Regulation of the neural crest cell fate by N-myc: promotion of ventral migration and neuronal differentiation

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SUMMARY

During neural crest development in avian embryos, transcription factor N-myc is initially expressed in the entire cell population. The expression is then turned off in the period following colonization in ganglion and nerve cord areas except for the cells undergoing neuronal differentiation. This was also recapitulated in the culture of Japanese quail neural crest, and the cells expressing N-myc eventually coincided with those expressing neurofilaments. These findings suggested that N-myc is involved in regulation of neuronal differentiation in the neural crest cell population. In fact, transient overexpression of N-myc in the neural crest culture by transfection resulted in a remarkable promotion of neuronal differentiation. An experimental procedure was developed to examine the effect of exogenous N-myc expression in the neural crest cells in embryos. Neural crest cell clusters still attached to the neural tube were excised from Japanese quail embryos, transfected and grafted into chicken host embryos. Using this chimera technique, we were able to analyze the consequence of transient high N-myc during the early phase of neural crest migration. Two effects were demonstrated in the embryos: first, high N-myc expression provoked massive ventral migration of the neural crest population and, second, those cells that migrated to the ganglion-forming areas underwent neuronal differentiation with the cell type determined by the nature of the ganglion. Thus, N-myc is involved in regulation of the neural crest fate in two different aspects: ventral migration and neuronal differentiation.

Key words: N-myc, neural crest, transfection, avian chimera, migration, neuronal differentiation

INTRODUCTION

The neural crest cells arise from the dorsal margin of the neural tube, and migrate away from the tube ventrally and laterally to various locations, where they give rise to a variety of derivatives including peripheral nervous system and melanocytes (Weston, 1970; LeDouarin, 1982). It has been indicated that the development of neural crest cells is under the influence of microenvironamental signals (for review, Stemple and Anderson, 1993; Dupin et al., 1993). However, little is understood regarding intracellular events, especially gene regulation in the nucleus. The present study was undertaken to examine the possible function of N-myc as a transcriptional regulator that has a determinative role in neural crest development.

The N-myc gene was first recognized by its similarity to c-myc and amplification in neuroblastomas derived from the neural crest (Kohl et al., 1983; Schwab et al., 1983). The N-myc gene codes for a DNA-binding transcriptional regulator (Wenzel et al., 1991), although its direct target genes are yet to be identified. Analysis of N-myc expression in mouse and chicken embryos indicated that it is expressed in early neural crest lineage, the central nervous system, endodermal epithelia and a subset of mesoderm derivatives (Mugrauer et al., 1988; Downs et al., 1989; Sawai et al., 1990; Hirning et al., 1991; Kato et al., 1991; Wakamatsu et al., 1993).

We have investigated N-myc function in the neural crest and its derivatives in mouse and chicken embryos (Sawai et al., 1990; Kato et al., 1991; Wakamatsu et al., 1993). The N-myc-deficient mouse embryos (Stanton et al., 1992; Charon et al., 1992; Sawai et al., 1993) showed great reduction in the number of mature neurons of the dorsal root ganglia (DRG) and sympathetic ganglia (SG), indicating the importance of N-myc expression in the production of neurons, especially those derived from the neural crest.

Avian embryos are advantageous for analysis of neural crest development because of established methodologies of tissue transplantation, of available cell markers and of the abundance of knowledge of the neural crest. We therefore examined N-myc protein expression in the trunk neural crest cells. As reported here, N-myc is expressed rather uniformly in the migrating crest cells, but then its expression becomes confined to cells undergoing neuronal differentiation, suggesting that N-myc expression has a determinative effect on the neuronal/non-neuronal choice of neural crest cells. This prompted us to test...
whether exogenous N-myc expression alters the developmental fate of neural crest cells in cultures in vitro and in embryos. The results demonstrate that high expression of exogenously introduced N-myc gene induces early ventral migration of neural crest cells and promotes neuronal differentiation.

MATERIALS AND METHODS

N-myc expression vector
cDNA library of day 13 chicken embryo brain (Funahashi et al., 1993) was screened with partial chicken N-myc cDNA clone C1 (Sawai et al., 1990). Clone Nc023s, which contained a full coding region (accession number D90071 in DDBJ/EMBL/GenBank Databases), was isolated and inserted in pmiwSV, and N-myc expression vector pmiwNc was constructed (Fig. 1A).

