

# Distinct and Different Effects of the Oncogenes *v-myc* and *v-src* on Avian Sympathetic Neurons: Retroviral Transfer of *v-myc* Stimulates Neuronal Proliferation Whereas *v-src* Transfer Enhances Neuronal Differentiation

H. Haltmeier and H. Rohrer

Max-Planck-Institut für Psychiatrie, Federal Republic of Germany

**Abstract.** Immature avian sympathetic neurons are able to proliferate in culture for a limited number of divisions albeit expressing several neuron-specific properties. The effect of avian retroviral transfer of oncogenes on proliferation and differentiation of sympathetic neurons was investigated. Primary cultures of 6-d-old quail sympathetic ganglia, consisting of 90% neuronal cells, were infected by Myelocytomatosis virus (MC29), which contains the oncogene *v-myc*, and by the *v-src*-containing Rous sarcoma virus (RSV). RSV infection, in contrast to findings in other cellular systems, resulted in a reduction of neuronal proliferation as determined by <sup>3</sup>H-thymidine incorporation (50% of control 4 d after infection) and in increased morphological differentiation. This is reflected by increased neurite production, cell size, and expression of neurofilament protein. In addition, RSV-infected neurons, unlike uninfected cells, are able to survive in culture for time periods up to 14 d in the absence of added neurotrophic factors. In contrast, retroviral

transfer of *v-myc* stimulated the proliferation of immature sympathetic neurons preserving many properties of uninfected cells. The neuron-specific cell surface antigen Q211 and the adrenergic marker enzyme tyrosine hydroxylase were maintained in MC29-infected cells and in the presence of chick embryo extract the cells could be propagated over several weeks and five passages. Within 7 d after infection, the number of Q211-positive neurons increased ~100-fold. These data demonstrate distinct and different effects of *v-src* and *v-myc*-containing retroviruses on proliferation and differentiation of sympathetic neurons: *v-src* transfer results in increased differentiation, whereas *v-myc* transfer maintains an immature status reflected by proliferation, immature morphology, and complex growth requirements. The possibility of expanding immature neuronal populations by transfer of *v-myc* will be of considerable importance for the molecular analysis of neuronal proliferation and differentiation.

**I**n higher vertebrates the neurons of the central and peripheral nervous system are generated during embryonic or early postnatal days. During this period of neuronal birth, which extends over a period of several days for a specific neuronal cell type, a defined number of neurons is produced by proliferation and differentiation of neuronal precursor cells. The final number of neurons results from the process of selective survival which leads to the degeneration and death of large numbers of postmitotic neurons in many regions of the peripheral and central nervous system. The analysis of the control of neuronal proliferation during these early stages has been hampered by the lack of available tissue culture systems to investigate this process, and by the lack of markers to identify precursor cells, present only transiently and in limited numbers during development.

We have recently demonstrated that neurons from 7-d-old

(E7)<sup>1</sup> chick paravertebral sympathetic ganglia, identified by several neuronal markers, are able to proliferate in vitro for a limited number of divisions, and thus represent a suitable model to investigate proliferation and differentiation (Rohrer and Thoenen, 1987; Ernsberger et al., 1989a,b). Cells with similar properties have also been described in cultures of embryonic rat adrenal medulla (Anderson and Axel, 1986) and of embryonic rat sympathetic ganglia (DiCocco-Bloom and Black, 1988). We observed, however, that these cells cease to proliferate and start to degenerate after several days

1. *Abbreviations used in this paper:* CEE, chicken embryo extract; CEF, chicken embryo fibroblast; CNTF, ciliary neurotrophic factor; E, embryonic day; MC29, Myelocytomatosis virus; NF, neurofilament; NF-IR, NF immunoreactivity; NGF, nerve growth factor; RSV, Rous sarcoma virus; TH, tyrosine hydroxylase; TH-IR, TH immunoreactivity.

in culture (Ernsberger et al., 1989b), thus, the number of cells available is still very limited.

Large numbers of undifferentiated cells of mesodermal and endodermal tissues have been obtained by transformation with retroviruses (Fizman and Fuchs, 1975; Graf and Beug, 1978; Falcone et al., 1985). In the hematopoietic system, retrovirus transduction of oncogenes has contributed considerably to the understanding of proliferation and differentiation, for example, by the expansion of rare, immature precursor cells of different cell lineages and the identification of growth factors using growth factor-dependent cell lines (Graf and Beug, 1978; Bazill et al., 1983; Leutz et al., 1984; Leutz et al., 1989).

Thus, it was of interest to investigate the impact of retroviral infection on cultures of immature avian sympathetic neurons with respect to the effects of viral oncogenes on neuronal proliferation and differentiation. In addition, these investigations should give indications on the possible roles of cellular proto-oncogenes during normal neural development.

In the present work the effects of the *v-myc* oncogene have been analyzed since in many cellular systems it results in immortalization (continued growth potential) rather than in tumorigenic transformation of infected cells (Palmieri et al., 1983; Gionti et al., 1985; Land et al., 1983; Ness et al., 1987; Casalbore et al., 1987), and thus seemed to be appropriate for the propagation of precursor cells. Recently, *v-myc* and *c-myc*-containing retroviruses (Frederiksen et al., 1988; Bartlett et al., 1988) have indeed been used to immortalize precursor cells from the neuroepithelium of embryonic mice and from early postnatal rat cerebellum. Although *v-myc*-infected cells do not produce a fully malignant phenotype, it is not clear to what extent the infected cells change their properties. In this context it seemed of considerable interest to investigate the effect of *v-myc* on the maintenance of differentiated functions in proliferating sympathetic neurons which have been described in detail previously (Ernsberger et al., 1989a,b).

Infection by Rous sarcoma virus (RSV) has been demonstrated to stimulate the proliferation of neural cells, i.e., neuroretinal cells (Pessac et al., 1983 a,b) and neural crest cells (Pessac et al., 1985). The expression of differentiated functions is suppressed by infection with *v-src*-containing retroviruses of immature cells, i.e., myoblasts (Fizman and Fuchs, 1975), chondroblasts (Pacifci et al., 1977), and retina melanoblasts (Boettiger et al., 1977). In the nervous system however, the effects of *v-src* expression on differentiation are strongly dependent on the cell type. In cultures of neural plate cells *v-src* expression interferes with the expression of neuronal, glial, and melanocyte-specific properties (Keane et al., 1984). In *v-src*-infected neuroretinal cultures, differentiated properties are maintained or induced (Crisanti et al., 1985), and, also, in the pheochromocytoma cell line PC12 infection by RSV results in the differentiation to neuron-like cells (Alema et al., 1985).

The cellular protooncogenes *c-myc* and *c-src* are both expressed in the nervous system. The time of expression is not strictly linked to proliferation since both protooncogenes are also present in postmitotic neurons (Cotton and Brugge, 1983; Levy et al., 1984; Sorge et al., 1984; Ruppert et al., 1986; Jaffredo et al., 1989), suggesting a role during differentiation or in the maintenance of differentiated traits.

Using primary cultures of E6 quail sympathetic neurons we observed in the present study that RSV infection results in a reduction of proliferation, induction of morphological differentiation, and the ability to survive in the absence of any added neurotrophic factor. In contrast, infection by *v-myc* results in a strong stimulation of neuronal proliferation. The cells still display factor-dependent survival and maintain several neuron-specific properties suggesting the usefulness of *myc*-containing retroviruses for the propagation of neuronal precursor cells.

## Materials and Methods

### Cell Culture and Retroviral Infections

Primary chicken embryo fibroblasts (CEFs) were obtained from 7 to 11-day old (E7-E11) embryos, and grown at 37°C in Iscove's Modified Dulbecco's Medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% (vol/vol) newborn calf serum (Gibco Laboratories) and 2% (vol/vol) chicken serum (Gibco Laboratories) in a humidified atmosphere containing 5% CO<sub>2</sub>. Primary cultures of quail sympathetic ganglion cells were established from E6 embryos. Paravertebral sympathetic chains were dissected, cleaned from attached nonganglionic tissue, and incubated at 37°C in 0.1% Trypsin/PBS (Worthington Biochemical Corp., Freehold, NJ) for 7 min. The reaction was terminated by the addition of 0.6 mg/ml Trypsin-Inhibitor from soybean (Sigma Chemical Co., St. Louis, MO), the solution replaced by serum-containing tissue culture medium, and a single cell suspension was generated by trituration of the tissue through a siliconized Pasteur pipette. Under standard conditions, 20,000 cells were plated in 4-well culture dishes (Greiner und Söhne, Nürtingen, FRG) coated with 0.5 mg/ml Poly-DL-ornithine (Sigma Chemical Co.) in borate buffer (0.15 M, pH 8.3) and 10 µg/ml laminin (BRL, Gaithersburg, MD) in PBS (see Ernsberger et al., 1989 for details). Neuronal cells were routinely cultured at 37°C in 1.5 ml F14 medium supplemented with 10% (vol/vol) horse serum and 5% (vol/vol) FCS (both from Gibco Laboratories) in a water-saturated atmosphere, containing 2.5% CO<sub>2</sub>. For retroviral infection, the Schmidt-Ruppin strain of *v-src*-containing RSV, Myelocytomatosis virus (MC29; containing the *v-myc* oncogene), and the helper virus RAV-1 were used. Viruses were generous gifts from H. Beug (Institut für Molekulare Pathologie, Wien) and T. Graf (European Molecular Biology Laboratory, Heidelberg). Virus-conditioned medium was prepared by harvesting the supernatant from subconfluent cultures of virus-infected CEF after 24 h at 37°C. After filtration through a 0.8-µm membrane (Nalgene Institut für Molekulare Pathologie) to remove detached cells, the filtrate was used immediately and undiluted for the infection procedure. Freshly trypsinized primary sympathetic ganglion cells were plated into 1.5 ml of virus-conditioned medium. Control cultures were treated with supernatant from virus-free CEF instead. After 3 h at 37°C, when cells were firmly attached to the Poly-DL-ornithine-laminin-substrate, virus-conditioned medium was replaced by 1.5 ml tissue culture medium. By day 3, the first virus-induced morphological changes were observed in neuronal cells. Chicken embryo extract (CEE) was added to the standard culture medium of respective cultures at concentrations of 1 and 2% (day 2 and 4 after infection, respectively) after filtration through a 0.2-µm membrane. The number of neurons was determined by counting cells with neuronal morphology in 10 randomly chosen visual fields with phase-contrast optics at 125-fold magnification. The area counted corresponded to 2.6% of the total area.

