,A.F. (1980) *J. Biomed.*, **187**, 1–20.
McGarvey,M.L., Baron-Van Evercooren,A., Kleinmann,H.K. and Dobois-Dalcq,M. (1984) *Dev. Biol.*, **105**, 18–28.
Meier,R., Becker-André,M., Heumann,R. and Thoenen, H. (1986) *EMBO J.*, **7**, 1489–1493.
Mobley,W.C., Rutkofski,J.L., Tennekoon,G.I., Buchanan,K. and Johnston,M.V. (1985) *Science*, **229**, 284–287.
Oppenheim,R.W. (1981) in Cowan,W.M. (ed.), *Studies in Developmental Biology*. Oxford University Press, pp. 74–133.
Rogers,A.W. (ed.) (1979) *Techniques of Autoradiography*. Biomedical Press, Amsterdam.
Rohrer,H. and Barde,Y.-A. (1982) *Dev. Biol.*, **89**, 309–315.
Rohrer,H. (1985) *Dev. Biol.*, **111**, 95–107.
Rush,R.A. (1984) *Nature*, **312**, 364–367.
Schwab,M.E. and Thoenen,H. (1983) In Lajtha,A. (ed.) *Handbook of Neurochemistry*, Vol.5, pp.381–404.
Schwab,M.E. and Thoenen,H. (1985) *J. Neurosci.*, **5**, 2415–2423.
Scott,J., Selby,M.J., Urdea,J., Quiroja,J., Bell,G.J. and Rutter, W.J. (1983) *Nature*, **302**, 538–540.
Shelton,D.L. and Reichardt,L.F. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7951–7955.
Shelton,D.L. and Reichardt,L.F. (1986) *J. Cell Biol.*, **102**, 1940–1948.
Singh,L. and Jones,K.W. (1984) *Nucleic Acids Res.*, **12**, 5627–5638.
Sommer,I. and Schachner,M. (1981) *Dev. Biol.*, **83**, 311–327.
Sun,T.-T. and Green,H. (1978) *Cell*, **14**, 469–476.
Taniuchi,M., Clark,H.B. and Johnson,E.M.,Jr (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 4094–4098.
Thoenen,H. and Barde,Y.-A. (1980) *Physiol. Rev.*, **60**, 1284–1335.
Wetmur,J.G. and Davidson,N. (1968) *J. Mol. Biol.*, **31**, 349–370.
Zimmermann,A. and Sutter,A. (1983) *EMBO J.*, **2**, 879–885.

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