Neural crest culture and transfection
Neural crest cells were prepared from stage 12-13 Japanese quail embryos (Coturnix coturnix japonica) (staged according to Hamburger and Hamilton (1951)) and cultured according to Duff et al. (1991). Neural tubes were placed in a culture dish for 18 hours and removed leaving the neural crest cells that had migrated out. After 24 hours, the neural crest cells were trypsinized and replated at a density (PF A) in Hepes-buffered saline (HBS) for 10 minutes and processed removed leaving the neural crest cells that had migrated out. After 24 hours, the neural crest cells were trypsinized and replated at a density around 10^5/cm^2. After 3 days, the cultures were cotransfected with pmiwZ coding for β-galactosidase (Suemori et al., 1990) and pmiwNc or insertless pmiwSV using a conventional calcium phosphate method. At intervals, cultures were fixed with 3.5% paraformaldehyde (PFA) in HBS-buffered saline (HBS) for 10 minutes and processed for immunostaining.

Gene transfer into neural crest cells in vivo
A method was established allowing the introduction of exogenous genes into neural crest cells in vivo by combining the technique of chicken-quail chimera (LeDouarin, 1973) with that of transfection using Lipofectin reagent (Gibco-BRL) (Fig. 1B). Isolated quail embryos of stage 12-13 were treated with 2000 units/ml Dispase (crystalline Dispase, Godo-Shusei) in HBS at room temperature for 3 minutes. After washing with HBS, the embryo was trimmed at the last somite, and the neural tube with premigratory neural crest cells adjacent to the last 5 somites was exposed by removing ectoderm, endoderm, somites and the notochord. Additional treatment with 0.2% DispaseII (crude Dispase, Godo-Shusei) in HBS was then administered at room temperature for 3 minutes to render the neural crest cells fully competent to be transfected. A total of 16 μg plasmid DNA, 8 μg of pmiwZ and 8 μg of pmiwNc or pmiwSV was diluted to 50 μl with distilled water and mixed with 100 μl of opti-MEM (Gibco-BRL). 50 μl of Lipofectin was also mixed with 100 μl of opti-MEM. These solutions were mixed together and left for 30 minutes to make the DNA-Lipofectin complex before transfection. Embryos, which had a segment of the neural tube and neural crest exposed, were incubated in the transfection mixture at 37°C for 3 hours with occasional gentle mixing. After washing in HBS, the naked portion of the tube was excised from the rest of the embryo and grafted into a stage 12-13 host chicken embryo from which the neural tube portion flanked by the most posterior 5 somites had been removed. The chimeric embryos were incubated at 37.5°C and fixed at intervals for examination. For 5-bromo-4-chloroindoly-β-D-galactopyranoside (X-gal) color reaction (Suemori et al., 1990), embryos were fixed in 1% glutaraldehyde in HBS. After examination as whole mounts, the embryos were processed for paraffin sectioning and counterstained by the Feulgen method (LeDouarin, 1973).

Immunofluorescence staining
Immunofluorescence staining was performed as described previously (Wakamatsu et al., 1993). Embryos were fixed with 3.5% PFA in HBS, sectioned at the thickness of 4-5 μm in a cryostat and processed for immunofluorescent and 4’,6-diamidino-2-phenylindole (DAPI) fluorescence. The following primary antibodies and appropriate secondary antibodies with fluorochromes (fluorescein isothiocyanate or Texas red) were used: anti-chicken N-myc (polyclonal anti-CN32 peptide, Wakamatsu et al., 1993), anti-160 kD neurofilament (polyclonal, InRto BioMedTek; monoclonal, Biomakor), anti-dopamine-β-hydroxylase (polyclonal, Eugene Technology); anti-HNK-1 (monoclonal, Beckton Dickinson, anti-β-galactosidase (polyclonal, Cappel; monoclonal, Boehringer-Mannheim).

RESULTS

N-myc expression in the trunk neural crest and its derivatives in embryos
N-myc expression in trunk neural crest and its derivatives was examined at the wing level. Neural crest cells were identified by immunostaining with HNK-1 antibody (Vincent et al., 1983) and N-myc protein was detected using anti-CN32 antibodies (Wakamatsu et al., 1993). Since no substantial difference was observed between chicken and quail embryos, the results are reported in a compiled form.