### Immunocytochemical Procedures

Cell surface antigens were identified by incubation of unfixed cultured cells with antibody. Neurons were stained with the monoclonal mouse antibody against the neuron-specific antigen Q211 (a generous gift from S. Henke-Fahle, MPI für Entwicklungsbiologie, Tübingen, FRG). Cells were washed and then incubated at room temperature in a 1:100 dilution of anti-Q211-immunoglobulin for 20 min, rinsed, and fixed with 4% paraformaldehyde in PBS for 20 min. Binding of the Q211 antibody was visualized by incubation with a 1:100 dilution of FITC-labeled goat anti-mouse antibody for another 20 min. Intracellular antigens (tyrosine hydroxylase [TH]), middle and high molecular mass neurofilament (NF<sup>68 kD</sup> and NF<sup>160 kD</sup>), pp60<sup>v-src</sup>, p110<sup>src-myc</sup>, and viral protein p19) were detected in formaldehyde-fixed cultures (4% paraformaldehyde in PBS for 20 min) after permeabilization of the cell membrane with PBT1 (PBS supplemented with 1% BSA and 0.1%

Triton X-100; Ernsberger et al., 1989a). Primary antibodies were used at the following dilutions for 30 min: anti-TH (1:200); anti-NF<sup>68 kD</sup> (1:3); anti-NF<sup>160 kD</sup> (1:3); anti-*v-src* (1:100); anti-*v-myc* (1:250); and anti-p19 (1:200). Anti-TH and anti-p19 antibodies were detected by a second incubation with rhodamine-conjugated goat anti-mouse antibody (1:100, 30 min). Neurofilaments and viral oncogene products pp60<sup>v-src</sup> and p10<sup>89g-myc</sup> were detected by a second biotinylated goat anti-mouse or donkey anti-rabbit, respectively, antibody (1:100, 30 min) followed by FITC-Streptavidin (1:100, 30 min) and analysis by fluorescence microscopy. Intracellular TH was also stained using the PAP method described by Ernsberger et al. (1989a). The mAb against TH has been described previously (Rohrer et al., 1986). Anti-NF antibodies were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) anti-*v-src* and anti-*v-myc* antibodies were purchased from Dianova, Hamburg, and Medac (Hamburg). The anti-*v-src* antibody is a mouse mAb raised against recombinant, bacterially expressed *v-src* (clone 327; Lipsich et al., 1983). The anti-*v-myc* is a polyclonal rabbit antiserum raised against bacterially expressed exon3 of the *v-myc* gene (Bunte et al., 1984). Anti-p19 was a generous gift from H. Beug (IMP, Wien). All stained cultures were embedded in PBS/Glycerin (1:1, vol/vol) and viewed with Leitz Orthoplan phase-contrast optics, bright-phase optics or epifluorescent illumination.

### <sup>3</sup>H-thymidine Labeling and Autoradiography

After 1-5 d in vitro, 1  $\mu$ Ci/ml methyl-<sup>3</sup>H-thymidine (Amersham Corp., Arlington Heights, IL; 42 Ci/mmol) were added to primary cultures of quail sympathetic chain ganglion cells. After a 3-h pulse at 37°C, cultures were fixed with 4% paraformaldehyde/PBS for 20 min, washed with PBS and double-distilled water, and subjected to dehydration by rinsing the dishes with 50%, 70%, and absolute ethanol in a rapid sequence. Dried cultures were covered with photographic emulsion (Kodak NTB 2) and exposed at 4°C for 6 d. Specimens were developed with Kodak D19, and neuronal cells labeled with silver grains were counted using phase-contrast and bright-field optics.

### Preparation of CEE

Total bodies of 10-11-d-old chicken embryos were, after removal of the eyes, passaged through a syringe, an equal amount of Ca<sup>2+</sup>-Mg<sup>2+</sup>-free Tyrode's solution was added to the fractured tissue, and the suspension was gently stirred for 30 min at 4°C. After centrifugation (4,000 rpm for 15 min) the supernatant was collected and aliquots were stored at -20°C. The protein

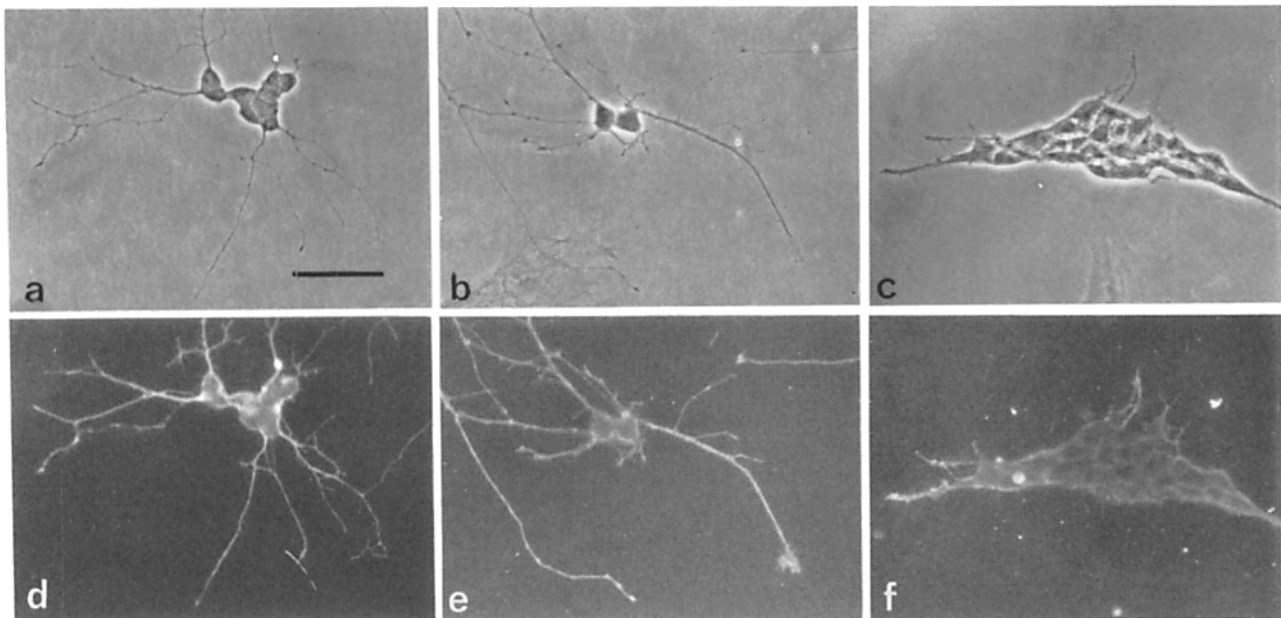
concentration was determined as 8.4 mg/ml by the Bradford procedure (Bradford, 1976).

## Results

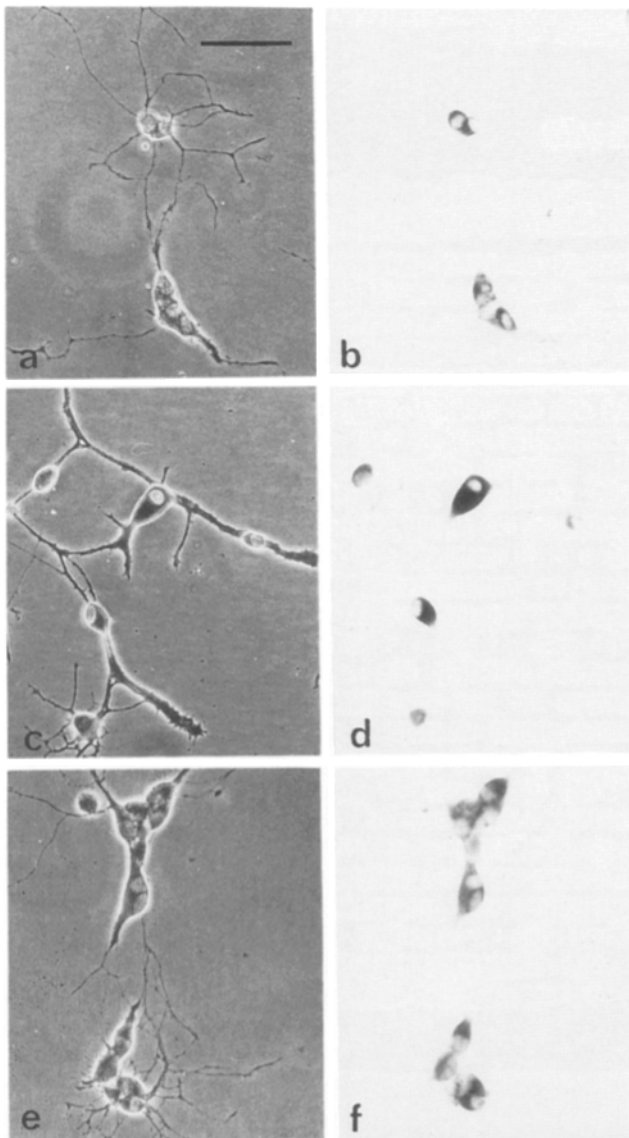
### Infection by RSV or MC29 Alters the Morphology of Neurons from Sympathetic Chain Ganglia

Primary cultures of immature sympathetic neurons were obtained from dissociated paravertebral sympathetic chain ganglia of E6 quail embryos. The stage of differentiation of 6-d-old quail embryos corresponds to that of 7-d-old chick embryos (Hamburger and Hamilton, 1951; Zacchei, 1961); and also the cellular composition of E6 quail lumbosacral paravertebral ganglia was virtually identical to that of E7 chick embryos analyzed previously (Rohrer and Thoenen, 1987; Ernsberger et al., 1989a). Under standard culture conditions, the population contained  $88 \pm 6.5\%$  (mean  $\pm$  SD,  $n = 3$ ) neuronal cells after 3 h in vitro, as was determined by immunocytochemical detection of the neuron-specific surface antigen Q211 (Rösner et al., 1985), and  $85 \pm 2\%$  (mean  $\pm$  SD,  $n = 3$ ) of the cells expressed TH immunoreactivity (TH-IR). The survival of E6 quail sympathetic neurons was also dependent on the presence of a laminin-coated culture substrate (data not shown), as described previously for chick sympathetic neurons (Ernsberger et al., 1989b). The majority of sympathetic ganglion cells acquire neuronal morphology, i.e., neuritic processes with growth cones (Ernsberger et al., 1989 a,b), on a laminin substrate after a few hours in culture and, due to proliferation and cell aggregation, small groups of Q211-positive neurons were observed after 3 d in culture (Fig. 1).

