

Gene Expression in Differentiating and Transdifferentiating Neural Crest Cells

D.J. ANDERSON,* R. STEIN, AND R. AXEL

Howard Hughes Medical Institute, Columbia University College of Physicians and Surgeons, New York, New York 10032

The neural crest is a transient population of cells that detach from the top of the neural tube and then disperse throughout the embryo giving rise to the peripheral nervous system, melanocytes, and the adrenal medulla, as well as a diverse array of other cell types. Transplantation experiments suggest that most premigratory cells throughout the neural crest have the same developmental potential, regardless of their actual fate (LeDouarin 1982; Weston 1982.) Thus, the environment encountered by crest cells during or after migration appears to be critical in determining their final phenotype.

One neural crest developmental decision that shows considerable environmental influence is the choice between an endocrine and a neuronal phenotype. Both adrenergic endocrine cells (chromaffin cells) of the adrenal medulla and noradrenergic neurons of the paravertebral sympathetic ganglia derive from the so-called sympathoadrenal (Landis and Patterson 1981) region of the crest, which originates in the caudal thoracic region (LeDouarin 1982). Transplantation experiments have shown that when this region is removed and replaced by a graft of more rostrally originating crest, histologically distinguishable donor-derived chromaffin cells develop in the adrenal medulla, despite the fact that the donor crest normally never gives rise to these cells but only to neurons (LeDouarin 1980). Thus, the embryonic environment ventral to the adrenomedullary crest appears to elicit the chromaffin phenotype from cells that migrate into it.

The effect of environment on the sympathoadrenal decision is apparent not only during development, but also in the "plasticity" of the fully differentiated derivatives. Thus, chromaffin cells, although expressing a terminally differentiated phenotype, retain the capacity to transdifferentiate into neurons both in vitro and in vivo, when exposed to nerve growth factor (NGF) (Unsicker et al. 1978; Aloe and Levi-Montalcini 1979; Doupe et al. 1985a,b). The in vitro conversion to a neural phenotype is blocked by glucocorticoids, suggesting that maintenance of the chromaffin phenotype may be a consequence of the ultimate location of the adrenal medullary cells, which are surrounded by adrenal cortical cells expressing large quantities of glucocorticoids (Unsicker et al. 1978). Taken together, these and other observations have suggested that NGF

and glucocorticoids may play opposing roles in promoting the neural and chromaffin phenotypes, respectively, from a common precursor cell (Landis and Patterson 1981; Doupe et al. 1985b). This developmental decision therefore provides an opportunity to understand the molecular mechanisms that underlie the expression of alternative phenotypes by a multipotential precursor, and the subsequent plasticity of these phenotypes, in response to defined environmental signals.

We have isolated cDNA clones for several mRNAs that are abundant in adult sympathetic neurons but not in adrenal chromaffin cells. Using RNA blot and in situ hybridization, we have analyzed the pattern of emergence of these messengers in embryonic development and their plasticity in cell culture. We find that these neural-specific genes are not synchronously induced but rather appear at recognizable stages in neurogenesis. Moreover, one of the genes is induced by NGF in PC12 cells and in adrenal medullary primary cultures, suggesting that the hormone may control the transcriptional activation of this gene early in development. The results suggest a mechanism in which the developmental history of some of these neural-specific genes could account for their plasticity in mature chromaffin cells.

RESULTS

The Appearance of Neural-specific mRNAs during Development

We have isolated several cDNA clones encoding mRNA transcripts expressed in ganglionic neurons but not in adrenal chromaffin cells by performing differential hybridization to a rat sympathetic ganglion cDNA library. The tissue-specificity of these clones was established by both Northern blot analysis and in situ hybridization. Clones SCG4, SCG5, and SCG10 detect abundant RNA species in both sympathetic ganglia and brain, which are either absent or detectable at much lower levels in the adrenal medulla and in nonneural tissues (Fig. 1). In situ hybridization to sections and cultures of superior cervical ganglion, using single-stranded RNA probes (Cox et al. 1984), revealed grain accumulations over the cell bodies of principal neurons, but not over the nonneural cells within the ganglion (data not shown). Consistent with the Northern analysis, no hybridization was observed to sections of adrenal chromaffin cells (Anderson and Axel 1985).

*Present address: Division of Biology, California Institute of Technology, Pasadena, California 91125.