Fig. 1. Expression vectors and transfection of neural crest cells to be grafted in a host embryo. (A) Schematic presentation of the expression vector and the inserted coding DNAs (open boxes). (B) Steps in production of chimera embryos with transfected neural crest cells. Donor quail embryos of stages 12-13 were treated with Dispase I, trimmed at the most caudal somite, and the neural tube and attached neural crest cells for the length of 5 somitomeres were exposed to the medium. The neural crest/tubes were further treated with Dispase II and transfected using Lipofectin medium. After 3 hours, the transfected neural crest cells were grafted into host chicken embryos replacing the host tissue and chimeras with transfected neural crest were produced.
At stages 14-18, when the neural crest cells were emigrating, the majority of the neural crest cells identified by expression of the HNK-1 antigen contained approximately constant moderate levels of N-myc protein in their nuclei (Fig. 2A-C). However, from stage 19 to 29, the stages after cessation of massive migration, the N-myc expression level became considerably heterogeneous. Some of the cells in the DRG and SG appeared to have an augmented level of N-myc expression, while some others had lowered level of N-myc expression (Fig. 2D-G for stage 19). Considering N-myc expression specificity in later stages, those with a high N-myc expression probably represented the neuron precursors. In the Schwann cell population, N-myc expression decreased and disappeared by the end of this period (Fig. 2D,E for stage 19; Fig. 2H,I for stage 31).

At stage 16, neural tube and dermomyotome also expressed a significant level of N-myc (Fig. 2C). Later in development, N-myc expression in the neural tube became confined to the ventricular zone as reported for mouse embryos (Wakamatsu et al., 1993), while the myotome maintained a high level of N-myc expression (data not shown).

At later stages of DRG development, N-myc protein gradually changed its localization from the nucleus to the cytoplasm, apparently in parallel with neuronal maturation as previously reported for mouse ganglia (Wakamatsu et al., 1993). At stage 31, DRG were divided into ventrolateral (early maturing) and dorsomedial (late maturing) compartments.

**Fig. 2.** N-myc expression in the neural crest cells in the quail embryo. (A-C) A section of stage 16 embryo showing a neural crest cell cluster indicated by surrounding arrowheads. (A) Phase-contrasted image. d, dermomyotome; nc, neural crest; nt, neural tube; sc, sclerotome. (B) Immunofluorescence of HNK-1 showing the neural crest cell cluster (arrowheads). (C) N-myc immunofluorescence showing that neural crest cells had a moderate level of N-myc protein. (D,E) A section of stage 19 embryo through a juvenile DRG (drg); (D) HNK-1 immunofluorescence, (E) N-myc immunofluorescence. N-myc level in the nuclei of the ganglion became heterogeneous and became higher in some of the nuclei (e.g., arrows) than the cells of surrounding tissues [neural tube (nt) and somite]. (The matrix encapsulating the neural tube was strongly stained in this particular specimen, which does not usually occur.) Along the ventral root (vr), nuclei of HNK-1-positive cells, putative Schwann cell precursors (e.g., arrowheads), were only weakly stained for N-myc. (F,G) A section of stage 19 embryo showing an area of SG; (F) HNK-1 immunofluorescence, (G) N-myc immunofluorescence. The neural crest cells located at the center of the SG showed a level of N-myc protein higher than others (arrows in F,G). (H,I) A section of stage 31 embryo showing a more mature DRG. (H) NF immunofluorescence. Note that the ventrolateral (vl) compartment was slightly higher in NF expression than the dorsomedial (dm) compartment. (I) N-myc immunofluorescence of the same section. Schwann cells along the dorsal root (dr) and ventral root (vr) (arrowheads in H and I) were totally devoid of N-myc protein (I). N-myc protein in the cells of the ventrolateral compartment was cytoplasmic and that in the dorsomedial compartment was nuclear. (J,K) An enlarged view of the ventrolateral compartment of a DRG; (J) N-myc immunofluorescence, (K) DAPI fluorescence showing nuclei. Sensory neurons characterized by large nuclei and pale DAPI staining (arrows in K) had N-myc protein in their cytoplasm (arrows in J). Satellite cells with small nuclei and bright DAPI staining lacked N-myc (arrowheads). The bars indicate 20 μm except for H,I where the bar indicates 100 μm.
The sensory neurons of the former strongly expressed neurofilaments (NF) (Fig. 2H) and possessed a higher level of N-myc protein almost exclusively in the cytoplasm (Fig. 2I,J). The latter compartment, which expressed NF less strongly, still contained nuclear N-myc (Fig. 2I). Satellite glial cells, recognizable by their small nuclei and absence of anti-NF immunoreactivity, were totally negative in N-myc expression (Fig. 2J,K).

