

# MASH1 activates expression of the paired homeodomain transcription factor Phox2a, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity

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## SUMMARY

We have investigated the genetic circuitry underlying the determination of neuronal identity, using mammalian peripheral autonomic neurons as a model system. Previously, we showed that treatment of neural crest stem cells (NCSCs) with bone morphogenetic protein-2 (BMP-2) leads to an induction of MASH1 expression and consequent autonomic neuronal differentiation. We now show that BMP2 also induces expression of the paired homeodomain transcription factor Phox2a, and the GDNF/NTN signalling receptor tyrosine kinase c-RET. Constitutive expression of MASH1 in NCSCs from a retroviral vector, in the absence of exogenous BMP2, induces expression of both Phox2a and c-RET in a large fraction of infected colonies, and also promotes morphological neuronal differentiation and expression of pan-neuronal markers. **In vivo**, expression of Phox2a in autonomic ganglia is strongly

reduced in *Mash1*  $-/-$  embryos. These loss- and gain-of-function data suggest that MASH1 positively regulates expression of Phox2a, either directly or indirectly. Constitutive expression of Phox2a, by contrast to MASH1, fails to induce expression of neuronal markers or a neuronal morphology, but does induce expression of c-RET. These data suggest that MASH1 couples expression of pan-neuronal and subtype-specific components of autonomic neuronal identity, and support the general idea that identity is established by combining subprograms involving cascades of transcription factors, which specify distinct components of neuronal phenotype.

Key words: MASH1, Phox2a, Transcription factor, Neuronal identity, Differentiation, Neural crest stem cell, Retroviral transduction, Basic helix-loop-helix protein

## INTRODUCTION

A central problem in developmental neurobiology is to understand the control of neuronal identity. Neuronal identity can be defined by the coordinated expression of a set of genes or functional properties: some, like neurofilament or voltage-gated sodium channels, are expressed by most or all neurons; others, like neurotransmitter synthesis, trophic factor dependence and connectivity, are specific to subsets of neurons. Extensive work in both vertebrate and invertebrate systems has indicated that transcription factors are crucial determinants of neuronal identity (Shankland and Macagno, 1992). The actual role these transcription factors play in specifying expression of particular components of neuronal identity is, however, not well understood.

The complexity of transcription factor expression in the nervous system is staggering, befitting the diversity of cell types in the brain. Members of dozens of different subfamilies of transcription factors, such as basic-helix-loop-helix (bHLH) (Guillemot, 1995), LIM (Tsuchida et al., 1994), POU (Ryan and Rosenfeld, 1997), homeobox (Lumsden and Krumlauf,

1996), zinc finger (Tamura et al., 1996) and paired domain proteins (Mansouri et al., 1996), display specific patterns of expression in developing and mature neurons (reviewed in He and Rosenfeld, 1991; Struhl, 1991). How members of these different subfamilies interact to specify the constellation of phenotypic properties that defines a particular neuronal identity is still not clear, although some details have emerged from studies of invertebrate systems (Duggan and Chalfie, 1995). The expression of these properties, and therefore the activity of the transcription factors that specify them, must be coordinated in each type of neuron. Yet this coordination must be sufficiently flexible so that various properties can be expressed together in some neurons, and separately in others.

We have focused on the role of bHLH proteins in vertebrate neurogenesis (reviewed in Anderson, 1995; Guillemot, 1995; Lee, 1997). Genetic experiments in *Drosophila* have indicated that these proteins are important determinants of neuronal fate and identity (reviewed in Jan and Jan, 1993). For example, *achaete-scute* and *atonal* function not only as proneural genes, but also help to specify different peripheral sense organ identities (Jarman et al., 1993). Within a given neural lineage,

moreover, bHLH proteins appear to act in cascades in both invertebrates (Jan and Jan, 1993) and vertebrates (Cau et al., 1997; Ma et al., 1996). Which aspect(s) of neuronal identity these cascades specify, and how they interact with members of other subfamilies of transcription factors, is poorly understood.

The most extensively studied vertebrate neuronal bHLH gene is *Mash1*, a mammalian homolog of the *Drosophila achaete-scute* genes (Johnson et al., 1990). *Mash1* is expressed in both the CNS and PNS (Guillemot and Joyner, 1993; Lo et al., 1991), and is essential for the development of both olfactory and peripheral autonomic (sympathetic, parasympathetic and a subset of enteric) neurons (Guillemot et al., 1993; Blaugrund et al., 1996). Loss-of-function experiments have revealed that, within the autonomic lineage, *Mash1* is required for expression of both subtype-specific and certain pan-neuronal properties (Guillemot et al., 1993; Sommer et al., 1995). Whether MASH1 is sufficient for the expression of these properties has been more difficult to address, because of the lack of gain-of-function data for this gene.