After infection by oncogenic retrovirus MC29 or RSV, the in vitro appearance of neuronal cells changed in a way char-



**Figure 1.** Neuronal morphology and expression of Q211 antigen in sympathetic neurons infected by oncogenic retroviruses. Cells were either treated with virus-free medium (a and d), or infected with RSV (b and e) or MC29 (c and f) at the time of plating. After 3 d in vitro, expression of neuron-specific morphology was documented (a-c; phase-contrast optics) and Q211 antigen was detected by indirect immunofluorescence (d-f; fluorescence optics). Bar, 50  $\mu$ m.



**Figure 2.** Adrenergic phenotype of cultured sympathetic neurons. Cells were either treated with virus-free medium (*a* and *b*), or infected with RSV (*c* and *d*) or MC29 (*e* and *f*) at the time of plating. After 3 d *in vitro*, intracellular tyrosine hydroxylase was detected in all cultures by staining with the PAP method (*b*, *d*, *f*). (*a*, *c*, *e*) Phase-contrast; (*b*, *d*, *f*) bright-field optics. Bar, 50  $\mu\text{m}$ .

acteristic for the infecting virus species. First alterations could be detected on day 3 after infection. In cultures inoculated with RSV, neuronal cells developed enlarged cell somata, an increased number and length of processes, and enlarged growth cones (see Fig. 1, *b* and *e*). Somatic hypertrophy and neurite formation is most obvious in long-term cultures of RSV-infected neurons (see Fig. 7). These hypertrophic cells could be identified as neurons by morphology and Q211 immunoreactivity (Fig. 1, *b* and *e*), and the adrenergic phenotype was demonstrated by staining for the enzyme TH, the key enzyme for catecholamine synthesis (Fig. 2 *d*).  $91 \pm 2\%$  (mean  $\pm$  SD,  $n = 3$ ) of RSV-infected neuronal cells expressed TH-IR 3 d after infection. A similar proportion of TH-IR positive neurons (87%) was detected in control cultures that had been treated with virus-free super-

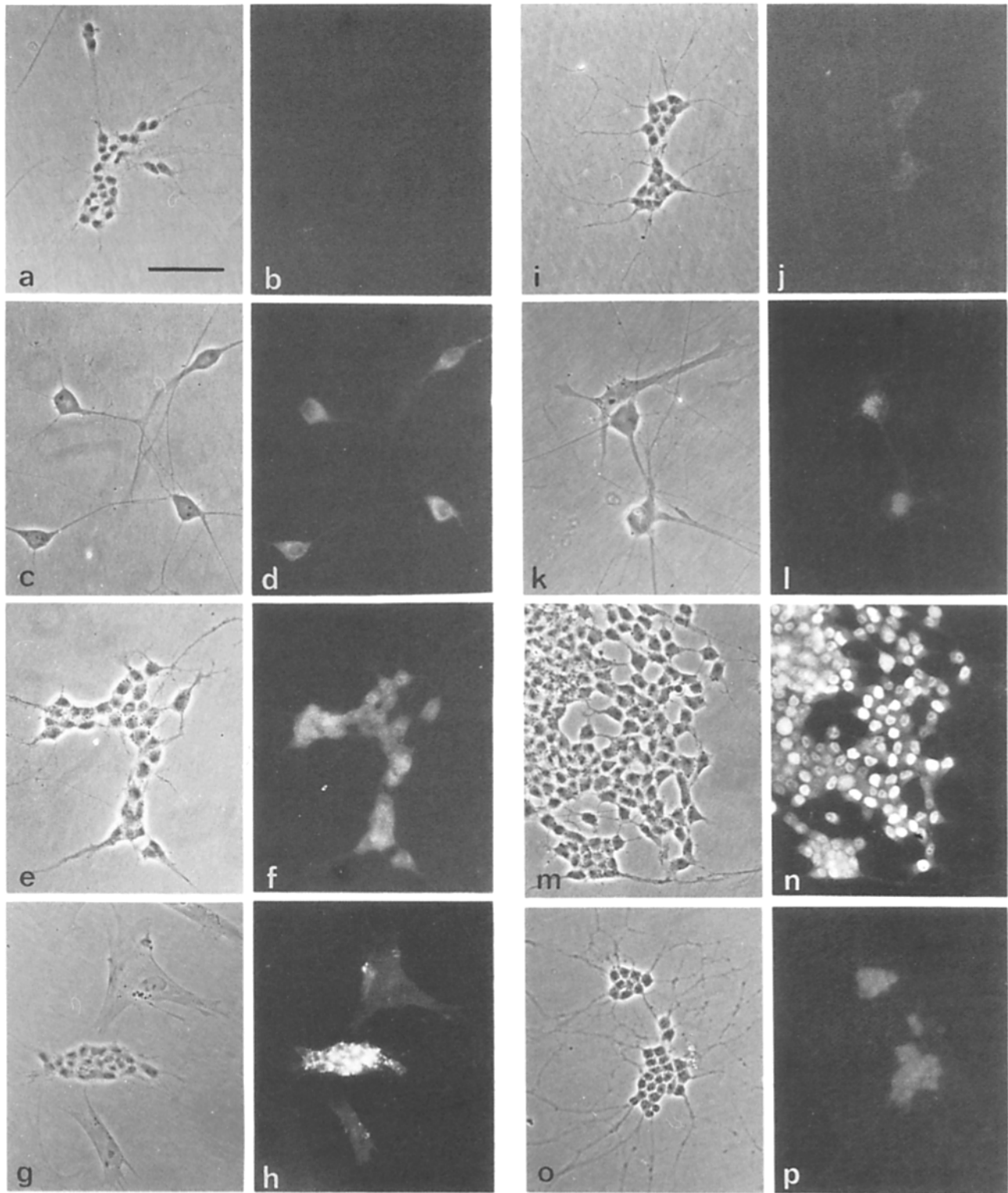
natant from CEFs. RSV-infected neurons, characterized by their hypertrophic morphology, were found preferentially as single cells, in contrast to the small groups of neurons found in cultures of noninfected sympathetic neurons after 3–4 d. Nonneuronal cells, characterized by their flat and phase-dark morphology, and by the absence of processes or growth cones, were negative for both Q211 and TH.

After infection of primary sympathetic ganglion cells with MC29 many clusters of small, phase-bright neurons were observed after a minimum of 3 d in culture. MC29-induced cell clusters contained exclusively Q211- and TH-positive neurons (Figs. 1 *c* and *f*, and 2 *f*). Of the neuronal cells,  $82 \pm 2\%$  (mean  $\pm$  SD;  $n = 3$ ) expressed TH-IR 3 d after infection. Cell somata were not enlarged as compared to controls, and the extent and length of neurite outgrowth from these clusters was observed to be variable. Typical clusters consisted either of loosely aggregated cells with round, spherical cell bodies, or densely packed cells with a more flattened appearance. Cell clusters grew rapidly due to intensive proliferation (see below), and after 6 to 7 d in culture under appropriate conditions (see below) initially sparse cultures ( $2 \times 10^3$  cells/cm<sup>2</sup>) formed nearly confluent sheets. The cells infected by MC29 did still depend for their survival and growth on the presence of a laminin substrate which could not be replaced by plastic, collagen, or poly-ornithine (data not shown). Nonneuronal cells that slowly increased their proliferation rate during the first week after infection, thereby gradually adopting a transformed phenotype, could be clearly distinguished from the neuronal population by the lack of neurites.

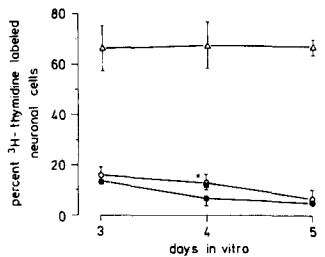
To control for the specificity of the virus-induced effects, cells were also infected by the transformation-defective virus RAV-1. This did not produce any of the morphological alterations observed after infection by RSV or MC29 (Fig. 3 *g*). To document the specific expression of oncogene proteins the cultures were stained with antibodies against *v-myc* and *v-src*, respectively, 4 d after infection. In addition, the presence of retroviral protein p19 was analyzed. P19 was detected exclusively in virus-infected cultures, but not in noninfected controls (Fig. 3, *b*, *d*, *f*, and *h*). In cultures infected by RSV, MC29, or RAV-1, more than 80% of cells with neuronal morphology could be shown to produce intracellular p19. After staining for *v-src*, 71% of neurons in RSV-infected cultures showed a typical pattern of intracellular pp60<sup>v-src</sup> expression (patchy somatic and faint membrane staining, Fig. 3 *l*), which is in accordance with published data (Rohrschneider, 1979; Krueger et al., 1983; Resh and Erikson, 1985). *Myc* expression was documented by immunocytochemical detection of the oncogene product p10<sup>src-myc</sup>. 74% of neurons showed a distinct nuclear staining, indicating that the oncoprotein is accumulating in the nuclei of MC29-infected sympathetic neurons, as was shown for other cell types (Fig. 3 *n*; Donner et al., 1982; Abrams et al., 1982; Bunte et al., 1982; Cañalobre et al., 1987). Noninfected control cultures did not express detectable amounts of *v-myc* (Fig. 3 *p*) or *v-src* oncoprotein (Fig. 3 *j*).

### **RSV and MC29 Influence the Proliferation of Sympathetic Neurons in Culture**

Chick sympathetic neurons, identified by the neuron-specific surface marker Q211 and by the expression of several adre-



**Figure 3.** Evidence for retroviral infection and expression of *v-src* and *v-myc* oncoproteins in cultured sympathetic neurons. Primary cultures were either treated with virus-free medium (*a, b, i, j, o, p*), or either infected with RSV (*c, d, k, l*), MC29 (*e, f, m, n*), or RAV-1 (*g, h*) at the time of plating. After 3 d in culture, retroviral protein p19 could be detected by indirect immunofluorescence after infection with RSV (*d*), MC29 (*f*), or RAV-1 (*h*), but not in noninfected controls (*b*). Note that p19 was also found in the nucleus after MC29 infection, reflecting the nuclear localization of the *gag-myc* fusion protein. 4 d after infection with RSV, *v-src* expression resulted in a distinct pattern of cytoplasmic and faint membrane staining (*l*), whereas *v-myc* oncoprotein could be visualized in the nuclei of MC29-infected neurons (*n*). Noninfected cells did not express *v-src* or *v-myc* immunoreactivity (*j, p*). (*a-h*) Phase-contrast; (*i-p*) fluorescence optics. Bar, 50  $\mu\text{m}$ .