Thus, among neural crest derivatives, those in neuronal cell lineages were characterized by persistent and incremental expression of N-myc, and those in glial and Schwann cell lineages by early cessation of N-myc expression. However, N-myc expression in neurons of the peripheral nervous system also became undetectable by stage 40 (data not shown).

**N-myc expression in the primary culture of the neural crest**

The change of N-myc expression during differentiation of neural crest cells was also examined in vitro. Cultures of trunk neural crest cells were prepared from stage 12-13 quail embryos and those that migrated out of the neural tubes were replated after one day. After this replating, the cell number was roughly doubled after 6 days of the total culture period and reached a plateau.

At 2 days of culture, virtually all neural crest cells had N-myc protein (Fig. 3A) in their nuclei (Fig. 3B,C). After 4 days, when cell differentiation became recognizable, the cell fraction expressing N-myc protein decreased (Fig. 3D,E). In these N-myc-positive cells, N-myc protein was found mostly in the nuclei up to 7 days, but after 8 days those possessing N-myc protein in their cytoplasm became a significant fraction (Fig. 3F). At 10 days of culture, 92% of N-myc-positive cells had N-myc protein in their cytoplasm (Fig. 3H and data not shown).

Melanoblasts were clearly distinguished by their pigmentation from 4 days of culture and constituted one quarter of the cell population after 6 days. At 4 days, roughly half of the melanoblasts expressed nuclear N-myc, but N-myc-positive melanoblasts gradually decreased and became undetectable by 10 days of culture.

NF-expressing cells also became detectable at 4 days and gradually increased thereafter to 7% of the cell population at 10 days (Fig. 3A). All these NF-positive cells had N-myc protein in their cytoplasm and represented a subfraction of the cells with cytoplasmic N-myc protein (Fig. 3F,G). At 10 days, NF-positive cells accounted for most of the N-myc-positive cells (Fig. 3H,I). Half of the NF-positive cells had a fibroblastic morphology (Fig. 3G), but the remaining half assumed the

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**Fig. 3.** N-myc and NF expression in neural crest cells in culture.

(A) The change of the fraction of N-myc- and NF-expressing cells with culture days. Quail neural crest cells in culture were fixed at intervals and processed for immunofluorescence. A total of 2500 cells in a number of fields arbitrarily chosen from triplicate cultures was analyzed to generate each point. At 10 days in culture, N-myc-positive cells and NF-positive cells coincided. In two other experiments analogous to this, essentially the same kinetics of the decrease of N-myc-expressing cells was obtained. (B,C) 2 day culture showing N-myc immunofluorescence (B) and DAPI fluorescence (C) of the same field. All the cells had N-myc protein in their nucleus. (D,E) 6 day culture showing N-myc immunofluorescence (D) and DAPI fluorescence (E) of the same field. Note that the majority of the cells had lost N-myc. (F,G) A part of 8 day culture. At this stage, N-myc-expressing cells were a minor fraction of the entire cell population. In this particular field rich in N-myc-positive cells, there are 6 cells, three of them expressing N-myc and having the protein in the cytoplasm (F). Of the three N-myc-expressing cells, only one expressed NF (G). (H-J) An example of cells assuming the morphology of pseudounipolar sensory neurons observed in 10 day cultures. They had N-myc protein in the cytoplasm (H), contained the neurofilament in the cell bodies and neurites (I), and occurred sporadically (J, DAPI fluorescence). The bar indicates 50 μm in B-E and 20 μm in F-J.
morphology of neurons, either pseudounipolar (Fig. 3H,I) or multipolar.

Thus, the culture mimicked the in vivo situation as regards the temporal change of N-myc expression and the cytoplasmic localization of N-myc protein in the NF-positive cells.

Effect of N-myc overexpression in neural crest cells in culture

As described above, both in situ and in culture, N-myc was initially expressed more-or-less uniformly among the neural crest population but thereafter gradually decreased except for the subset of cells that eventually underwent neuronal differentiation. This may suggest that N-myc expression drives neuronal differentiation of neural crest cells. To test this, we transfected neural crest cells in culture with N-myc expression vector pmiwNc (Fig. 1) to give N-myc overexpression. Plasmid pmiwZ (Suemori et al., 1990) was cotransfected to identify transfected cells by their expression of β-galactosidase (β-gal). Insertless pmiwSV was used to control the effect of exogenous N-myc expression.