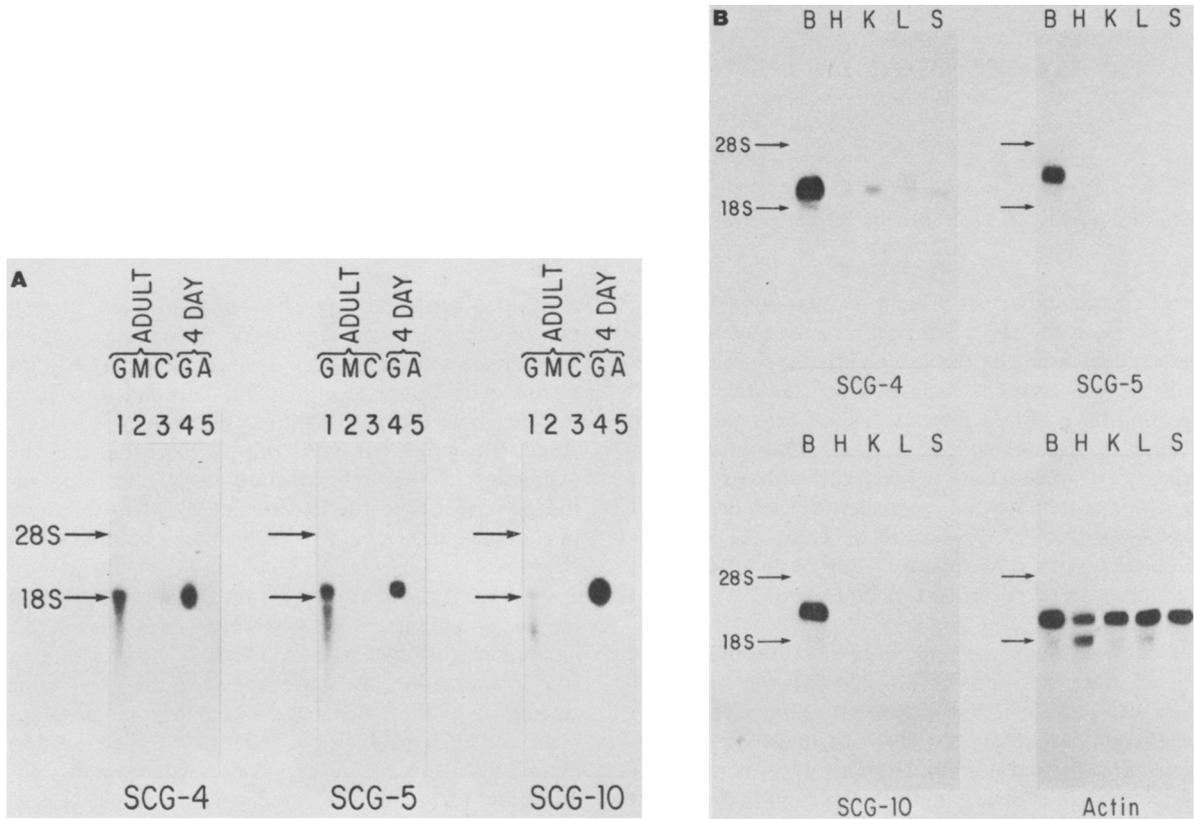


Figure 1. Tissue-specificity of clones SCG4, SCG5, and SCG10 expression. (A) Northern blots containing 10 μ g/lane of total RNA from adult SCG (G, lanes 1), adrenal medulla (M, lanes 2), adrenal cortex (C, lanes 3) or 4-day postnatal SCG (G, lanes 4), and whole adrenal (A, lanes 5) were hybridized with nick-translated probes as indicated below the panels. (B) As in A, except the lanes contained RNA from 6-day postnatal tissues: (B) brain; (H) heart; (K) kidney; (L) liver; (S) spleen. The probes used are indicated below the panels; "Actin" was a human β -actin probe that detects a smaller cardiac actin transcription in the heart RNA. All autoradiograms represent overnight exposures; we estimate the relative abundance of SCG4 and SCG10 in 4-day ganglia as 0.25 and 0.6%, respectively.

The availability of neuron-specific clones that detect abundant mRNAs now permitted us to relate the appearance of neural-specific mRNAs to the migratory events in neural crest differentiation. In the rat, neural crest cell migration begins around the ninth day of gestation (E9), proceeds along the embryo in a rostro-caudal gradient, and ends about E11.5–E12. At this stage, the sympathetic ganglia primordia have largely formed and consist of collections of primitive neuroblasts that continue to divide throughout gestation, enlarging the ganglia (Hendry 1977; Rothman et al. 1978). The formation of the adrenal medulla occurs somewhat later via a secondary ventral migration of crest cells from the caudal thoracic region to a position above the kidney tubules where the cells coalesce with the mesodermally derived cortical cells. This secondary migration occurs beginning around E13 and continues until birth. A discrete adrenal medulla is visible by E17.5 (Bohn et al. 1981; Teitelman et al. 1982).

In initial experiments we examined the developmental appearance of the neural-specific RNAs by performing Northern blot analyses on total embryo RNA. SCG5 mRNA is barely detectable between E10.5 and

E13.5, but undergoes at least a 20-fold induction at birth. Thus, significant levels of SCG5 mRNA do not appear to accumulate in sympathetic neurons or the CNS until long after the cessation of neural crest migration (Anderson and Axel 1985). In contrast to SCG5 mRNA, SCG4 and SCG10 mRNAs were both detectable at significant levels in midgestational embryos, although their patterns of expression during development are distinct (Fig. 2). SCG10 mRNA is barely detectable at E10.5, but undergoes a 30-fold induction over the next 48 hours (Fig. 2A). During this period, SCG4 is expressed at fairly constant levels but then declines significantly beginning on about E13.5 (Fig. 2B).