**Figure 4.** [ $^3\text{H}$ ]thymidine incorporation in cultured sympathetic neurons. Cells were either treated with virus-free medium (○), or infected with RSV (●), MC29 (Δ), or RAV-1 (■) at the time of plating, and a 3-h pulse of [ $^3\text{H}$ ]thymidine (1  $\mu\text{Ci/ml}$ ) was given after different time periods in vitro.

On day 4, the 50% reduction in the labeling index of RSV-infected neurons as compared to controls is significant ( $*p < 0.025$ ), whereas there is no significant difference between controls and RAV-1-infected cultures. Data points represent the average with SD from three independent experiments.

ergic properties, are able to proliferate both in vivo (Cohen, 1974; Rothman et al., 1978; Rohrer and Thoenen, 1987) and in vitro (Rohrer and Thoenen, 1987; Ernsberger et al., 1989a,b).

Quail sympathetic ganglion cells from 6-d-old (E6) embryos were also found to proliferate in vitro, as was demonstrated by the increase in cell number and by the incorporation of [ $^3\text{H}$ ]thymidine. The absolute cell number per dish increased to 180% within 3 d in culture (see Fig. 5 A). The fraction of labeled neurons after a 3-h pulse of [ $^3\text{H}$ ]thymidine decreased with time in culture from 28% at day 1 to  $16 \pm 3\%$  on day 3 and  $6.5 \pm 3.5\%$  on day 5 (Fig. 4).

The effects of retrovirus-mediated oncogene transfer on proliferation were analyzed 3–5 d after infection, allowing for infection and oncogene expression in all cells. Infection by RSV reduced the potential to divide. Within 4 d after infection, thymidine incorporation in sympathetic neuronal cells declined to 50% of the control level (Fig. 4). This reduction of neuronal proliferation after RSV infection is not reflected by a decrease of neuronal cell number (see Fig. 5 A). This can be explained by better survival of RSV-infected cells as compared to the control cultures (see below), which compensates for the decrease in cell number due to reduced proliferation.

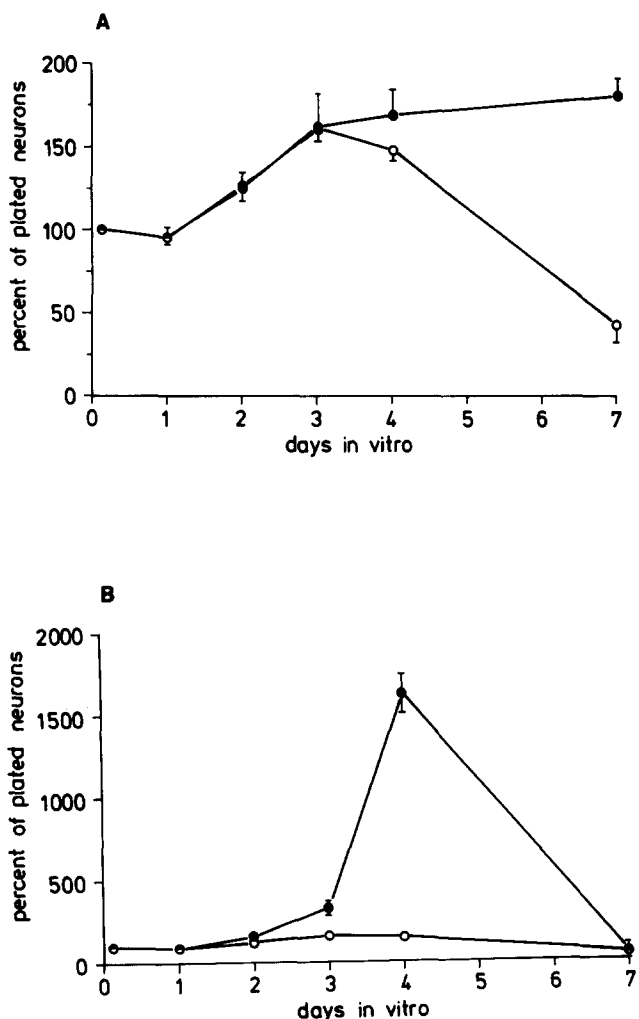
In contrast to RSV, infection by MC29 increased the neuronal proliferation as reflected by an increase in cell number and [ $^3\text{H}$ ]thymidine incorporation. 3 d after plating, a four-fold increase in the fraction of labeled neurons was observed by autoradiography. Respective values at 4 and 5 d in vitro were not changed, suggesting that maximal stimulation has been reached after 3 d and was maintained for at least 5 d after the infection (Fig. 4). The cell number also increased dramatically, reaching a peak 4–5 d after infection where the cell numbers had increased to  $\sim 16$  times the original number (Fig. 5 B). Thus, MC29 increased the proliferation of immature sympathetic neurons, enhancing an undifferentiated trait of this cell type, whereas RSV suppressed proliferation and induced a differentiated phenotype. In contrast to the strong effects of RSV and MC29, infection by RAV-1 affected neither [ $^3\text{H}$ ]thymidine incorporation nor cell numbers. 4 d after infection, the thymidine incorporation (3-h pulse) of RAV-1-infected cells and the total number of neurons amounted to 99 (Fig. 4) and 108% of the respective controls.

#### Effects of Infection by RSV or MC29 on the In Vitro Survival of Quail Sympathetic Neurons

Neurons from E6 quail sympathetic ganglia can be main-

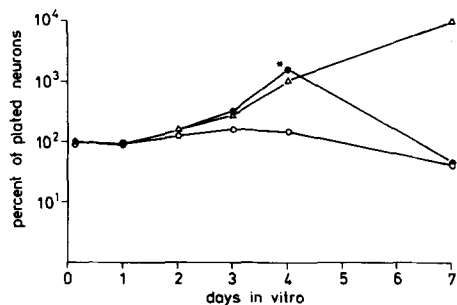
tained in culture only for a limited time period. After the initial increase in cell number due to cell proliferation, the numbers of sympathetic neurons start to decrease after 3–4 d in culture (Fig. 5), as observed previously for E7 chick sympathetic neurons (Ernsberger et al., 1989b). Addition of neurotrophic factors, nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF), did not prevent the death of neurons (data not shown), which parallels earlier findings using cultures of E7 chick sympathetic neurons (Ernsberger et al., 1989b) and also in the presence of 2% CEE the number of surviving neurons was reduced rather than increased (see below).

After infection by RSV, cultured neurons could be maintained in standard medium for at least two weeks in vitro. As shown in Fig. 5 A, absolute neuronal numbers of RSV-infected cultures reached a plateau after 4 d in vitro (180% of the neurons plated), whereas the amount of uninfected neurons declined within 7 d to  $\sim 40\%$  of the cells plated. The



**Figure 5.** Differential growth and survival of cultured sympathetic neurons after infection with oncogenic retrovirus. Similar cell numbers were plated on laminin substrate on day 0, and the number of neurons was determined after 3 h in vitro (corresponding to 100%). The number of neurons surviving without viral infection (A and B; ○), or after infection by RSV (A; ●) or MC29 (B; ●) was determined by cell counts (see Materials and Methods), and is expressed as a fraction of the respective 3-h value. Data points indicate the average with SD from three independent experiments.





**Figure 6.** Long-term survival of MC29-infected sympathetic neurons is dependent on CEE. Similar cell numbers were plated on laminin substrate (day 0) and the number of neurons was determined after 3 h in vitro (corresponding to 100%). The number of neurons surviving without viral infection (○), or after infection with MC29, with (△) or without (●) addition of CEE, was determined by direct cell counting (see Materials and Methods). Only on day 7, very dense cultures of MC29-infected cells supplemented with CEE were harvested and counted in a hemacytometer. The fraction of neuronal cells was determined by replating and immunocytochemical detection of neuronal Q211 antigen. About 20–30% of the cells were negative for the Q211 antigen on day 7. CEE was added on day 2 (1%) and day 4 (2%). On day 4, a significant decrease by 36% ( $*p < 0.01$ ) was observed in MC29-infected cultures with CEE, as compared to those without CEE. Note that cell numbers are expressed on a logarithmic scale. Data points represent the average from three independent experiments (SEM is below 5%).

number of surviving neurons after 14 d was 117% (RSV infected) and 25% (controls) as compared to 3-h cultures. Several control experiments were carried out to exclude the possibility that the survival effect is due to indirect effects of neurotrophic factors, known to be produced by nonneuronal cells (Burnham et al., 1972), rather than being due to a direct effect of the oncogene in the infected neurons. (a) Cultures were maintained at low cell density ( $1.3 \times 10^2$  cells/cm<sup>2</sup>), which is unfavorable for the growth of nonneuronal cells. Also, under these conditions survival of infected neurons was improved in addition to cellular hypertrophy and intensive neurite formation, as compared to uninfected neurons. (b) Neurotrophic factors NGF and CNTF, which are able to maintain sympathetic neurons from older chick embryos (Levi-Montalcini and Angeletti, 1963; Edgar et al., 1981; Manthorpe and Varon, 1985), did not improve the survival of E6 quail sympathetic neurons (data not shown). (c) To exclude the possibility that RSV infection induces the responsiveness of sympathetic neurons to NGF, infected cultures were also kept in the presence of anti-NGF antibodies, which are known to block the activity of avian NGF (Rohrer et al., 1988). Since the survival was not influenced under these conditions (106% of cultures without antibodies), the possibility that NGF is involved in the improved survival is excluded.

In contrast, MC29 infection did not improve the in vitro survival of sympathetic neurons. The strong proliferative increase in cell number during days 1–4, reaching a peak of ~16 times the original numbers, was followed by a sharp drop due to cell death between days 5 and 6 after infection (Fig. 5 B). Nearly all neuronal cells were eliminated within 1 d, independent of the cell density (range between  $1.25 \times 10^3$  to  $2.5 \times 10^4$  cells/cm<sup>2</sup> tested), and could not be prevented by alternative medium change protocols. By day

7, the number of MC29-infected neurons approached control levels. Thus, infection by RSV, but not by MC29, improves the survival of early sympathetic neurons in vitro demonstrating also that the survival effect is oncogene specific. RAV-1 infection did not improve the survival of sympathetic neurons. After 7 d, absolute cell numbers also declined to control levels.