The neural crest cells 2 days after replating (3 days of the culture) were transfected, and stained with anti-N-myc CN32 antibodies and anti-β-gal (Wakamatsu et al., 1993). After 1 day of transfection of the control cultures (cotransfected with pmiwSV and pmiwZ), 6.2% of the cells expressed β-gal (β-gal+) and 65% of β-gal+ cells expressed endogenous N-myc protein. When pmiwNc was used for transfection, a comparable population (5.4%) of cells was β-gal+ and those β-gal+ cells expressing N-myc increased to 92% (Fig. 4A, open bar at day 4); importantly, 51% of N-myc-positive nuclei of β-gal+ cells were scored higher in N-myc expression than those in β-gal- cells of the same culture (Fig. 4A, solid bar at 4 days; Fig. 4B,C). On the contrary, no β-gal- cells expressed N-myc higher than the control. These indicated that most of the transfected cells received both pmiwNc and pmiwZ. An important feature of this transfection was that the higher N-myc level in the pmiwNc-transfected cells was transient and, by 3 days of transfection, the cell fraction exhibiting higher than normal N-myc expression became negligible (Fig. 4A, solid bar at 6 days). This is ascribed to the short half life of the N-myc protein (Ramsay et al., 1986). There was no indication of a proliferative effect of exogenous N-myc: the increase of β-gal+ cells was comparable to control transfectants.

![Figure 4](image-url)
Sibling cultures were stained with anti-β-gal and anti-NF (Fig. 4A,E,F,H,I,K,L). In the control cultures transfected with pmiwSV, NF+ cells in the β-gal+ population was around 5% 7 days after transfection (10 days of culture), similar to the non-transfected cultures shown in Fig. 2. In pmiwNc transfected cultures, by contrast, β-gal+/NF+ double-positive cells increased dramatically. By 7 days after transfection, NF+ fraction in β-gal+ cells reached 42%, which was comparable to the high N-myc cell fraction shortly after transfection (Fig. 4A, solid bar at 4 days). In a separate experiment using DBH as a marker of sympathetic neuron (Duff et al., 1991), we observed that 22% of β-gal+ transfecants were DBH+ 7 days after transfection in contrast to 3% in control transfecants. These results indicated that exogenous N-myc expression supported neuronal differentiation in the neural crest cell population.

At the level of individual cells, tight association of NF+ cell phenotype and pmiwNc transfection was confirmed. 3 days after transfection, when a cell or a small cluster of cells was found β-gal+, a significant fraction of them were also NF+ (Fig. 4D-F). The NF+ phenotype did not extend to neighboring cells, thus the effect of transfection was cell autonomous. 5 days after transfection about half of the β-gal+/NF+ cells began to assume the bipolar morphology, indicative of neuronal differentiation (Fig. 4G-I), but the remaining half of the NF+ cells assumed a fibroblastic morphology (Fig. 4J-L). Therefore, high N-myc expression was very effective in inducing the NF+ state, but may or may not be sufficient for overt neuronal differentiation depending on the transfected cells.

**Development of transfected neural crest cells in the embryos**

To examine the effect of exogenous N-myc expression in vivo, we developed a technique for transfecting neural crest cells and allowing development of the transfected cells in the embryo. The basic design was to expose quail neural crest cells attached to a neural tube to transfection reagents and then to graft them in a host chicken embryo. Among a number of methods tested, transfection using Lipofectin proved to be successful.

In first trials, the neural tubes from stage 12-13 quail embryos were transfected and placed in culture, allowing the neural crest cells to emigrate on the culture dish. Staining with X-gal showed that β-gal+ cells were predominantly distributed among the neural crest cell population (Fig. 5A), indicating preferential transfection of the neural crest cells exposed outside the extracellular matrix. The efficiency of transfection of neural crest cells was estimated to be a few percent.

Chimeric embryos were produced by grafting the transfected neural crest/tube and were stained with X-gal in whole mount. The majority of X-gal-stained (β-gal+) cells were distributed along the dorsomedial region of the trunk in the clear stripes expected for the normal migratory pathway of the neural crest and for the areas of DRG and SG (Fig. 5B). There were a small number of β-gal+ cells close to the ectodermal surface, which may represent those of melanoblastic lineage. Histological sections of such embryos confirmed preferential distribution of β-gal+ cells in the ganglionic areas of the peripheral nervous system (Fig. 5C). Counterstaining by the Feulgen technique confirmed donor origin of the β-gal+ cells.