Regulated Expression of SCG10

The temporal appearance of SCG10 mRNA suggests that this gene is not expressed at high levels in the neural crest, but rather is induced in crest cells following migration and ganglion formation. To confirm this interpretation, we performed *in situ* hybridizations with an SCG10 probe to embryo sections at various stages of development. In very caudal sections of E11.5 em-

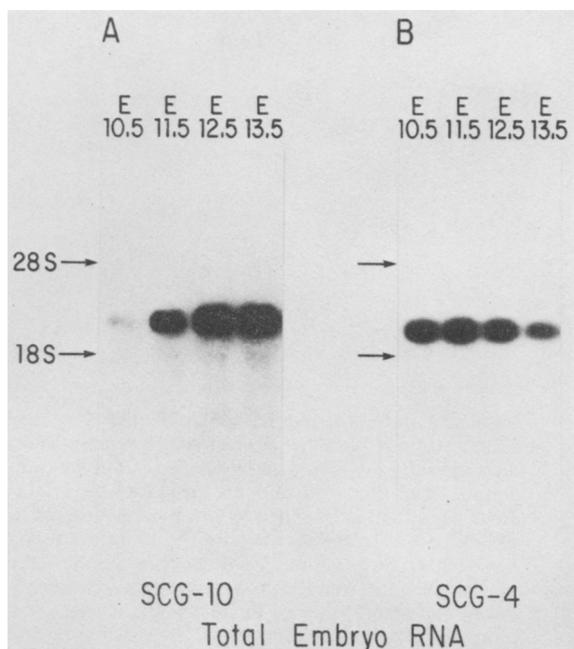


Figure 2. Early expression of SCG10 and SCG4 mRNAs. Total cellular RNA (10 μ g) isolated from embryos of the stages indicated was subjected to Northern analysis as described in Methods. Blots were hybridized with 32 P-labeled SP6 RNA probes labeled to a specific activity of 4×10^9 dpm/ μ g. Blots A and B were exposed for different times and therefore the signal intensities are not directly comparable. (A) SCG10 probe; (B) SCG4 probe.

bryos, significant hybridization is observed to the neurons of the ventrolateral neural tube, but not to cells in the migrating neural crest (Fig. 3D). In more rostral sections of the same embryo (Fig. 3B,C), much higher grain densities were apparent in the ventral and lateral margins of the neural tube, suggesting that expression of SCG10 mRNA occurs in the neural tube in a rostro-caudal gradient. Later, at E13.5, SCG10 transcripts were detectable at high levels in the sympathetic and sensory ganglia (Fig. 4A) and in parasympathetic primordia in the arterial walls (Fig. 4B). However, no hybridization is observed with the ependymal zone of the neural tube which contains mitotic undifferentiated neuroblasts (Fig. 4A). Thus, in both the central and peripheral nervous system, expression of SCG10 mRNA correlates with the earliest overt signs of commitment to a neuronal phenotype, occurring first in the ventrolateral neural tube and later in the peripheral ganglion primordia.

Comparison of the intensity of grains over developing ganglia suggest that a large component of the induction seen on Northern blots is likely to be due to an increase in the proportion of embryonal cells which are neurons as the consequence of either mitotic expansion (Rothman et al. 1978) or conversion of cells. Quantitatively, however, a 30-fold relative increase in the neural population (needed to account for the magnitude of SCG10 mRNA induction) would exceed that actually generated in this 48-hour interval, so that induction is

probably occurring on a per cell basis as well. In support of this, we observe an increase in grain density between E11.5 and E13.5 in the ventrolateral neural tube.

The observation that SCG10 mRNA appears in sympathetic neurons subsequent to neural crest migration next led us to ask whether any known environmental factors could influence the expression of this gene. Previous studies have suggested that the choice of a neuronal phenotype by sympathoadrenal precursors may be influenced by NGF in the cell's local environment (Unsicker et al. 1978; Aloe and Levi-Montalcini 1979; Landis and Patterson 1981; Doupe et al. 1985b). We therefore examined the ability of this polypeptide hormone to influence levels of SCG10 mRNA in vitro. We observed that SCG10 mRNA was induced almost 10-fold by NGF in PC12 cells (Fig. 5A), an adrenal medullary pheochromocytoma tumor line (Greene and Tischler 1976) that has many properties of a committed adrenergic precursor and which responds to NGF with neurite outgrowth. This induction is also observed in primary cultures of dissociated neonatal adrenal chromaffin cells exposed to NGF. In situ hybridization to adrenal chromaffin cell cultures in the presence of NGF revealed that those cells that converted to a neuronal morphology (Unsicker et al. 1978; Naujoks et al. 1982; Ogawa et al. 1984; Doupe et al. 1985a) also expressed SCG10 mRNA at high levels (Fig. 6A,B).

Thus, SCG10 mRNA is induced by NGF exposure in both primary cultures of chromaffin cells and in an established pheochromocytoma cell line. These results suggest that the dramatic appearance of this RNA early in the development of sensory and sympathetic neurons may result from the migration of these cells to an environment containing high local concentrations of NGF, or from the synchronous appearance of NGF in the embryo.