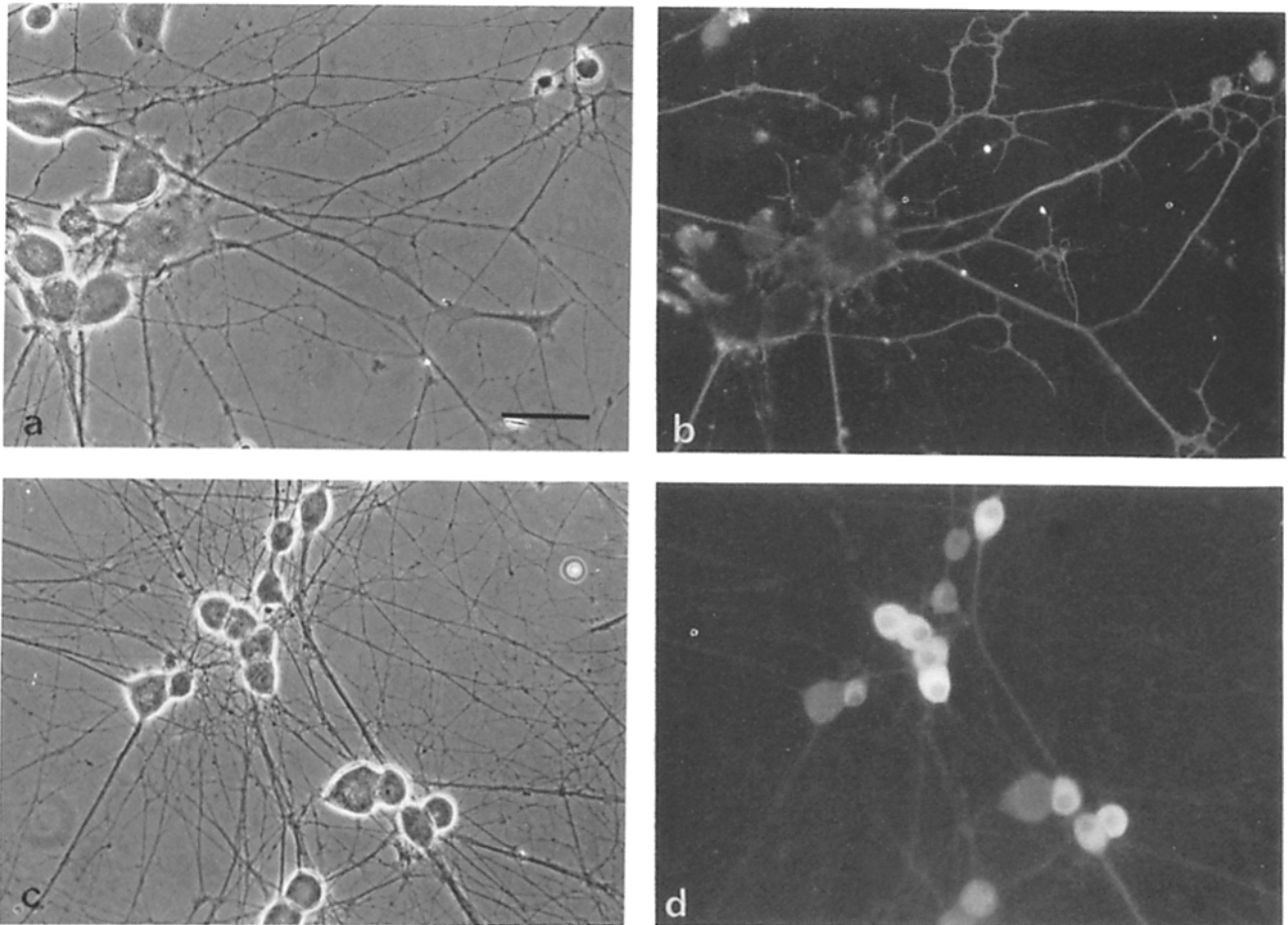
### CEE Prevents Cell Death of MC29-infected Sympathetic Neurons In Vitro

The capacity of MC29-infected sympathetic neurons to proliferate in vitro at a higher rate could offer a way to generate large amounts of immature neurons of a defined developmental stage. Due to their limited life span, however, experimental investigation would be restricted to culture periods of no longer than a few days. We therefore attempted to find culture conditions that support long-term survival of proliferating MC29-infected neurons. CEE turned out to include such (a) survival factor(s). After the addition of 2% CEE (final concentration) to the standard culture medium, MC29-infected neurons could be maintained in a proliferative state (Fig. 6). The neuronal number increased during the first 7 d after infection by a factor of ~100 (Fig. 6). Cultures have been kept under these conditions for up to 4 wk, but neuronal cell number is not given because of the increasing proportion of nonneuronal cells. However, growing cells in clonal cultures demonstrated an increase in neuronal cell number during several weeks in cultures (see below). A reduction in absolute cell numbers ( $36 \pm 9\%$ ) was observed 4 d after infection in MC29-transformed cultures after CEE exposure (Fig. 6). A similar reduction in cell number (by  $50 \pm 10\%$ ) was found after 4 d in cultures of uninfected cells. Since CEE did not affect the proliferation of neuronal cells ( $65 \pm 4\%$  of cells grown with CEE incorporate <sup>3</sup>H-thymidine during a 3-h pulse 3 d after infection, as compared to  $66 \pm 9\%$  of cells grown without CEE), it is concluded that this supplement, while providing components obligatory for the survival of MC29-infected sympathetic neurons, eliminates a fraction of these neurons.

### Effect of Infection by RSV and MC29 on the Expression of Neuron-specific Properties

The usefulness of retroviral oncogene transfer depends strongly on the ability of infected cells to retain a cell type-specific set of morphological, cell biological, and biochemical properties. Since the properties of the proliferating neuronal cell population from E7 chick and E6 quail sympathetic chain ganglia have been characterized in some detail (Ernsberger et al., 1989a), it was of interest to investigate the effects of MC29 infection and, for comparison, of RSV infection.

Sympathetic neurons infected by RSV were analyzed morphologically and the expression of the neuronal cell surface marker Q211 as well as the expression of TH-IR was investigated after 2 wk in culture. Although at this time period the number of nonneuronal cells had increased, the neuronal cells could easily be recognized and distinguished from the nonneuronal cells. Due to the hypertrophy induced by RSV, neuronal cell somata by that time reach a mean diameter of  $25 \pm 6.8 \mu\text{m}$  (SD), as compared to the mean size of  $9.5 \pm 2.1 \mu\text{m}$  (SD) in neurons after 3 d in culture (compare also Fig. 1 and Fig. 7). Most cells expressing neuronal morphology



**Figure 7.** Expression of Q211 antigen and TH in long-term cultures of RSV-infected neurons. Sympathetic ganglion cells were inoculated with RSV at the time of plating and stained by indirect immunofluorescence with antibodies against Q211 (*b*) or TH (*d*) after 14 d in vitro. Note the somatic hypertrophy and intensive neurite formation, as compared to noninfected controls after 3 d in culture (see Fig. 1). (*a* and *c*) Phase-contrast optics; (*b* and *d*) fluorescence optics. Bar, 50  $\mu$ m.

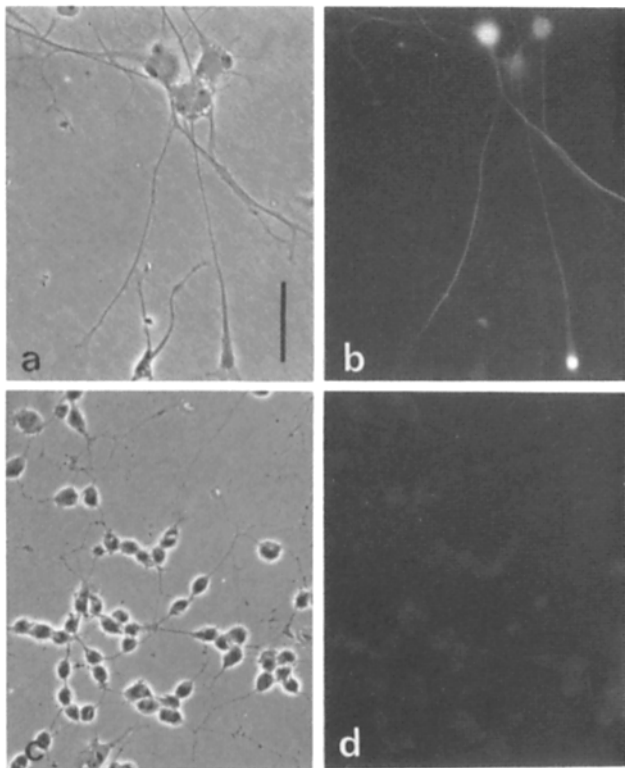
expressed also TH-IR (70%, Fig. 7 *d*). The Q211 antigen was maintained only by a subpopulation of sympathetic neurons (10%, Fig. 7 *b*), which is in agreement with an advanced state of neuronal differentiation (Rösner et al., 1988). Neuronal differentiation was also reflected by a rapid increase in the proportion of neurofilament-positive neurons after RSV infection. In controls, the fraction of NF-immunoreactive cells declined from 16% (NF<sup>68 kD</sup>) and 30% (NF<sup>160 kD</sup>) on day 1 to less than 1% (NF<sup>68 kD</sup> and NF<sup>160 kD</sup>) on day 4. In contrast, RSV infection rapidly raised the fraction of NF-expressing neurons to 50% (NF<sup>68 kD</sup>) and 70% (NF<sup>160 kD</sup>) after 4 d in culture (Fig. 8). These levels were maintained for at least 7 d in culture.

