myc products induce the expression of catecholaminergic traits in quail neural crest-derived cells

(proliferation/differentiation/oncogenes)

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ABSTRACT The avian myelocytomatosis virus strain MC29 *v-myc* oncogene transforms a wide panel of avian cells *in vitro* and either blocks or maintains differentiation, depending on the cell type. In the present work, we have investigated the effect of this oncogene on the differentiation of early embryonic cells, neural crest cells, grown *in vitro*. We report that the MC29 *v-myc* gene product induces a strong cellular proliferation of 2-day quail neural crest with the appearance of catecholaminergic traits. Other *v-myc* as well as the *c-myc* gene products also trigger this phenotype. Retroviruses carrying some other oncogenes do not elicit this phenotypic expression, although they activate cell multiplication. Thus, our results indicate that *myc* gene products induce (directly or indirectly) a differentiated phenotype in a subpopulation of neural crest cells.

The avian myelocytomatosis virus strain MC29 induces *in vivo* myelocytomatosis, lymphoid leukemia, kidney liver carcinomas, and tumors of myogenic origin (1–4). MC29 virus expresses in the infected cells a gag-myc fusion protein of 110 kDa (P110^{gag-myc}) (5). MC29 alters the competence for terminal differentiation of myogenic cells, but transformation of chondroblasts or macrophages by this virus does not suppress the appearance of differentiated traits (3, 6–8).

Neural crest cells are the progenitors of neuronal and glial derivatives of the peripheral nervous system (9). Here, we have focused on the catecholaminergic phenotype, which is found *in vivo* in neural crest-derived structures such as sympathetic ganglia and adrenal medulla (10, 11). *In vitro*, 2-day neural crest cells, cultured in the absence of other cell types, only express catecholaminergic traits after addition of 10–15% chicken embryo extract (CEE) (12–15).

Our results indicate that the *v-myc* and the *c-myc* gene products greatly induce cell proliferation with the appearance of catecholaminergic characteristics in a subpopulation of neural crest cells grown in medium lacking CEE [nonpermissive (NP) medium]. This effect is specific for the catecholaminergic pathway, since no acetylcholine synthesis can be found in these cultures, whereas early crest cells preferentially synthesize this neurotransmitter *in vitro* (15, 16). No tyrosine hydroxylase (TyrOHase)-positive cells were detected in neural crest cultures infected with retroviruses that carry other viral oncogenes. Surprisingly, none of the TyrOHase-positive cells of sympathetic ganglia or adrenal gland was induced to proliferate after MC29 infection, suggesting that the *myc* protein is effective before, but not after, catecholaminergic differentiation has occurred.

MATERIALS AND METHODS

Culture Methods. Embryonic cells. Two-day trunk neural crest cells were obtained from neural tubes isolated from quail embryos with 20–24 somite pairs as described (17). Ciliary ganglia were dissected from four 5- to 7-day embryos, sensory ganglia from 5- to 9-day embryos, sympathetic ganglia and adrenal gland from 6- to 9-day embryos, and neural tubes from 3- to 7-day embryos.

Culture condition. Two-day trunk neural crests were grown in Dulbecco's modified minimum essential medium (GIBCO) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (GIBCO). This medium represents the NP basal medium.

In some experiments, 15% of 11-day CEE was added to the neural crest culture medium to allow the differentiation of catecholamine-expressing cells as described (12).

Viruses and cell infection. Supernatants of 7-day quail or chicken neuroretina cells infected by the avian retrovirus MC29 produced from the molecular clone pMC38 (18, 19), by avian carcinoma virus MH2 (20), by MH2 mutant virus MH2-PA 200 (19), and by the human *c-myc* gene inserted in a retroviral vector, vSX-AHM (21), were used in these experiments. All viruses were pseudotyped with Rous-associated RAV-1 helper virus produced from a molecular clone.

Immunolabeling of Cells. Antibodies. The identification of TyrOHase was performed by using a monoclonal antibody (15). *Myc*-encoded products were revealed with a rabbit antiserum (22). The presence of dopamine and laminin was detected with rabbit antisera given respectively by Dr. Tramu and Dr. Päivi Liesi. Fibronectin was detected with a commercial antibody (BRL).

Immunostaining procedures. Cells, grown on collagen-coated coverslips, were fixed 1 hr in 4% paraformaldehyde in phosphate-buffered saline (PBS). Cells were permeabilized with 0.25% Triton X-100 for 10 min, washed, and incubated for 3 hr with the different antibodies.