The Regulated Expression of SCG4

The pattern of expression of SCG4 mRNA during development is distinct from that of SCG10. SCG4 mRNA is highly enriched in adult neurons. Early in development, however, this RNA is abundant in virtually all tissues of the embryo. Expression of SCG4 mRNA declines precipitously in all tissues during late gestation but then returns to high levels after birth, specifically in the nervous system. Northern blot analysis of total embryo RNA reveals that SCG4 mRNA is expressed as an abundant species in midgestational embryos and is already quite abundant at E10.5, a time when SCG10 mRNA is barely detectable (Fig. 2B). This RNA is maintained at high levels (~ 10 times the abundance of SCG10) until E14 and then declines gradually to low levels at birth. In situ hybridizations yielded an unexpected result: although the SCG4 mRNA was clearly abundant in sympathetic ganglia, dorsal root ganglia, and the neural tube, hybridization was also detected in several other nonneural tissues, particularly

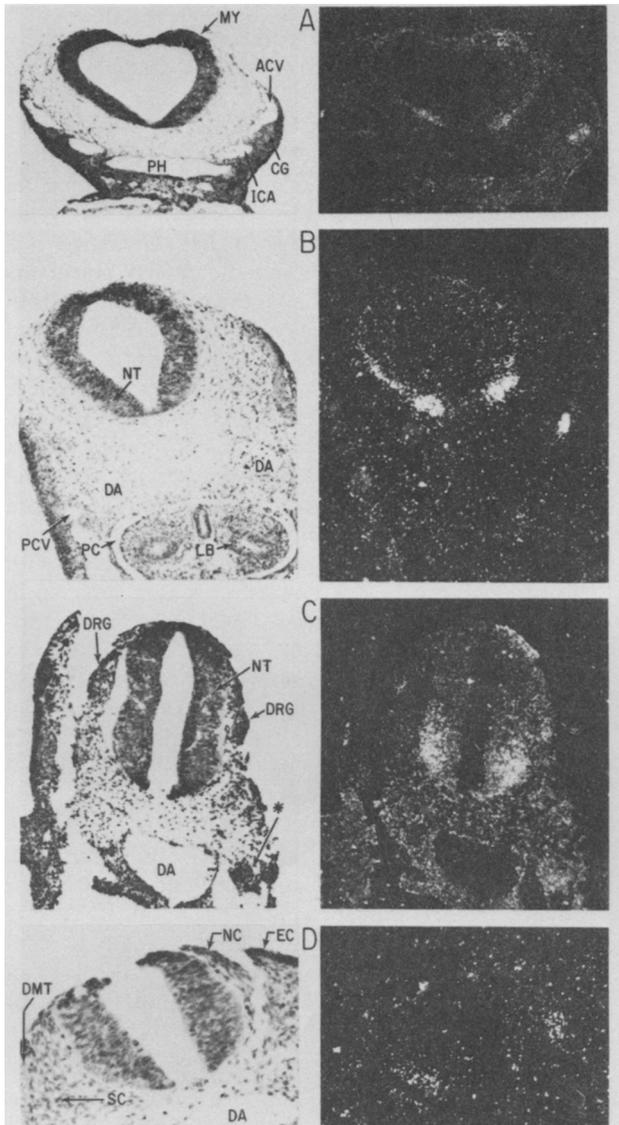


Figure 3. In situ distribution of SCG10 mRNA at stage E11.5. Sections obtained from a single embryo series were hybridized in situ with ^{35}S -labeled SP6 RNA transcripts from clone 10 and are displayed as bright-field-dark-field pairs. The sections proceed from A to D in a rostral to caudal direction. Sections are transverse but slightly oblique, thus structures ventral to the neural tube in a given section appear slightly more caudal in characteristics. (A) Region of the myelencephalon (MY). A cranial ganglion (CG) is visible ventral to the anterior cardinal vein (ACV) and dorsolateral to the internal carotid artery (ICA), which shows strong hybridization signal in dark field. Note also the high grain density in the ventrolateral neural tube. (PH) Pharynx. (B) Region of the posterior myelencephalon. (DA) Dorsal aorta; (NT) neural tube; (PCV) posterior cardinal vein; (PC) pleural cavity; (LB) lung bud. (C) Trunk region. Due to the curvature of the embryo, this section shows a more tangential appearance. (NT) Neural tube; (DRG) dorsal root ganglia; (DA) dorsal aorta; (*) putative sympathetic ganglion primordium. Note the relative absence of grains over this structure relative to the ventrolateral neural tube and the lateral aspect of the DRG. (D) More caudal region. Migrating neural crest cells (NC) are visible between the neural tube and a somite, which is divided into dermomyotome (DMT) and sclerotome (SC) regions. (DA) Dorsal aorta; (EC) ectoderm. B and D were hybridized in parallel and thus the grain densities are directly comparable. Exposure times were 3–4 days. Magnifications: A, $36\times$; B and C, $45\times$; D, $93\times$.

the lung (not shown). Northern blot analysis however confirmed this observation: SCG4 mRNA is abundant in heart, kidney, liver, lung, adrenal, and brain from E16 embryos (Fig. 7), declines neonatally, and reappears postnatally in neurons.