**Effect of exogenous N-myc expression on neural crest migration in vivo**

Graft of transfected neural crest cells was utilized to modulate N-myc expression level in vivo by exogenous N-myc expression. Quail neural crest cells were transfected with pmiwNc and pmiwZ and grafted to the corresponding region of chicken host embryos. Immunofluorescent staining of grafted embryos confirmed the occurrence of N-myc overexpression in β-gal+ transfected cells (see Fig. 8A-C, below) analogous to the cells in culture (Fig. 4B,C).

The embryos were fixed and stained for β-gal expression with X-gal in toto and compared with control embryos in which neural crest cells were transfected with pmiwSV and pmiwZ (Fig. 6). The first difference between pmiwNc-transfected and control embryos was observed at 16 hours after grafting. Most of the β-gal+ cells remained close to the dorsal midline in the controls (Fig. 6A), but pmiwNc transfection resulted in the spreading of the β-gal+ cells in more ventrolateral locations (Fig. 6D). At 24 hours after the graft, it was noted that β-gal+ cells with exogenous N-myc expression often made dense clusters deep in the trunk in the areas expected for SG (Fig. 6E), which was not observed in control embryos (Fig. 6B). At 48 hours when massive migration was over, the distinction between pmiwNc-transfected and control neural crest cells became less conspicuous (Fig. 6C,F). This result indicates that high N-myc expression by transfection of pmiwNc caused accelerated migration of the neural crest cells, but did not cause ectopic colonization of the cells.

To compare in more detail the tissue distribution of pmiwNc-transfected neural crest cells with that of controls, histological sections were examined. Inspection of sections of X-gal-stained embryos shown in Fig. 6 confirmed the conclusion based on the examination in whole mount. However, diffusion of the stain (e.g., Fig. 5C) hampered precise counting of β-gal+ cells and generating quantitative data. Thus, serial sections prepared from chimeras 16 and 36 hours after grafting were...
stained with an anti-β-gal antibody and examined. Data of several embryos scanning a number of somite units were compiled to give an overall view of the distribution of graft-derived, transfected neural crest cells (Fig. 7). 16 hours after grafting, before formation of ganglionic structures, distribution of β-gal+ neural crest cells was scored in the five areas of cross sections shown in Fig. 7A. In control embryos, the β-gal+ cells largely remained dorsally as observed in whole mount but, in pmiwNc-transfected embryos, the major fraction of β-gal+ cells had already located more ventrally. In embryos 36 hours after grafting, the location of β-gal+ cells was assigned by the tissues in which they resided (Fig. 7B). In DRG, neural crest cells transfected with pmiwNc constituted a smaller cell fraction than the controls, while β-gal+ cell fraction in SG was more than twice as large in pmiwNc-transfected groups. Moreover, the putative Schwann cell population along the spinal nerve cord located more ventral to DRG was larger in pmiwNc-transfected groups. Thus, even after being assigned to each structure/tissue 36 hours after graft, overall distribution of the β-gal-labeled neural crest cells still indicated the more ventrally driven migration of pmiwNc-transfected cells.

**Expression of neuronal traits in pmiwNc-transfected neural crest cells in vivo**

Developmental fate of transfected neural crest cells in vivo was examined by immunohistological analysis of chimeric embryos (Figs 8, 9). The majority of transfectant neural crest cells (β-gal+, Fig. 8B) expressed N-myc higher than the surrounding non-transfected cells (Fig. 8C).

**Fig. 6.** Whole-mount view of migration and colonization of transfected neural crest cells in chimeric embryos. Shown here are representatives of sample groups each consisting of more than six embryos and showing qualitatively the same migratory behavior of transfected neural crest cells. 16 (A,D), 24 (B,E), 48 (C,F) hours after grafting, embryos were fixed and processed for X-gal staining. (A-C) Control embryos, which received pmiwZ and pmiwSV. (D-F) Embryos carrying neural crest cells that received pmiwZ and pmiwNc. Arrows in C,E,F indicate clusters of pmiwNc-transfected cells in the area of SG. The bar indicates 360 μm in A,B,D,E, and 250 μm in C,F.