Fluorescein isothiocyanate- or tetramethylrhodamine-conjugated antibodies (Biosys, Compiègne, France) were used as second antibodies. Cells were washed in PBS, mounted in glycerol, examined with a Zeiss IM35 fluorescence microscope, and photographed on Tri X-Pan Film.

Neurotransmitter Synthesis and Accumulation. Synthesis of acetylcholine and catecholamine by cultured cells was measured by incorporation of [³H]choline (Amersham; specific activity, 10 Ci·mmol⁻¹, 1 Ci = 37 GBq) and [³H]tyrosine

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Abbreviations: CEE, chicken embryo extract; NP, nonpermissive; TyrOHase, tyrosine hydroxylase.

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(Amersham; specific activity, $33 \text{ Ci}\cdot\text{mmol}^{-1}$) for 4 hr followed by high-voltage paper electrophoresis (14).

[^3H]Thymidine Incorporation and Autoradiography. Neural crest cultures were labeled by the addition of [^3H]thymidine (specific activity, $25 \text{ Ci}\cdot\text{mmol}^{-1}$; CEA, Gif-sur-Yvette) to the medium (final concentration, $0.2 \mu\text{Ci}/\text{ml}$). The cells were then washed extensively in PBS and treated for immunocytochemistry as described below. The cultures were coated with NTB₂ emulsion (Kodak). Autoradiographs were exposed for 3 days, developed with the D19 developer (Kodak), and mounted in glycerol.

Protein Labeling, Immunoprecipitation, and Immunoblotting (Western Blotting). Cells were labeled, lysed, and immunoprecipitated as described (19) with a rabbit anti-myc antiserum (22), a rabbit anti-mil antiserum (23), and a rabbit anti-gag antiserum. Immunoprecipitated proteins were analyzed by SDS/PAGE, followed by fluorography (19). For Western blotting, cells were trypsinized, and lysates were made as described (23). A mouse antibody labeled with diaminobenzidine (Biosys, Compiègne, France) was used to reveal the binding of the monoclonal antibody to the TyrOHase.

RESULTS

Expression of Catecholamine Traits Exhibited by MC29-Infected Neural Crest Cells. Two-day neural crest cells grown in NP medium divided poorly and never expressed TyrOHase. In the presence of 15% CEE, the growth of neural crest cells was slightly increased, and after at least 1 week, some catecholamine-expressing cells were found (13). They represented less than 1% of the total cell population.

Infection of 2-day neural crest cells in NP medium with MC29 (RAV-1) viruses resulted within 3–4 days in the appearance of several foci of actively dividing cells. Beginning with some 100 cells, we obtained several-100,000 cells after 3 weeks. These cells could be passaged at least 30 times. Two cell types were seen by phase-contrast observation in infected culture. The first type was of a polygonal shape and appeared flattened on the Petri dish, whereas the second type showed a more ovoid morphology and a reduced substrate adherence (Fig. 1a).

Examination of the phenotypic properties of MC29-infected neural crest cells indicated that no cell expressed neurofilament proteins (data not shown), whereas melanocytes differentiated in about 10% of the cultures (Fig. 1b). TyrOHase-immunoreactive cells appeared as early as 5 days after infection (Fig. 1c). The percentage of TyrOHase-positive cells increased until generations 4–5, where they represented about 16% of the total population. These cells also converted [^3H]tyrosine into dopamine and noradrenaline or adrenaline (see below). Subsequently, the percentage of TyrOHase-positive cells began to decrease, and after passage 15–17, no TyrOHase-expressing cells could be found. In cultures where the TyrOHase-expressing cells were disappearing, the TyrOHase-positive cells incorporated less [^3H]thymidine compared with the surrounding cells (Fig. 1d and e).

The P110^{gag-myc} product could be detected by immunoprecipitation with either the anti-myc or the anti-gag antibodies (Fig. 2) in lysates of MC29-infected neural crest cells. In TyrOHase-positive cells infected by MH2, we also detected the P100^{gag-mil} and p61-63^{v-myc} proteins (20). The presence of TyrOHase, previously detected by immunofluorescence, was confirmed by Western blot analysis, in which a single 63-kDa protein was recognized by the specific anti-TyrOHase antibody (data not shown). TyrOHase-immunoreactive cells as well as TyrOHase-negative cells expressed the myc protein localized in the nucleus, as shown by double immunolabeling experiments (Fig. 3).