To analyze the effects of MC29 infection on the expression of cell type-specific properties of sympathetic neurons, clonal cultures were investigated rather than mass cultures, since after  $\sim$ 1 wk in culture the identity of neurons cannot be unequivocally determined simply by morphological criteria in very dense cultures. Thus, MC29-infected cells were seeded at clonal cell density in microtiter plates. Several cell colonies of presumably single cell origin were grown in the presence of CEE, passaged four to five times, and analyzed after 3–4 wk for the expression of neuronal phenotypes. Only colonies containing a major fraction of

cells with neuronal morphology were analyzed. Virtually all ( $95 \pm 8\%$ , SD) of the cells of each colony (eight colonies analyzed) were found to be positive for TH-IR and  $74 \pm 14\%$  (SD) of the cells (3 colonies analyzed) expressed the Q211 antigen (Fig. 9 and Table I). None of the colonies of MC29-infected cells showed NF immunoreactivity (NF-IR) after long-term culture. This lack of NF expression could be already observed after 7 d in culture (Fig. 8, *c* and *d*), resembling the situation in noninfected controls, where the fraction of NF-positive cells declined to less than 1% after that time period. The ability to generate clones of MC29-infected sympathetic neurons that continue to proliferate indicates that this is due to a direct effect of the transferred oncogene on the infected neurons, rather than due to indirect effects via infected nonneuronal cells. The maintenance of adrenergic properties in long-term cultures may be influenced to some extent by the presence of CEE, which supports the development of adrenergic precursor cells in neural crest cultures (Howard and Bronner-Fraser, 1985; Ziller et al., 1987), although the concentrations necessary for this effect to occur are much higher than the concentrations used in the present study.

These data indicate that *v-myc* expression, albeit stimulating neuronal proliferation, is compatible with the main-



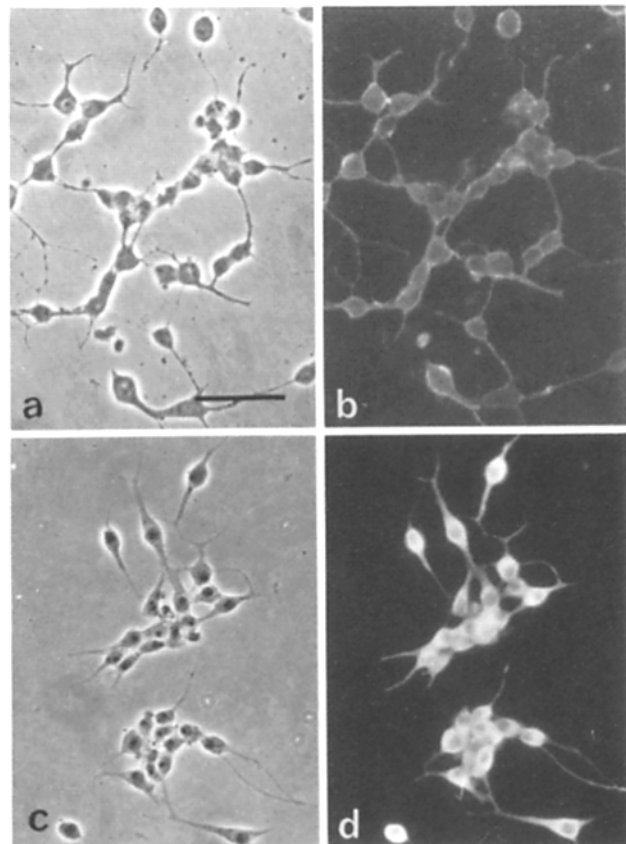


**Figure 8.** Expression of neurofilament protein in sympathetic neurons after retroviral infection. Primary cultures were infected with RSV (*a* and *b*) or MC29 (*c* and *d*) at the time of plating. After 4 d in vitro, the majority of RSV-infected cells strongly expressed NF<sup>160 kD</sup> in the soma, neurites, and growth cones (*b*). MC29 infection did neither induce nor maintain the expression of NF<sup>160 kD</sup>, as is documented by the lack of NF-IR after 7 d in culture (*d*). (*a* and *c*) Phase-contrast; (*b* and *d*) fluorescence optics. Bar, 50  $\mu$ m.

tenance of several neuronal traits. In addition to the expression of TH and Q211 it was observed that survival and the production of neurites depended on the presence of laminin substrate (data not shown), properties also displayed by the uninfected control cells. Also the lack of NF expression in MC29-infected cells parallels the situation in cultures of uninfected immature sympathetic neurons.

## Discussion

Avian sympathetic neurons from early developmental stages proliferate in vitro while expressing several neuron-specific properties. In this report we describe the effects of retrovirus transduction of oncogenes *v-src* and *v-myc* on proliferation and differentiation of E6 quail sympathetic neurons. Infection by RSV (*v-src*) inhibited proliferation and resulted in a more differentiated neuronal phenotype, reflected by the increase in the size of cell bodies and growth cones, and by an increase in neurofilament expression. These neurons could be maintained in culture for a prolonged time period in the absence of any neurotrophic factor. In contrast, infection by MC29 (*v-myc*) both maintained the proliferative state and the expression of neuronal morphology and several neuron-specific properties; i.e., the neuron-specific cell surface antigen Q211 and the adrenergic marker enzyme TH. Retroviral



**Figure 9.** Expression of Q211 antigen and TH in long-term cultures of MC29-infected neurons. Sympathetic ganglion cells were inoculated with MC29 at the time of plating, subsequently separated, and grown as discrete colonies for a period of 3–4 wk. 24 h before staining of Q211 antigen (*a* and *b*) or TH (*c* and *d*) by indirect immunocytochemistry, cells were replated on laminin substrate. (*a* and *c*) Phase-contrast optics; (*b* and *d*) fluorescence optics. Bar, 50  $\mu$ m.

transfer of *v-myc* resulted in an increase in neuronal cell number by a factor of 100 within 7 d after infection.

Although retroviral transduction of *v-src* did not result in an increased number of immature sympathetic neurons,

**Table I.** Analysis of Neuron-specific Q211 and TH Immunoreactivity in Discrete Colonies of MC29-infected Sympathetic Ganglion Cells After Long-term Culture

Colony no.	Days in vitro	Fraction of total cells expressing neuronal antigens	
		Q211	TH
		%	%
1	30	74	ND
2	30	60	ND
3	30	88	ND
4	24	ND	99
5	24	ND	95
6	24	ND	75
7	24	ND	100
8	24	ND	99
9	24	ND	98
10	24	ND	92
11	24	ND	100

which had been one of the goals of the present study, the observed effects of RSV infection are of considerable interest both with respect to *src* function and to the control of neuronal proliferation and differentiation. The transforming gene of RSV, *v-src*, has been shown to stimulate proliferation of a variety of different cell types, mainly of mesenchymal origin *in vitro* (for review, see Golden and Brugge, 1988). This increase in the proliferation rate was often accompanied by a block in differentiation. Similar results were obtained using cells of neural origin, like neural plate cells (Keane et al., 1984) or neuroretinal cells (Brackenbury et al., 1984). However, in rat pheochromocytoma cells (PC12) *v-src* expression stimulated neuronal differentiation (i.e., neurite production) in the absence of nerve growth factor (Alema et al., 1985).

In the present study we demonstrate in primary cultures of sympathetic neurons, a strong induction of differentiation caused by retroviral transduction of *v-src* as illustrated by the inhibition of proliferation, increase in the size of cell body, neurites, and growth cones, and the expression of neurofilament protein. Also, the loss of Q211 antigen can be considered as evidence for increased differentiation, as the Q211 antigen *in vivo* is also lost during neuronal differentiation (Rösner et al., 1988). These differentiation effects of RSV infection are of considerable interest, since the proto-oncogene *c-src* has been implied in neuronal differentiation and maintenance of function because it is expressed in early differentiating and postmitotic neurons (Sorge et al., 1984; Levy et al., 1984; Fults et al., 1985; Cotton and Brugge, 1983) in addition to an early phase of *c-src* expression in proliferating neural cells during neural tube formation (Maness et al., 1986). This correlation between the biological effects of *v-src* and the mode of expression of *c-src* suggests the possibility that sympathetic neuronal differentiation may be stimulated by both *v-src* and *c-src*. However, with respect to the *src* effects on cellular proliferation, it has been demonstrated that overexpression of *c-src* does not induce the growth effects of *v-src* in fibroblasts (Iba et al., 1984; Parker et al., 1984; Shalloway et al., 1984) and neuroretina cells (Iba et al., 1985). Thus, it has been concluded that the functional role of *c-src* in normal cells is different from the role of *v-src*. The *src* effects on neuronal differentiation could, however, represent an exception to that rule as the protein encoded by neuronal *c-src* is also a different form of pp60<sup>*c-src*</sup> with higher specific activity (Brugge et al., 1985, 1987; Lynch et al., 1986; Martinez et al., 1987). The difference between the presently described differentiation effects of RSV infection and the observed proliferative effect on neural cultures (Péssac et al., 1983; Keane et al., 1984; Iba et al., 1985) may reflect the stage of differentiation of the cell types infected, the sympathetic neuron being an exception by its expression of differentiated traits during proliferation (Cohen, 1974; Rothman et al., 1978; Rohrer and Thoenen, 1987).

The infection by RSV does not only induce morphological differentiation but also leads to the long-term survival of infected neurons. The sympathetic neurons from E6 quail or E7-E8 chick neurons depend for their survival on a suitable substrate like laminin, and their survival cannot be influenced by neurotrophic factors on these substrates (Ernsberger et al., 1989b; the present study). However, the neurons cannot be maintained for long time periods in culture,

after ~4 d in culture the cells start to degenerate and neuronal death cannot be prevented by NGF (Ernsberger et al., 1989b). In contrast, during *in vivo* development sympathetic neurons acquire responsiveness to neurotrophic factors. The effect of *v-src* on neuronal survival may thus indicate that (a) the infected cells are now able to respond to NGF produced by nonneuronal cells; (b) expression of *v-src* maintains the differentiation status the cells have during the initial culture period; and (c) expression of *v-src* (i.e., pp60<sup>*v-src*</sup> kinase activity), results in the activation of a neurotrophic factor signal transduction pathway. The first possibility has been excluded since survival of RSV-infected neurons is not influenced by the presence of anti-NGF antibodies, which were previously shown to block the activity of avian NGF (Rohrer et al., 1988). It is however not possible to distinguish between the latter two possibilities since neither the mode of action of *src* proteins nor the signal transduction pathway of neurotrophic factors after binding to their cell surface receptors is understood (but see Borasio et al., 1989), and the mode of NGF-independent survival of less mature cells is completely unknown.