If, as we have suggested previously, the induction of clone 10 is in part mediated by the appearance of NGF, we would not predict that the expression of clone 4 is also mediated by NGF, as it is not coordinately expressed in time with clone 10 and, more importantly, it is expressed at high levels in tissues such as lung and heart which are not thought to contain NGF receptors. Indeed, we observed that SCG4 mRNA is expressed at high levels in naive P12 cells and is not affected by NGF treatment (Fig. 5B). This finding was therefore consistent with the presumed embryonic origin of this cell line. A different result is obtained when primary adrenal chromaffin cells are exposed to NGF. Chromaffin cells cultured from 7-day postnatal animals exhibited

little or no in situ hybridization signals over background with an SCG4 probe (Fig. 6A, bottom), a finding consistent with previous data from Northern blot analyses (cf. Fig. 1). When similar cultures were then exposed to nerve growth factor for 1 month, those cells that morphologically converted to a neural phenotype showed strong hybridization signals (Fig. 6B, bottom) similar in intensity to that observed for bona fide sympathetic neurons treated in parallel (not shown).

These results suggest that the expression of clone 4 mRNA may be regulated by distinct mechanisms at different times in development. Early in embryogenesis, a high level of constitutive synthesis of clone 4 RNA is observed in a host of neural and nonneural tissues and this synthesis is unlikely to be regulated by NGF. Postnatally, the expression of clone 4 declines in all tissues and increases solely in tissues of neural origin where its expression may be regulated by exposure to NGF.

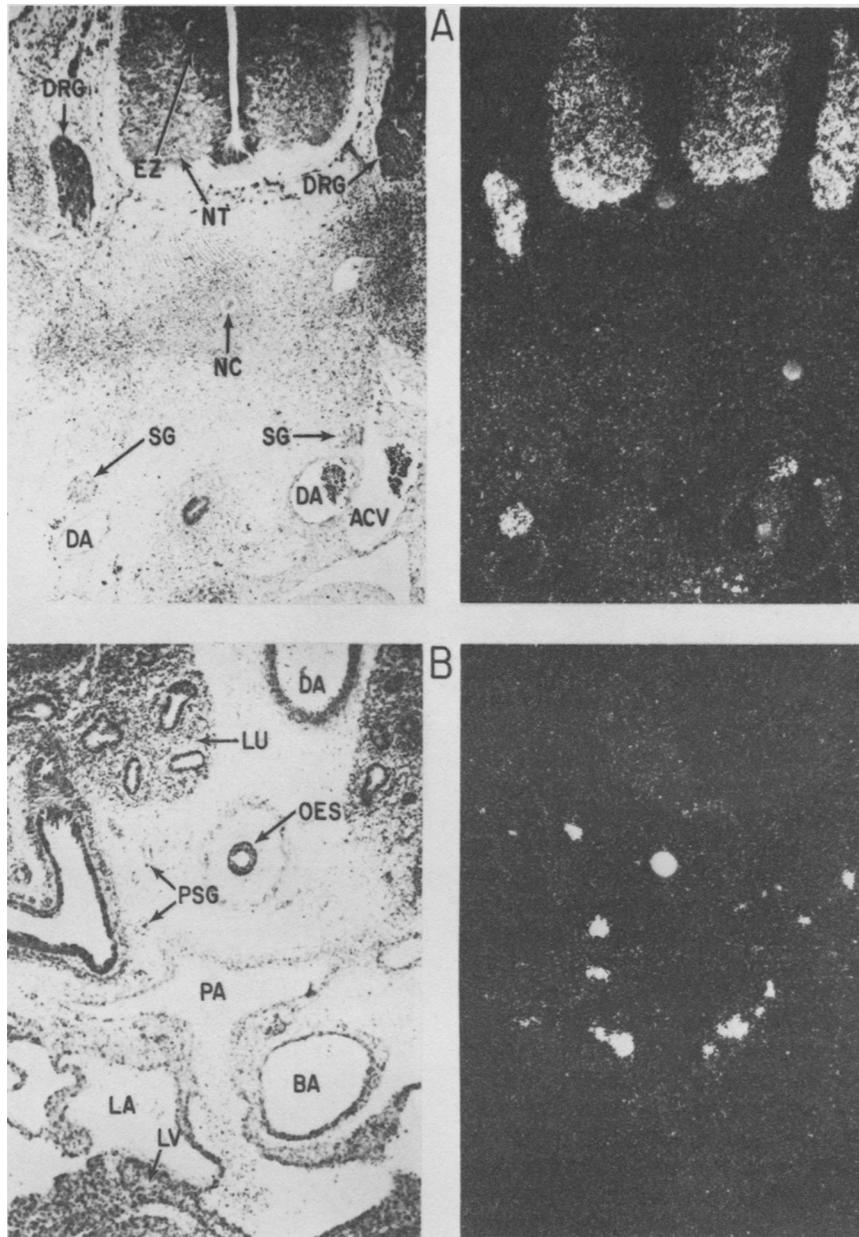


Figure 4. In situ distribution of SCG10 at later stages of development. (A) Section through trunk region E13.5. Note intense hybridization to neural tube (NT), dorsal root ganglia (DRG), and sympathetic ganglia (SG), while the ependymal zone (EZ) containing undifferentiated neuroblasts is negative. (NC) Notochord; (DA) dorsal aorta; (ACV) anterior cardinal vein. Magnification, $40\times$. (B) Section through ventral trunk E15. (DA) Dorsal aorta; (LU) lung; (BA) bulbus arteriosus; (LA) left atrium; (LV) left ventricle. Note strong signal to putative parasympathetic ganglia (PSG) surrounding the pulmonary artery (PA). The circular white spot in dark field (*right panel*) is an artifact.