FIG. 1. Morphology and TyrOHase expression of trunk neural crest cells differentiated in the presence of myc-encoded products. (a) Phase-contrast microphotography of neural crest cells infected by MC29 virus (passage 10). (Bar = $30 \mu\text{m}$.) (b) Melanocytes differentiated in MC29-infected neural crest cell cultures (passage 6). (Bar = $30 \mu\text{m}$.) (c) TyrOHase-expressing cells in MC29-infected neural crest cell cultures (passage 5). (Bar = $60 \mu\text{m}$.) (d and e) TyrOHase-expressing cells and [^3H]thymidine incorporation: a TyrOHase-positive cell (arrow) is completely devoid of silver grains over its nucleus, whereas the cells of the underlayer are heavily labeled. (Bars = $40 \mu\text{m}$.) (f) TyrOHase-expressing cells in MH2-infected neural crest cell cultures (passage 6). (Bar = $62 \mu\text{m}$.) (g) TyrOHase-positive cells in vSX-AHM-infected neural crest cell cultures. (Bar = $62 \mu\text{m}$.)

Does the MC29 Virus Act on Autonomic Precursors To Allow the Expression of TyrOHase Protein? In the above experi-

FIG. 2. Expression of the oncogene products in neural crest-derived cells infected by MC29 (lanes 1–4) and MH2 (lanes 5 and 6) viruses. The cell proteins labeled *in vitro* were immunoprecipitated with the anti-myc antiserum (lanes 1–5) or with the anti-mil antiserum (lane 6). Lanes: 1, 2-day neural crest cells; 2, 5-day sensory ganglia; 3, 7-day sympathetic ganglia; 4, 7-day adrenal medulla; 5 and 6, 2-day neural crest cells.

FIG. 3. *myc* expression in neural crest cells. (a and b) Labeling of noninfected (a) and MC29-infected (b) neural crest cells by the anti-*myc* antibody. (c and d) MC29-infected neural crest cells were double-labeled with the anti-TyrOHase antibody (c) and the anti-*myc* antibody (d). The nuclei of the two TyrOHase-positive cells (arrows) are also decorated by the anti-*myc* antiserum. (Bars = 30 μ m.)

ments, we used the culture medium conditioned by quail neuroretina cells that had been infected by MC29 (RAV-1) virus and were producing viral particles. To determine whether viral particles were implicated in the appearance of TyrOHase-positive cells, we centrifuged (45 min at $210,000 \times g$) the culture medium of MC29 (RAV-1)-infected quail neuroretina cells as well as the culture medium of the MC29 (RAV-1)-infected neural crest cells.

After addition of viral pellets (each resuspended in a fresh NP medium), the morphology of neural crest cells was rapidly modified. Foci of fusiform cells emerged in the cultures and, among them, TyrOHase-expressing cells differentiated (Fig. 4 *a* and *b*). No morphologically modified and no TyrOHase-expressing cells appeared when cells were grown in the supernatant of centrifuged mediums. In a set of control experiments, we centrifuged in the same manner the medium enriched with 15% CEE. When grown in the supernatant of 15% CEE-enriched medium, neural crest cells in contrast were able to differentiate into TyrOHase-expressing cells.

We have examined the effect of some extracellular matrix components on the induction of the observed phenotype. We cultured uninfected neural crest cells in NP medium on ghosts of MC29-transformed neural crest cells lysed by distilled water or previously fixed by ethanol. None of these substrates was able to promote catecholaminergic differentiation. In addition, as shown in Fig. 4 *e* and *f*, the distribution of laminin appeared similar, as judged by the immunohistochemistry experiment, in MC29-infected neural crest cell cultures and control noninfected neural crest cell cultures. In contrast, fibronectin immunoreactivity was somewhat lower in infected cultures (Fig. 4 *c* and *d*).

Characterization of the Phenotype Displayed by TyrOHase-Positive, MC29-Infected Neural Crest Cells. TyrOHase converts tyrosine into dopa. Dopa is then transformed into dopamine, noradrenaline, or adrenaline depending on the type of catecholaminergic cell. To determine whether the whole catecholaminergic biosynthetic pathway was activated in neural crest cells after MC29 infection, we looked first for the storage of catecholamine and second for neurotransmitter biosynthetic activity. TyrOHase-positive cells were doubly labeled with an antibody directed against dopamine, the first

FIG. 4. Influence of MC29 viral particles on the expression of the TyrOHase protein and on the composition of the extracellular matrix. (a and b) Expression of the TyrOHase protein in neural crest cells grown in the presence of the pellet of either centrifuged MC29 NR medium (a) or of centrifuged MC29 neural crest medium (b). (c and d) Labeling of control noninfected neural crest cell culture (c) and MC29-infected neural crest cell culture (d) by the anti-fibronectin antibody. The infected culture appears less labeled than the control. (e and f) Labeling of control noninfected neural crest cell culture (e) and MC29-infected neural crest cell culture (f) by the anti-laminin antiserum. (Bars = 60 μ m.)

biogenic amine produced by the catecholamine metabolic pathway (Fig. 5 *a* and *b*).