Infection of sympathetic neurons by *v-myc*-containing retrovirus resulted in a dramatic increase in the proportion of proliferating cells as has been described for other avian cell types. Since the levels of *c-myc* mRNA (Kelly et al., 1983) increase when cells are stimulated by mitogens and growth factors, a role for the *myc* proteins in the transduction of mitogenic signals from growth factor receptors, i.e., in the control of cell proliferation, has been assumed. Since in several cellular systems differentiation processes are accompanied by a decrease in *c-myc* expression (Westin et al., 1982; Reitsma et al., 1983; Campisi et al., 1984; Lachmar and Skoultchi, 1984), and since high levels of *c-myc* or *v-myc* expression prevent or delay differentiation of cells (Falcone et al., 1985; Denis et al., 1987; Schneider et al., 1987), it has been suggested that *myc* expression would also be involved in the control of differentiation. In the present study, we observed that MC29-infected cells, although proliferating at high rates, maintained many functions of uninfected cells, i.e., neuron-like morphology, a neuron-specific surface antigen, the adrenergic marker enzyme TH, requirement for a laminin substrate, and limited ability to survive in culture. As in cultured uninfected cells, NF expression in immature MC29-infected cells is still unstable and NF-IR is no more detectable after several days in culture. These results support and extend previous observations that *v-myc* expression in avian neural retina cells is compatible with the expression of differentiated properties (Casalbore et al., 1987). Although the proportion of <sup>3</sup>H-thymidine-labeled neurons is increased after MC29 infection, it is not clear if this is only due to a stimulation of neuronal proliferation. Since the cell cycle length of neural crest derivatives increases with increasing differentiation (Maxwell, 1976; Maxwell and Sietz, 1985), it is both conceivable that MC29 infection maintains the high proliferation of immature sympathetic neurons and/or stimulates their proliferation.

Since in MC29-infected cultures nonneuronal cells start to proliferate and tend to overgrow the cultures, the phenotype of MC29-infected cells was analyzed in discrete cell colonies obtained by limiting dilution. It was observed that in some of these homogenous colonies the majority of cells still maintained neuronal morphology, TH, and the Q211 antigen

after the fifth passage. The *in vitro* survival of MC29-infected cells always depended on the presence of CEE. Since the sympatho-adrenal precursor cells present in neural crest cultures were found to depend for their differentiation and/or survival on the presence of CEE (Howard and Bronner-Fraser, 1985; Ziller et al., 1987), it is suggested that the present infection protocol selects for the less mature neuronal subpopulation of E6 sympathetic ganglia.

In summary, these results clearly demonstrate that infection by *myc*-containing avian retroviruses can be used to produce large numbers of immature sympathetic neurons which maintain many neuron-specific properties. It is possible to expand rare populations of dividing precursor cells, making these cells available for biochemical and molecular analysis. This approach seems to be limited by the complex growth and survival requirements of the infected cells, rather than by gross changes of phenotype induced by the infection.

We thank Hans Thoenen for continuous support; Hartmut Beug, Thomas Graf, and Klaus von der Helm for advice and help with retroviral techniques; and Peter-Hans Hofschneider and Wolfgang Neubert for lab facilities. Thanks are due for helpful comments on the manuscript to Hartmut Beug, Manfred Schartl, Christiana Rüdell, and Patrick Carroll.

This work has been supported by the Deutsche Forschungsgemeinschaft (SFB 220).

Received for publication 18 December 1989 and in revised form 15 February 1990.

## References

- Abrams, H. D., L. R. Rohrschneider, and R. N. Eisenman. 1982. Nuclear location of the putative transforming protein of avian Myelocytomatosis virus. *Cell*. 29:427-439.
- Alema, S., P. Casalbore, E. Agostini, and F. Tato. 1985. Differentiation of PC12 pheochromocytoma cells induced by *v-src* oncogene. *Nature (Lond.)*. 316:557-559.
- Anderson, D. J., and R. Axel. 1986. A bipotential neuroendocrine precursor whose choice of cell fate is determined by NGF and glucocorticoids. *Cell*. 47:1079-1090.
- Bartlett, P. F., H. H. Reid, K. A. Bailey, and O. Bernard. 1988. immortalization of mouse neural precursor cells by the *c-myc* oncogene. *Proc. Natl. Acad. Sci. USA*. 85:3255-3259.
- Bazill, C. W., M. Haynes, J. Garland, and T. M. Dexter. 1983. Characterization and partial purification of a haemopoietic cell growth factor in WEHI-3 cell conditioned medium. *Biochem. J.* 210:747-759.
- Boettiger, D., K. Roby, J. Brumbaugh, J. Biehl, and H. Holtzer. 1977. Transformation of chicken embryo retinal melanoblasts by a temperature-sensitive mutant of Rous Sarcoma Virus. *Cell*. 11:881-890.
- Borasio, G. D., J. John, A. Wittinghofer, Y.-A. Barde, M. Sendtner, and R. Heumann. 1989. *ras* p21 protein promotes survival and fiber outgrowth of cultured embryonic neurons. *Neuron*. 2:1087-1096.
- Brackenbury, R., M. E. Greenberg, and G. M. Edelman. 1984. Phenotypic changes and loss of N-CAM-mediated adhesion in transformed embryonic retinal cells. *J. Cell Biol.* 99:1944-1954.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Brugge, J. S., P. C. Cotton, A. E. Queral, J. N. Barrett, D. Nonner, and R. W. Keane. 1985. Neurons express high levels of a structurally modified activated form of pp60<sup>src</sup>. *Nature (Lond.)*. 316:554-557.
- Brugge, J., P. Cotton, A. Lustig, W. Yonemoto, L. Lipsich, P. Coussens, J. N. Barrett, D. Nonner, and R. W. Keane. 1987. Characterization of the altered form of the *c-src* gene product in neuronal cells. *Genes & Dev.* 1:287-296.
- Bunte, T., I. Greiser-Wilke, and K. Moelling. 1982. Association of *gag-myc* proteins from avian myelocytomatosis virus wild-type and mutants with chromatin. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:919-927.
- Bunte, T., P. Donner, E. Pfaff, B. Reis, I. Greiser-Wilke, H. Schaller, and K. Moelling. 1984. Inhibition of DNA binding of purified p55<sup>src</sup> *in vitro* by antibodies against bacterially expressed *myc* protein and a synthetic peptide. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1919-1924.
- Burnham, P., C. Raiborn, and S. Varon. 1972. Replacement of nerve growth factor by ganglionic non-neuronal cells for the survival *in vitro* of dissociated ganglionic neurons. *Proc. Natl. Acad. Sci. USA*. 69:3556-3560.
- Campisi, J., H. E. Gray, A. B. Pardee, M. Dean, and G. E. Sonenshein. 1984. Cell-cycle control of *c-myc* but not *c-ras* expression is lost following chemi-

- cal transformation. *Cell*. 36:241-247.
- Casalbore, P., E. Agostini, S. Alema, G. Falcone, and F. Tato. 1987. The *v-myc* oncogene is sufficient to induce growth transformation of chick neuroretina cells. *Nature (Lond.)*. 326:188-190.
- Cohen, A. 1974. DNA synthesis and cell division in differentiating avian adrenergic neuroblasts. In Wenner-Gren Center International Symposium Series. Vol. 22. K. Fuxe, L. Olson, and Y. Zotterman, editors. Pergamon Press, Oxford. 359-370.
- Cotton, P. C., and J. S. Brugge. 1983. Neural tissues express high levels of the cellular *src* gene product pp60<sup>src</sup>. *Mol. Cell. Biol.* 3:1157-1162.
- Crisanti, P., A. M. Lorinet, G. Caloty, and B. Pessac. 1985. Glutamic acid decarboxylase activity is stimulated in quail retina neuronal cells transformed by Rous sarcoma virus and is regulated by pp60<sup>src</sup>. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1467-1470.
- Denis, N., M. P. Blanc, N. Leibovitch, F. Nicolaiw, F. Dautry, M. Raymond-jean, J. Kruh, and A. Kitzis. 1987. *c-myc* oncogene expression inhibits the initiation of myogenic differentiation. *Exp. Cell Res.* 172:212-217.
- DiCocco-Bloom, E., and I. B. Black. 1988. Insulin growth factors regulate the mitotic cycle in cultured rat sympathetic neuroblasts. *Proc. Natl. Acad. Sci. USA*. 85:4066-4070.
- Donner, P., I. Greiser-Wilke, and K. Moelling. 1982. Nuclear localization and DNA binding of the transforming gene product of avian myelocytomatosis virus. *Nature (Lond.)*. 296:262-266.
- Edgar, D., Y.-A. Barde, and H. Thoenen. 1981. Subpopulations of cultured chick sympathetic neurons differ in their requirements for survival factors. *Nature (Lond.)*. 289:294-295.
- Ersnerberger, U., D. Edgar, and H. Rohrer. 1989a. The survival of early chick sympathetic neurons *in vitro* is dependent on a suitable substrate but independent on NGF. *Dev. Biol.* 135:250-262.
- Ersnerberger, U., M. Sendtner, and H. Rohrer. 1989b. Proliferation and differentiation of embryonic chick sympathetic neurons: effects of ciliary neurotrophic factor. *Neuron*. 2:1275-1284.
- Falcone, G., F. Tato, and S. Alema. 1985. Distinctive effects of the viral oncogenes *myc*, *erb*, *fps*, and *src* on the differentiation program of quail myogenic cells. *Proc. Natl. Acad. Sci. USA*. 82:426-430.
- Fiszman, M. Y., and P. Fuchs. 1975. Temperature-sensitive expression of differentiation in transformed myoblasts. *Nature (Lond.)*. 254:429-431.
- Frederiksen, K., P. S. Jat, N. Valtz, D. Levy, and R. McKay. 1988. Immortalization of precursor cells from the mammalian CNS. *Neuron*. 1:439-448.
- Fults, D. W., A. C. Towle, J. M. Lauder, and P. F. Maness. 1985. pp60<sup>src</sup> in the developing cerebellum. *Mol. Cell. Biol.* 5:27-32.
- Gionti, E., G. Pontarelli, and R. Cancedda. 1985. Avian myelocytomatosis virus immortalizes differentiated quail chondrocytes. *Proc. Natl. Acad. Sci. USA*. 82:2756-2760.
- Golden, A., and J. S. Brugge. 1988. The *src* oncogene. In The Oncogene Handbook. E. P. Reddy, A. M. Skalka, and T. Curran, editors. Elsevier Science Publishing Co. Inc., New York. 149-173.
- Graf, T., and H. Beug. 1978. Avian leukemia viruses: interaction with their target cells *in vivo* and *in vitro*. *Biochim. Biophys. Acta*. 516:269-299.
- Hamburger, V., and H. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88:49-92.
- Howard, M. J., and M. Bronner-Fraser. 1985. The influence of neural tube-derived factors on differentiation of neural crest cells *in vitro*. I. Histochemical study on the appearance of adrenergic cells. *J. Neurosci.* 5:3302-3309.
- Iba, H., T. Takeya, F. Cross, T. Hanafusa, and H. Hanafusa. 1984. Rous sarcoma virus variants which carry the cellular *src* gene instead of the viral *src* gene cannot transform chicken embryo fibroblasts. *Proc. Natl. Acad. Sci. USA*. 81:4424-4428.
- Iba, H., R. Jove, and H. Hanafusa. 1985. Lack of induction of neuroretinal cell proliferation by rous sarcoma virus variants that carry the *c-src* gene. *Mol. Cell. Biol.* 5:2856-2859.
- Jaffredo, T., B. Vandenbunder, and F. Dieterlen-Lievre. 1989. *In situ* study of *c-myc* protein expression during avian development. *Development (Camb.)*. 105:679-695.
- Keane, R. W., L. A. Lipsich, and J. S. Brugge. 1984. Differentiation and transformation of neural plate cells. *Dev. Biol.* 103:38-52.
- Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder. 1983. Cell-specific regulation of the *c-myc* gene by lymphocyte mitogens and platelet-derived growth factor. *Cell*. 39:339-348.
- Krueger, J. G., E. A. Garber, and A. R. Goldberg. 1983. Subcellular localization of pp60<sup>src</sup> in RSV-transformed cells. *Curr. Top. in Microbiol. Immunol.* 107:51-124.
- Lachman, H. M., and A. I. Skoultchi. 1984. Expression of *c-myc* changes during differentiation of mouse erythroleukemia cells. *Nature (Lond.)*. 310:592-594.
- Land, H., L. F. Parada, and R. A. Weinberg. 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature (Lond.)*. 304:596-602.
- Leutz, A., H. Beug, and T. Graf. 1984. Purification and characterization of cMGF, a novel chicken myelomonocytic growth factor. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3191-3197.
- Leutz, A., K. Damm, E. Sterneck, E. Kowenz, S. Ness, R. Frank, H. Gausepohl, Y.-C. E. Pan, J. Smart, M. Hayman, and T. Graf. 1989. Molecular cloning of the chicken myelomonocytic growth factor (cMGF) reveals relationship to interleukin 6 and granulocyte colony stimulating factor.