**Fig. 7.** Distribution of transfected neural crest cells in chimeric embryos. The results of pmiwSV transfection (control, left) and of pmiwNc transfection (right) are compared. Data are from the number of β-gal+ cells, indicated by ‘100%’, in the number of chimera embryos (n) examined. (A) Distribution 16 hours after grafting. Location of each β-gal+ cell in cross section was assigned to one of the areas schematically shown. (B) Distribution 36 hours after grafting. Location of each β-gal+ neural crest cell was assigned to one of the areas: LP (lateral pathway), DRG, SNC (spinal nerve cord), SG and DV (deep ventral area).
In addition to NF, DBH expression was scored to detect differentiation of sympathetic neurons (Duff et al., 1991). 16 hours after grafting, expression of neither NF nor DBH was detectable in any embryos (data not shown). After 24 hours of graft, only 13.6% of β-gal+ cells were DBH+ in the control SG (Figs 8D-F, 9). In contrast, as many as 38.5% of β-gal+ cells in pmiwNc-transfected SG were DBH+ (Figs 8G-I, 9). It is to be noted that in all cases the scores of NF+ cells approximated those of DBH+ cells. Antibody staining of adjacent sections, one with anti-NF plus anti-β-gal and the other with anti-DBH plus anti-β-gal, indicated that NF+ cells and DBH+ cells were identical (data not shown). Thus, as the result of high exogenous N-myc expression, neuronal differentiation as sympathetic neurons was provoked in the SG area.

In the DRG areas also, NF+ cells increased significantly among β-gal+ cells after pmiwNc transfection (Fig. 9). At 24 hours after grafting, NF+ cells amounted to 28.4% of β-gal+ cells in the DRG of pmiwNc-transfected chimeras, while 17.3% of β-gal+ cells were NF+ in the control. Even at 48 hours after grafting, the same degree of difference between the pmiwNc-transfected chimeras and control chimeras was observed in DRG. All the β-gal+/NF+ cells in pmiwNc-transfected chimeras assumed neuronal morphology, with large cell bodies.

Outside the ganglion-forming areas, however, ectopic expression of NF or DBH was never observed in the pmiwNc-transfected or control chimeras.

**DISCUSSION**

**Endogenous N-myc expression during the neural crest differentiation**

Expression of N-myc protein in the trunk neural crest cells was examined in chicken and quail embryos. N-myc protein was detected at a moderate level in the nuclei of all the neural crest cells before and during their migration. After the migration, however, the levels of N-myc expression differed among the cell types. In neurons of both DRG and SG, as identified by the characteristic morphology and neurofilament expression, N-myc expression lasted long and was up-regulated, and, in the phase of neuronal maturation, N-myc protein became localized in the cytoplasm (Wakamatsu et al., 1993), although the significance of cytoplasmic N-myc protein in gene regulation has not been fully understood. These obser-
vations suggested that N-myc expression has important roles in the neuronal differentiation of neural crest cells.

**Effect of N-myc expression on directed migration of the neural crest cells**

Migratory behaviors of neural crest cells are dependent on their locomotive activity, and cell-cell and cell-matrix interactions. In the initial emigration stage, neural crest cells alter their cell adhesion molecules (Duband et al., 1988; Akitaya and Bronner-Fraser, 1992; Nakagawa and Takeichi, 1995). Later, the cells interact with different matrices depending on the route of migration (Erickson and Perris, 1993). Finally, they colonize at the appropriate locations possibly in response to external signals. Overall, migratory behavior is under the regulation of N-myc, since massive ventral migration of neural crest cells was provoked by N-myc overexpression at much earlier stages than those that would exhibit migration in normal embryos (Figs 6, 7), although the molecular events underlying the alteration of migratory behavior have not been elucidated. These prematurely migrating neural crest cells colonized normal ganglion-forming regions, indicative of normal response to environmental signals.

**Role of N-myc in neuronal differentiation of the neural crest cells**

This study also demonstrated that transient expression of N-myc protein above the normal level induced neuronal phenotypes in the neural crest cells in culture and in the embryos. In culture, nearly half of the pmNcNe transfectants expressed NF and a significant fraction also expressed DBH. In the embryo, premature expression of neuronal traits was provoked in the transfectant cells.