Infected MC29 neural crest cells also exhibited a catecholaminergic metabolism, converting [3 H]tyrosine into dopamine and noradrenaline or adrenaline (Fig. 6).

Catecholaminergic Differentiation of Neural Crest Cells in NP Medium Is Not a Simple Consequence of the Proliferating State. To assess if other *myc* products are able to induce TyrOHase phenotype, we tested the effect of the human c-*myc* produced from vSX-AHM virus (21) and the effect of the v-*myc* protein of the MH2 virus. Both products effectively induced the TyrOHase phenotype, as shown in Fig. 1 *f* and *g*. Transformation alone is not able to induce this phenotype since v-*src*-containing retroviruses, RSV-PRA and RSV-SRA, previously reported to be efficient transforming agents for neural crest cells (24, 25), were found ineffective for the TyrOHase expression. No TyrOHase-positive cells were found with other viral oncogene products, all able to induce neural crest cell proliferation, such as *erbB* and

FIG. 5. Double immunostaining of MC29 neural crest cells. Infected neural crest cells were double-labeled with the anti-TyrOHase antibody (a) and the anti-dopamine antiserum (b). (Bars = 30 μ m.) All the dopamine-positive cells are recognized by the anti-TyrOHase antibody.

FIG. 6. Synthesis and storage of neurotransmitters by MC29 neural crest cells. Results are expressed as fmol of neurotransmitters synthesized (mean $\pm$ SEM) by 10^5 cells for 4 hr. Four independent cultures were tested for each passage (P). DA, dopamine; NA, noradrenaline + adrenaline; ACh, acetylcholine.

v-erbA, v-mil, v-myb, v-erbA, or v-myb-ets (data not shown).

Is MC29 Able to Activate the Catecholamine-Related Phenotype in Other Cell Types? Trunk neural crest cells are the progenitors of the peripheral nervous system and the adrenal medulla. We examined the influence of MC29 on adrenal medulla and on sympathetic and sensory ganglia taken at different stages of development. We also tested the ciliary ganglion (a cholinergic structure arising from the cranial neural crest), which has been shown to contain cells able to express the catecholaminergic pathway under certain conditions only. Central neuronal cells arising from neural tube were also tested. Only some MC29-infected cells arising from sensory and ciliary ganglia were recognized by the anti-TyrOHase antibody (data not shown). The labeled cells represented less than 0.1% of the total population in 4.5- to 6-day ciliary cultures, whereas they amounted to 10% in 5-day sensory ganglion cultures. Taken at later stages, the proportion of positive cells decreased significantly. No TyrOHase-positive cells were obtained in MC29-transformed adrenal medulla or sympathetic ganglion cultures after five passages. No TyrOHase-expressing cells were induced to differentiate in 3- to 9-day neural tube cultures.

DISCUSSION

In the present study we report that *myc* products enhance the proliferative capacity of 2-day quail trunk neural crest cells cultured *in vitro* and that some of these cells express the TyrOHase protein as well as a complete catecholaminergic metabolism. Previous reports have already shown that the infection by retroviruses carrying the *src* oncogene induces the proliferation of neural crest cells *in vitro* and a very faint neurotransmitter synthesis (24–26). Our data extend this finding, since avian viruses expressing *myc* or several oncogenes also stimulate the cell multiplication of this embryonic population. But the active catecholamine metabolism that occurs in the presence of the *myc* oncogene is not simply due to the modification of the proliferative rate, since the other tested oncogenes do not trigger this kind of differentiation.