DISCUSSION

Regulated Expression of Specific RNAs during Neural Development

We have followed the emergence of neurons during developmental time by performing in situ and blot hybridization with three neural-specific cDNA probes. Our data suggest that the induction of neural-specific genes does not occur synchronously but rather occurs in at least two major stages in vertebrate neurogenesis, one coinciding with the earliest overt signs of commitment to a neural phenotype (SCG10) and the other cor-

responding to maturation events that occur early in postnatal life (SCG4 and SCG5).

The observation that each clone appears at different times in development raises the question of what factors control these patterns of expression. NGF has previously been implicated in the chromaffin-neuron decision that occurs early in development (Aloe and Levi-Montalcini 1979; Landis and Patterson 1981). Clone SCG10 was inducible by NGF both in primary cultures of neonatal chromaffin cells and in the NGF-responsive pheochromocytoma tumor cell line PC12. It is therefore possible that NGF may be responsible for

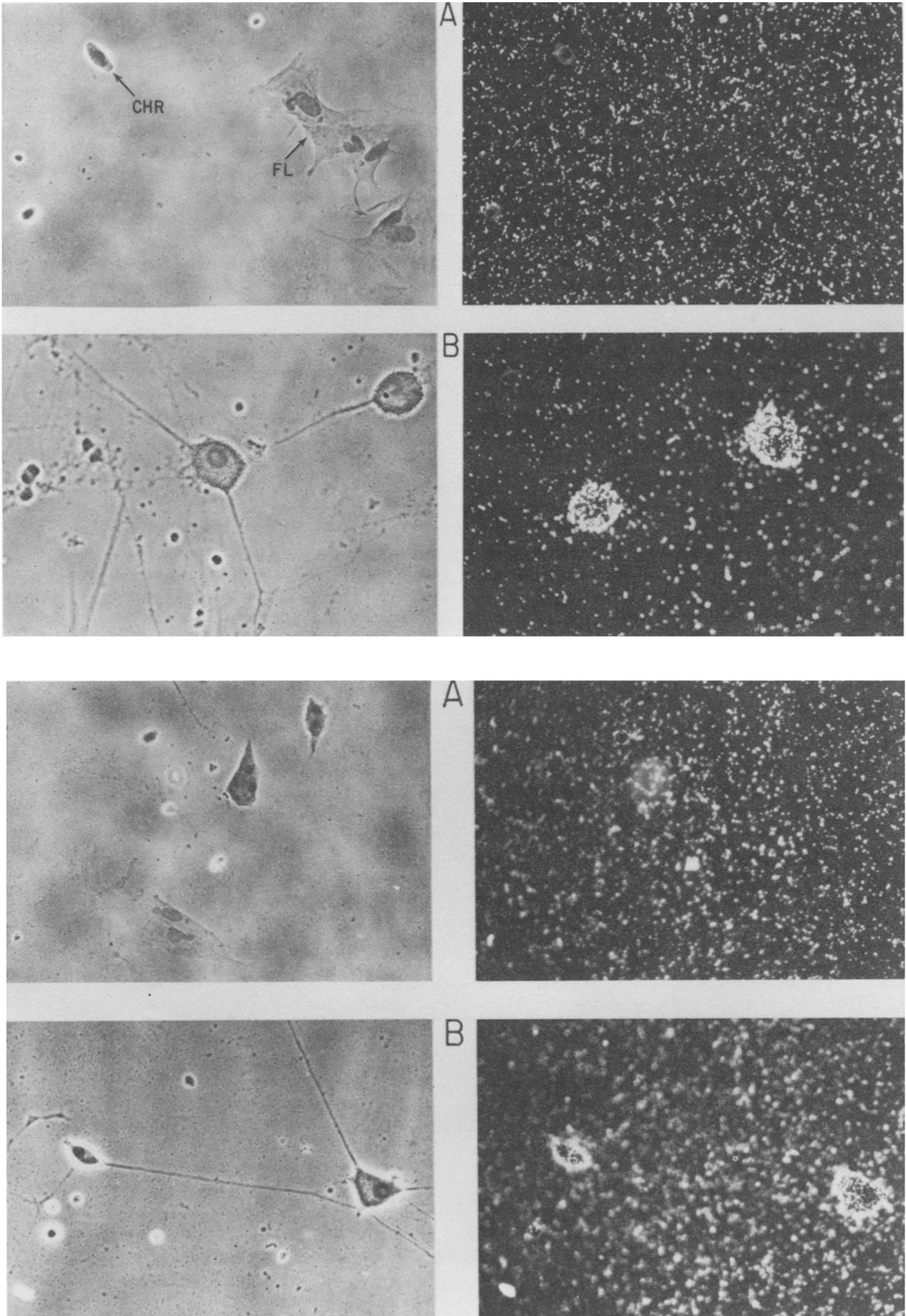


Figure 6. (See facing page for legend.)