We looked at neuronal differentiation in the neural crest cell population in N-myc-null mutant mouse embryos. We previously reported that peripheral ganglia of mutant embryos were significantly smaller and contained fewer neurons than those of wild type (Sawai et al., 1993). For instance, DRG of 11.5 day mutant embryo at the forelimb level contained only 20% of normal neuron number, while the same ganglia contained a normal number of supporting cells. It appears that N-myc expression is not an absolute requirement for neuronal differentiation in the neural crest, although the possibility remains that a requirement of N-myc function was alleviated by the action of other myc family genes. It is under investigation whether the neuronal deficiency in the mutant ganglia is ascribable to inferior neuronal differentiation or to selective inhibition of the migration of a neuron-oriented cell population. In either case, the observations on N-myc-null mutant embryos corroborate the proposed effect of N-myc expression in the neural crest, promotion of neuronal differentiation.

It was observed in culture that, although pmNcNe transfection induced expression of neurofilaments, half of the NF+ transfectants did not assume a typical neuronal morphology. It seems that other conditions must be fulfilled to give rise to a proper neuronal phenotype. It is interesting to note that a similar cell phenotype is produced by Mash-1 null mutant mice (Sommer et al., 1995). Mutant mice lacking transcription factor Mash-1 are devoid of expression of sympathetic markers in SG (Guillemot et al., 1993) and the neural crest cells from the mutant ganglia produced cells positive in neurofilament expression but morphologically fibroblastic in culture condition (Sommer et al., 1995). Therefore, it is possible that the differentiation of sympathetic neurons, for instance, is established by cooperative action of N-myc and Mash-1.

One possible cause of the differences in the response of the cells in culture to exogenous N-myc expression is the heterogeneity of the neural crest cells, already demonstrated to be present from the early period of their development (Sellecke et al., 1993; Sieber-Blum et al., 1993; LeDouarin et al., 1994; and references therein). It is likely that some of the cells had already been committed to non-neuronal lineages at the time of transfection. Although exogenous high N-myc expression induced NF synthesis in these cells, it may have failed in induction of overt neuronal differentiation.

Even in vivo, where transfection was performed when most of the neural crest cells remained attached to the neural tube and transgene expression took place during the migration, heterogeneity among neural crest population might have caused different responses at the level of individual cells. Lineage analysis of single cells labeled with a tracer dye suggested that commitment occurs progressively during and after migration (Fraser and Bronner-Fraser, 1991). Culture experiments have shown the existence of uncommitted cells in ganglion areas at early stages of development (Duff et al., 1991; Sieber-Blum et al., 1993). In contrast, specification to Schwann cells may occur at a very early stage, since a possible Schwann cell marker cadherin7 mRNA is expressed in the subpopulation of migrating crest cells (Nakagawa and Takeichi, 1995). Labeling of premigratory neural crest cells showed that later migrating cells contribute to progressively more dorsal derivatives (Serbedzija et al., 1989), indicating the occurrence of a stage-dependent bias of the developmental fate of neural crest cells. Considering these observations, exogenous N-myc expression presumably acted on the population of neural crest cells still not totally committed to a non-neuronal lineage, directed them to ganglion-forming areas of an embryo and then promoted on-site neuronal differentiation.

The microenvironment of neural crest cells after their emigration then seems to restrict the final cell type. Even when ventral cell migration and overall neuronal differentiation were promoted by exogenous N-myc, no cells outside the ganglia expressed any neuronal traits. Those that migrated early to the spinal nerve cord area seem to have differentiated into Schwann cells (Fig. 7). In addition, no neurons (NF+ cells) in DRG expressed DBH and no neurons in SG assumed the morphology of sensory neurons. It was also noted that premature neuronal differentiation in SG did not increase the neuron content of the ganglion at a later stage (Fig. 9). Thus, although high N-myc expression potentially drives neuronal differentiation, the final phenotype of individual cells and final cell composition of a ganglion, SG at least, is determined within the allowance of the microenvironment. The importance of proper environmental signals for neural crest development has been well documented (Stemple and Anderson, 1993; Henion and Weston, 1994; Reissmann et al., 1996; Shah et al., 1996). In the case of SG, BMP family proteins derived from the dorsal aorta have been shown to be important signal molecules for sympathoadrenal lineage and to induce Mash-1 (Reissmann et al., 1996; Shah et al., 1996), consistent with the model of cooperative action of N-myc and Mash-1 in the generation of sympathetic neurons.

In summary, N-myc exerts its effect in the following two phases of neural crest development. In the early phase, N-myc
gene regulates migratory potential and a high N-myc expression promotes overall ventral migration. High N-myc expression in a subfraction of the cells during this migratory period promotes their neuronal differentiation in a later phase, in cooperation with other intracellular factors and with environmental signals.

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