Recently, we obtained a gain-of-function phenotype for *Mash1*, by constitutively expressing this gene in a population of non-neurogenic, post-migratory crest cells isolated from E14.5 fetal rat gut (Lo and Anderson, 1995). Constitutive expression of exogenous MASH1 in these cells maintains competence for neurogenesis elicited by bone morphogenetic protein-2 (BMP2) (Lo et al., 1997). These studies demonstrated a role for continued expression of MASH1 in maintaining neurogenic competence in post-migratory precursor cells, but did not address the issue of how the initial expression of MASH1 affects the development of more primitive, migratory neural crest cells.

We have now constitutively expressed MASH1 in primary cultures of neural crest stem cells (NCSCs) (Stemple and Anderson, 1992), to address two questions: is *de novo* expression of exogenous *Mash1* sufficient to promote neurogenesis; and is it sufficient to promote expression of any components of neuronal subtype identity? As neuronal subtype markers we have used two pan-autonomic genes: the paired homeodomain transcription factor *Phox2* (now renamed *Phox2a*) (Tiveron et al., 1996; Valarché et al., 1993), and the GDNF signalling receptor *c-ret* (Pachnis et al., 1993; reviewed in Lindsay and Yancopoulos, 1996); both of these genes are also essential for the development of subsets of autonomic neurons (Durbec et al., 1996; Morin et al., 1997; Schuchardt et al., 1994). Our results indicate that *de novo* expression of exogenous MASH1 is sufficient to promote expression of *Phox2a* (as well as *c-ret*) in NCSCs. Moreover, in vivo, MASH1 is necessary for expression of *Phox2a* in subsets of autonomic precursors (see also Hirsch et al., 1998). Thus, MASH1 positively regulates expression of *Phox2a*, either directly or indirectly. Surprisingly, we also found that MASH1 is sufficient to promote overt neurogenesis and expression of pan-neuronal genes in a subset of NCSCs. In contrast, forced expression of *Phox2a* is not sufficient to promote neurogenesis, but is sufficient to promote expression of *c-ret*. Taken together, these data suggest that MASH1 couples the expression of both pan-neuronal and subtype-specific components of neuronal identity. This coordination is achieved, at least in part, by activating expression of a more specifically expressed paired homeodomain transcription factor, *Phox2a*, which in turn controls expression of a restricted subset of neuronal properties.

## MATERIALS AND METHODS

### Genotyping and in situ hybridization

*Mash1* +/- mice were maintained on a 129SvEv × C57Bl6/J hybrid background. Mouse embryos from *Mash1* +/- × *Mash1* +/- crosses were genotyped by PCR as previously described (Sommer et al., 1995). Non-radioactive in situ hybridization was carried out using *Phox2a* cRNA probes (Valarché et al., 1993) according to our published procedure (Birren et al., 1993) (a detailed protocol is available on request).

### Neural crest cultures and retroviral infection

Primary rat neural crest cultures were established from explants of E10.5 rat embryos as previously described (Stemple and Anderson, 1992), except that Dispase (Gibco/BRL) and collagenase (Worthington Biochemical Corp.) instead of collagenase alone were used for enzymatic dissociation of the tissue (Sommer et al., 1995). 24-hour explants were trypsinized and replated at clonal density (100 cells/35 mm plate) on a fibronectin/poly-D-lysine substrate and growth factors added 2-3 hours after plating, as described (Shah et al., 1996). Purified recombinant BMP2 was obtained from Genetics Institute, Inc. and used at 50 ng/ml, as previously described (Shah et al., 1996).