- EMBO (Eur. Mol. Biol. Organ.) J.* 8:175-181.
- Levi-Montalcini, R., and P. U. Angeletti. 1963. Essential role of the nerve growth factor in the survival and maintenance of dissociated sensory and sympathetic embryonic nerve cells *in vitro*. *Dev. Biol.* 7:653-659.
- Levy, B. T., L. K. Sorge, A. Meymandi, and P. F. Maness. 1984. pp60<sup>src</sup> kinase is in embryonic tissues of chick and human. *Dev. Biol.* 104:9-17.
- Lipsich, L. A., A. J. Lewis, and J. S. Brugge. 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruses. *J. Virol.* 48:352-360.
- Lynch, S. A., J. S. Brugge, and J. M. Levine. 1986. Induction of altered *c-src* product during neural differentiation of embryonal carcinoma cells. *Science (Wash. DC)*. 234:873-876.
- Maness, P. F., L. K. Sorge, and D. W. Fufts. 1986. An early developmental phase of pp60<sup>src</sup> expression in the neural ectoderm. *Dev. Biol.* 117:83-89.
- Manthorpe, M., and S. Varon. 1985. Regulation of neuronal survival and neuritic growth in the avian ciliary ganglion by trophic factors. In *Growth and Maturation Factors*. Vol. 3. G. Guroff, editor. John Wiley & Sons Inc., New York. 77-117.
- Martinez, R., B. Mathey-Prevot, A. Bernards, and D. Baltimore. 1987. Neuronal pp60<sup>src</sup> contains a six-amino acid insertion relative to its non-neuronal counterpart. *Science (Wash. DC)*. 237:411-415.
- Maxwell, G. D. 1976. Cell cycle changes during neural crest cell differentiation *in vitro*. *Dev. Biol.* 49:66-79.
- Maxwell, G. D., and P. D. Sietz. 1985. Development of cells containing catecholamines and somatostatin-like immunoreactivity in neural crest cultures: relationship of DNA synthesis to phenotypic expression. *Dev. Biol.* 108:203-209.
- Ness, S. A., H. Beug, and T. Graf. 1987. *V-myc* dominance over *v-myc* in doubly transformed chick myelomonocytic cells. *Cell*. 51:41-50.
- Pacifici, M., D. Boettiger, K. Roby, and H. Holtzer. 1977. Transformation of chondroblasts by Rous Sarcoma Virus and synthesis of the sulfated proteoglycan matrix. *Cell*. 11:891-899.
- Palmieri, S., P. Kahn, and T. Graf. 1983. Quail embryo fibroblasts transformed by four *v-myc*-containing virus isolates show enhanced proliferation but are non-tumorigenic. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2385-2389.
- Parker, R., H. E. Varmus, and J. M. Bishop. 1984. Expression of *v-src* and chicken *c-src* in rat cells demonstrates qualitative differences between pp60<sup>src</sup> and pp60<sup>c-src</sup>. *Cell*. 37:131-139.
- Pessac, B., A. Girard, G. Romey, P. Crisani, A.-M. Lorinet, and G. Calothy. 1983a. A neuronal clone derived from a Rous sarcoma virus-transformed quail embryo neuroretina established culture. *Nature (Lond.)*. 302:616-618.
- Pessac, B., A. Girard, G. Romey, P. Crisanti, M. Wassef, A. Privat, and G. Calothy. 1983b. Cells with neuronal properties in permanent cultures of quail embryo neuroretinas infected with Rous sarcoma virus. *Brain Res.* 275:53-59.
- Pessac, B., C. Ziller, J. Vautrin, A. Girard, and G. Calothy. 1985. Quail neural crest cells transformed by Rous sarcoma virus can be established into differentiating permanent cell cultures. *Dev. Brain Res.* 20:235-239.
- Reitsma, P. H., P. G. Rothberg, S. M. Astrin, J. Trial, Z. Bar-Shavit, A. Hall, S. L. Teitelman, and A. J. Kahn. 1983. Regulation of *c-myc* gene expression in HL60 leukemia cells by a vitamin D metabolite. *Nature (Lond.)*. 306:492-494.
- Resh, M. D., and R. L. Erikson. 1985. Highly specific antibody to Rous Sarcoma Virus *src* gene product recognizes a novel population of pp60<sup>src</sup> and pp60<sup>c-src</sup> molecules. *J. Cell Biol.* 100:409-417.
- Rohrer, H., and H. Thoenen. 1987. Relationship between differentiation and terminal mitosis: chick sensory and ciliary neurons differentiate after terminal mitosis of precursor cells, whereas sympathetic neurons continue to divide after differentiation. *J. Neurosci.* 7:3739-3748.
- Rohrer, H., A. L. Acheson, J. Thibault, and H. Thoenen. 1986. Developmental potential of quail dorsal root ganglion cells analyzed *in vitro* and *in vivo*. *J. Neurosci.* 6:2616-2624.
- Rohrer, H., M. Hofer, R. Hellweg, S. Korsching, A. D. Stehle, S. Saadat, and H. Thoenen. 1988. Antibodies against mouse nerve growth factor interfere *in vivo* with the development of avian sensory and sympathetic neurones. *Development (Camb.)* 103:545-552.
- Rohrschneider, L. R. 1979. Immunofluorescence on avian virus-transformed cells: localization of the *src* gene product. *Cell*. 16:11-24.
- Rösner, H., M. Al-Aqtum, and S. Henke-Fahle. 1985. Developmental expression of GD3 and Polysialogangliosides in embryonic chicken nervous tissue reacting with monoclonal antiganglioside antibodies. *Dev. Brain Res.* 18:85-95.
- Rösner, H., C. Greis, and S. Henke-Fahle. 1988. Developmental expression in embryonic rat and chicken brain of a polysialoganglioside-antigen reacting with the monoclonal antibody Q211. *Dev. Brain Res.* 42:161-171.
- Rothman, T. P., M. D. Gershon, and H. Holtzer. 1978. The relationship of cell division to the acquisition of adrenergic characteristics by developing sympathetic ganglion cell precursors. *Dev. Biol.* 124:331-346.
- Ruppert, C., Goldowitz, D., and W. Willie. 1986. Proto-oncogene *c-myc* is expressed in cerebellar neurons at different developmental stages. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1897-1901.
- Schneider, M. D., M. B. Perryman, P. A. Payne, G. Spizz, R. Roberts, and E. N. Olson. 1987. Autonomous expression of *c-myc* in BC<sub>3</sub>H1 cells partially inhibits but does not prevent myogenic differentiation. *Mol. Cell. Biol.* 7:1973-1977.
- Shalloway, D., P. M. Coussens, and P. Yaciuk. 1984. Overexpression of the *c-src* protein does not induce transformation of NIH-3T3 cells. *Proc. Natl. Acad. Sci. USA.* 81:7071-7075.
- Sorge, L. K., B. T. Levy, and P. F. Maness. 1984. pp60<sup>src</sup> is developmentally regulated in the neural retina. *Cell*. 36:249-257.
- Westin, E. H., F. Wong-Staal, E. P. Gelmann, R. Dalla-Favera, T. S. Papas, J. A. Lautenberger, A. Eva, E. P. Reddy, S. R. Tronick, S. A. Aaronson, and R. C. Gallo. 1982. Expression of cellular homologues of retroviral oncogenes in human hematopoietic cells. *Proc. Natl. Acad. Sci. USA.* 79:2490-2494.
- Zacchei, A. M. 1961. Lo sviluppo embrionale della quaglia giapponese (*Coturnix coturnix japonica*, T.e.S.) *Arch. Ital. Anat. Embriol.* 66:36-62.
- Ziller, C., M. Fauquet, C. Kalcheim, J. Smith, and N. M. LeDouarin. 1987. Cell lineages in peripheral nervous system ontogeny: medium-induced modulation of neuronal phenotypic expression in neural crest cell cultures. *Dev. Biol.* 120:101-111.