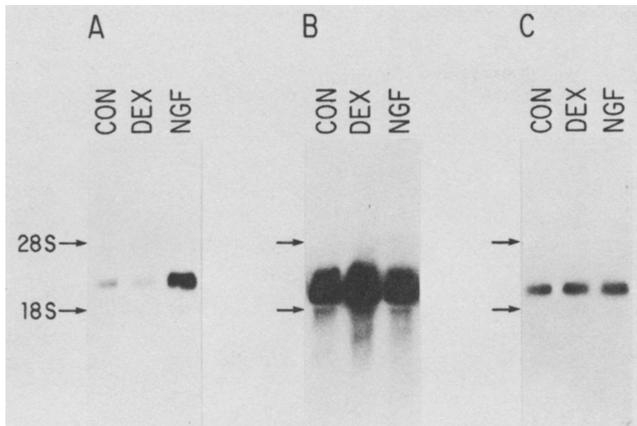


Figure 5. SCG10 mRNA is induced by NGF in PC12 cells. PC12 cells, at $\sim 0.5\text{--}1.0 \times 10^6$ cells/10-cm dish, were treated for 4 days with medium containing no additives (lanes CON), $5 \mu\text{M}$ dexamethasone (lanes DEX), or $50 \mu\text{g/ml}$ β -NGF (lanes NGF). Shown are three identical Northern blots containing $10 \mu\text{g/lane}$ of total RNA prepared from the indicated cultures. Probes used were SCG10 (A), SCG4 (B), or human γ -actin (C). A and B were hybridized with ^{32}P -labeled SP6 probes of the same specific activity, and the blot was exposed for the same length of time to show the relative abundance of the two mRNAs.

the transcriptional activation of SCG10 mRNA in sensory and sympathetic neurons during early development. This induction may be a consequence of the appearance of NGF, NGF receptors, or alternatively, the migration of crest cells to an environment enriched in NGF. However, we also observe ubiquitous expression of SCG10 mRNA in parasympathetic and central neurons, most of which are not responsive to NGF by classical criteria. We therefore think it unlikely that NGF is the sole mediator of SCG10 mRNA induction in the central and peripheral nervous system. Rather, expression of SCG10 mRNA may be controlled by different factors in different types of neurons.

SCG4 mRNA was also induced by NGF in postnatal chromaffin cells, but was constitutively expressed by PC12 cells. Our *in situ* hybridization data for SCG4 indicated that in the embryo it is abundant in both neural and nonneural cells, but is then extinguished and reexpressed selectively in neurons after birth. The NGF induction of SCG4 in chromaffin cells may thus reflect the reexpression of the gene that normally occurs in neurons of the same age. Likewise, the high level of expression of SCG4 mRNA in PC12 cells is consistent with the presumed embryonic nature of this tumor cell line (Greene and Tischler 1976).

The observation that glucocorticoids inhibit the NGF-induced conversion of chromaffin cells to neurons has led to the suggestion that these hormones may promote endocrine differentiation by blocking the NGF-induced expression of the neuronal phenotype by embryonic adrenal medullary pheochromoblasts. Preliminary *in situ* hybridization data suggest that, at E14, SCG10 mRNA is not expressed by, or is present at low levels in, immature chromaffin cells compared to neighboring sympathetic neurons (not shown.) Yet, the adrenal cortex does not secrete glucocorticoids until E17 (Bohn et al. 1981). The implication is that adrenal

environmental factors other than glucocorticoids may suppress (or permit only transient, low-level) expression of SCG10 mRNA by E14 pheochromoblasts. Consistent with this, dexamethasone only weakly (\sim two-fold) inhibited NGF induction of SCG10 mRNA in PC12 cells (not shown), and had no apparent effect on neurite outgrowth from organ-cultured E17 adrenal glands (Unsicker et al. 1985). *In vivo*, then, glucocorticoids may serve to maintain the differentiated chromaffin phenotype rather than to prevent initial differentiation of pheochromoblasts along the neuronal pathway.

Molecular Mechanism of Chromaffin Cell Plasticity

Chromaffin cells, expressing the phenotype of a mature endocrine cell, convert at high frequency to a neuronal phenotype both *in vitro* and *in vivo* in response to NGF (Unsicker et al. 1978; Aloe and Levi-Montalcini 1979; Doupe et al. 1985a). Our data illustrate this phenotypic plasticity at the level of RNA: mRNAs whose expression is normally restricted to neurons are induced to high levels in chromaffin cells exposed to NGF. Although the induction of neural-specific mRNAs by NGF in primary chromaffin cells may differ mechanistically from their initial induction *in vivo*, we believe that the plasticity of these genes in response to NGF nevertheless is a consequence of developmental changes in their chromatin structure in the sympathoadrenal precursor.