We have presented evidence that, among the different oncogenes that activate the cellular cycle of neural crest cells, only *myc* specifically elicits the appearance of the catecholaminergic traits. The *myc* gene product itself appears to be required to induce the catecholaminergic phenotype in autonomic precursors, since in our case no diffusible factor

or matrix-associated compounds were found to elicit TyrOHase expression in NP medium. In contrast, diffusible factor and matrix-associated compounds are required for induction of the catecholaminergic phenotype in noninfected neural crest cells cultured *in vitro* with CEE. The differentiation-promoting substance of CEE, which is present in extracts of central nervous system or neural tube-conditioned medium (27), remains unidentified, although it was found to be a low molecular weight component (<10 kDa) (27). Extracellular matrix of somites also constitutes a favorable substrate for adrenergic development (28, 29), and the active component was identified as fibronectin (29, 30). More recently, molecules associated with the reconstituted basement membrane of Engelbreth-Holm-Swarm sarcoma were described as being potent enhancers of adrenergic development of neural crest cells (31).

The different *myc* gene products used were found to be equally effective in TyrOHase induction: the *gag-myc* fusion gene product of MC29, the *myc* gene product of MH2, or the human *c-myc* gene product overexpressed through sX-AHM retroviral vector. The role of the endogenous *c-myc* proteins in the physiological TyrOHase induction is difficult to assess; the amount of neural crest recovered from one neural tube (2×10^3) precludes any direct measurement of the *c-myc* proteins. However, from *in situ* study of *c-myc* protein expression during avian development, the *c-myc* signal is stronger in neural crest cells than in the surrounding tissues at 2 days of development (32).

The TyrOHase protein was not induced alone, since dopamine and noradrenaline were synthesized and accumulated in MC29-infected cells. Thus, the entire catecholaminergic biosynthetic pathway was activated. We could first hypothesize that, as DNA binding proteins (and probably by their effects on gene expression), the *myc* products act at the beginning of the cascade of events leading to the expression of the catecholaminergic phenotype. It remains to be ascertained whether the expression of the genes for TyrOHase and the other catecholaminergic enzymes is directly controlled by the *myc* gene product. On the other hand, one cannot exclude that the *myc* gene products do not act directly in catecholamine precursors but in a subpopulation of cells that then become able to interact with the catecholamine precursors via cell-cell or cell-substrate interactions, allowing the latter to express the catecholamine phenotype.

TyrOHase expression and catecholamine synthesis and accumulation decreased with cell amplification, and only low activity remained after 11 passages. This effect could be the result of two distinct, nonmutually exclusive phenomena: first, the number of catecholaminergic precursors is low, and *myc*-encoded products are only able to elicit the phenotype but not to maintain the population of differentiated cells. Second, the catecholamine-expressing cells could replicate more slowly than the other cells in the dish, and catecholamine-positive cells would be rapidly lost during the propagation of the culture. The fact that these cells incorporate [³H]thymidine to a lesser extent than the cells that do not express TyrOHase suggests that the mitogenic properties of catecholaminergic cells are reduced. On the other hand, TyrOHase-positive cells are included in the population of cells that lack substrate adhesivity. Thus, they could gradually detach from the dish.

The effect of the *myc* gene is restricted to catecholaminergic metabolism, since no acetylcholine was produced after infection with MC29. Acetylcholine is nevertheless the first known neurotransmitter elaborated, to our knowledge, by neural crest cells (16).

Neural crest is composed of morphologically undifferentiated cells that generate several structures, including peripheral ganglia and adrenal medulla. To assess how more differentiated neural crest-derived cells react to MC29 virus, we

infected sympathetic, ciliary, and sensory ganglia and adrenal medulla culture *in vitro*. Neuronal cells of the central nervous system were also tested. In all these cultures, cells were induced to proliferate after MC29 infection. No TyrOHase-positive cells were seen in neural tube cultures. These data suggest strongly that the *myc*-encoded product cannot trigger catecholaminergic traits in all cell types. Among the neural crest-derived structures, the sensory ganglia and the cholinergic ciliary ganglia were found to produce detectable numbers of TyrOHase-positive cells after infection with MC29. These two ganglionic structures contain catecholaminergic precursors that differentiate *in vivo* after ectopic graft in the avian embryo and migration into a favorable environment (33–35). In our experiments, it is highly probable that these latent autonomic precursors are also induced to differentiate in the presence of *myc* gene products.

Sympathetic ganglion cells continue to divide, *in vivo*, after the acquisition of neuronal properties (36, 37) and express catecholaminergic traits when cultured *in vitro*. Surprisingly, no TyrOHase-expressing cells were detected in infected cultures of sympathetic ganglia and adrenal medulla after 4–5 passages. Therefore, it seems that during the differentiation of autonomic cells, the positive influence of the *myc* gene products can only act on a very early step, before the establishment of catecholaminergic metabolism.

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