Specifically, the common precursor may maintain a battery of genes, specific to either sympathetic neurons or chromaffin cells, in an "open" chromatin configuration that is poised for transcription (Groudine and Weintraub 1982). The selection of those genes to be expressed would depend upon environmental signals, such as NGF or glucocorticoids, encountered by the

Figure 6. SCG10 and SCG4 mRNAs are induced by NGF in primary chromaffin cells. Shown are phase/dark-field pairs of *in situ* hybridizations to cultured cells. (Top) SCG10 probe; (bottom) SCG4 probe. (A) Chromaffin cells (CHR) grown in $5 \mu\text{M}$ dexamethasone. Note fibroblastic flat cells (FL) that are also negative with both probes. (B) Chromaffin cells switched after 1 week in dexamethasone to medium containing 50 ng/ml NGF and 10^{-5} M each of cytosine arabinoside and fluorodeoxyuridine. Cultures were maintained in this medium for 3 weeks prior to fixation. The photomicrographs are focused on the upper surface of the cells and thus the background is out of focus. Magnification, $215 \times$.

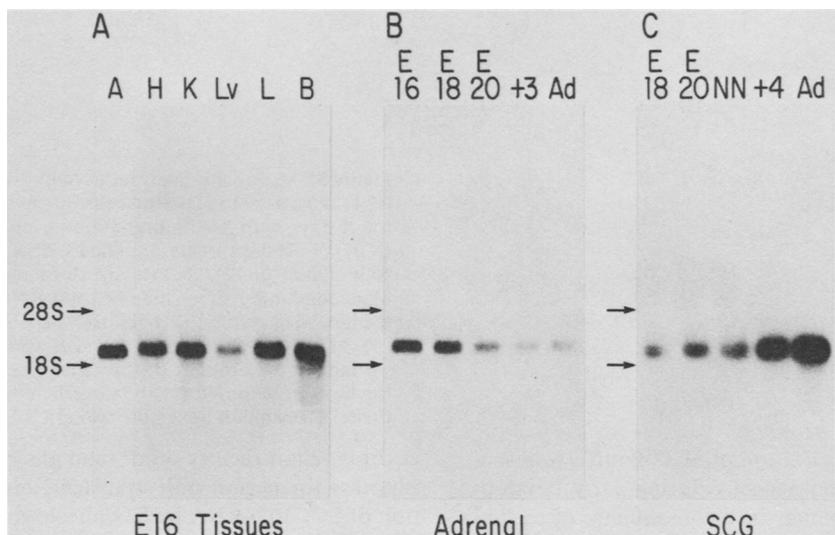


Figure 7. Developmental regulation of SCG4 mRNA. (A) Expression of SCG4 mRNA in nonneural tissues from E16 embryos. Each lane contains 10 μ g of total RNA from adrenal gland (lane A), heart (lane H), kidney (lane K), liver (lane Lv), lung (lane L), and brain (lane B). Compare with Fig. 1B. (B and C) SCG4 mRNA declines in late gestation and is reexpressed selectively in nervous tissue. (B) Each lane contains 10 μ g of total RNA from adrenal glands of the ages indicated. The amount detectable in adult (Ad) tissue corresponds to \sim 30–40 copies of mRNA/cell, assuming 2×10^5 mRNA molecules/cell. (C) RNA from developing superior cervical ganglion (SCG). Adult levels correspond to about 1400 copies/cell.

cell during or after migration. The inducibility of neural-specific genes in chromaffin cells may therefore reflect the persistence of these genes in such a transcriptionally competent configuration, despite the fact that they are not expressed as part of the mature chromaffin phenotype. In support of this model, studies of the structure of several tissue-specific genes have demonstrated that a nuclease-sensitive, “open” chromatin configuration developmentally precedes active transcription (Hofer et al. 1982; Sheffery et al. 1982; Burch and Weintraub 1983). In this way, the precursor may actively differentiate along one pathway while retaining the potential to switch to another pathway in response to a changed environment.

Why Have Plasticity?

The cells of the neural crest undergo extensive migrations before terminally differentiating into derivatives of widely varying phenotypes. The migratory pathways followed by neural crest cells are complex and may depend largely upon the cell's starting position (Le-Douarin 1982; Thiery et al. 1982). While “homing” mechanisms may play a role in specifying a crest cell's final destination in advance of migration, the available data nevertheless suggest the process involves a fair amount of chance and randomness. If the ultimate environment of a crest cell cannot be completely determined in advance of migration, and this environment determines the cell's fate, then premigratory neural crest cells must face considerable developmental uncertainty. The multipotentiality apparent within the sympathoadrenal lineage, at least, constitutes one evolutionary strategy for accommodating this uncertainty.

This multipotentiality may be exploited to generate the phenotypic diversity displayed by the nervous system, and its persistence may permit changes in neuronal phenotypes in the mature organism (Furshpan et al. 1976; Patterson 1978; Black 1982; Landis and Keefe 1983; Black et al. 1984; Doupe et al. 1985a).

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