Retroviral constructs were made in a modified (Lo et al., 1997) pBABE vector (Morgenstern and Land, 1990) and packaged by transient transfection into BOSC 23 cells (Pear et al., 1993) as described (Lo et al., 1997). The full-length coding sequence of *Phox2a* was amplified from plasmid pRC/CMV 903S (the generous gift of Drs J.-F. Brunet and C. Goridis), using PFU polymerase (Stratagene) in a reaction mix modified to include 10% (v/v) DMSO. All constructs contained six myc-epitope tags at the N terminus to permit the retrovirally encoded product to be distinguished from the endogenous gene product (Lo et al., 1997). For retroviral infection of crest cells, approximately 5,000 cells were plated overnight in a cloning ring (3 mm inner diameter) on a fibronectin-coated substrate. The following day they were subjected to three rounds of infection with viral supernatants diluted 1:1 with standard medium (Stemple and Anderson, 1992) (3 hours infection plus 1 hour recovery period in between; see Lo et al., 1997 for further details). The infected cells were then replated at 100 cells/35 mm dish, on fibronectin/poly-D-lysine. Cells were fixed at various times thereafter and analyzed by immunocytochemical staining (see below). The fact that infected cells were replated at clonal density prior to analysis ensured that if a given colony contained any myc-tag<sup>+</sup> cells, it was derived from an infected founder cell (Lo et al., 1997). This method of analysis is important, because some of the progeny of infected cells subsequently down-regulate or lose expression of the transgene as they differentiate (L. L. and D. J. A., unpublished observations). Therefore, analysis of infected mass-cultures would systematically misidentify some myc-tag<sup>-</sup> cells as uninfected. The colony analysis method circumvents this problem.

### Immunocytochemistry

Monoclonal antibodies to MASH1 (Lo et al., 1991), c-RET (Lo and Anderson, 1995) and rabbit polyclonal anti-*Phox2a* (Tiveron et al., 1996) have been described previously. Monoclonal antibody to the human c-myc epitope tag (clone Myc1-9E10.2) was obtained from the ATCC. Monoclonal antibody to NF160 (clone NN18) was purchased from Sigma and rabbit polyclonal anti-peripherin and monoclonal anti-DBH antibodies from Chemicon.

To carry out double labeling for *Phox2a* and endogenous MASH1 or c-RET, fixed cells were first stained with anti-*Phox2a* and FITC-conjugated goat anti-rabbit IgG. The position of the stained cells was marked by encircling them with a grease pencil on the bottom of the dish, and each cell was photographed, assigned a number and scored as *Phox2a*<sup>+</sup> or *Phox2a*<sup>-</sup>. The cultures were then processed for staining with anti-MASH1 or anti-c-RET followed by HRP-conjugated secondary antibodies, and developed using nickel intensification of the DAB

reaction product (NiDAB, as described (Shah et al., 1994). As this reaction product quenched the FITC signal, the proportion of single- and double-positive cells was measured by comparison to the data on Phox2a expression collected prior to counter-staining for MASH1 or c-RET.

For detection of endogenous Phox2a or peripherin in retrovirally infected cultures, fixed cells were first stained with anti-Phox2a or peripherin antibody followed by FITC-conjugated goat anti-rabbit secondary antibody, and subsequently stained with monoclonal antibody to the myc epitope tag (anti-myc tag) followed by phycoerythrin (PE)-conjugated goat anti-mouse IgG secondary antibody. In some experiments, the cells were first stained with anti-myc and PE-linked secondary antibody, then circled, numbered and photographed and subsequently stained with anti-Phox2a antibody followed by HRP-conjugated secondary antibody and developed with NiDAB. For detection of endogenous c-RET in retrovirally infected cultures, cells were first stained with anti-RET followed by biotinylated secondary antibody (Jackson ImmunoResearch, Inc.) and ABC complex (Vectastain ABC kit, Vector Labs, Inc.), and then developed with NiDAB. The cultures were then restained with anti-myc antibody followed by PE-conjugated secondary antibody.

To perform double-labeling for NF160 and the myc-tag, since both primary antibodies are mouse IgG1 monoclonals the following staining protocol was employed. Retrovirally infected cultures were first stained with anti-myc tag followed by HRP-conjugated secondary antibody, and developed for the DAB reaction. Following identification, quantification and photography, the cultures were incubated with anti-NF160 followed by PE-conjugated secondary antibody. Although both primary antibodies are of the same subclass, double-labeling was possible because the NF160 antigen is cytoplasmic and the myc-tag antigen nuclear. In the case of E12-infected cells, the cultures were first stained with anti-NF160 followed by HRP-conjugated antibody and developed with DAB, then restained following quantification and photography with anti-myc tag and PE-conjugated secondary antibodies.

## RESULTS

### Phox2a expression is reduced in *Mash1*<sup>-/-</sup> mutant autonomic ganglia in vivo

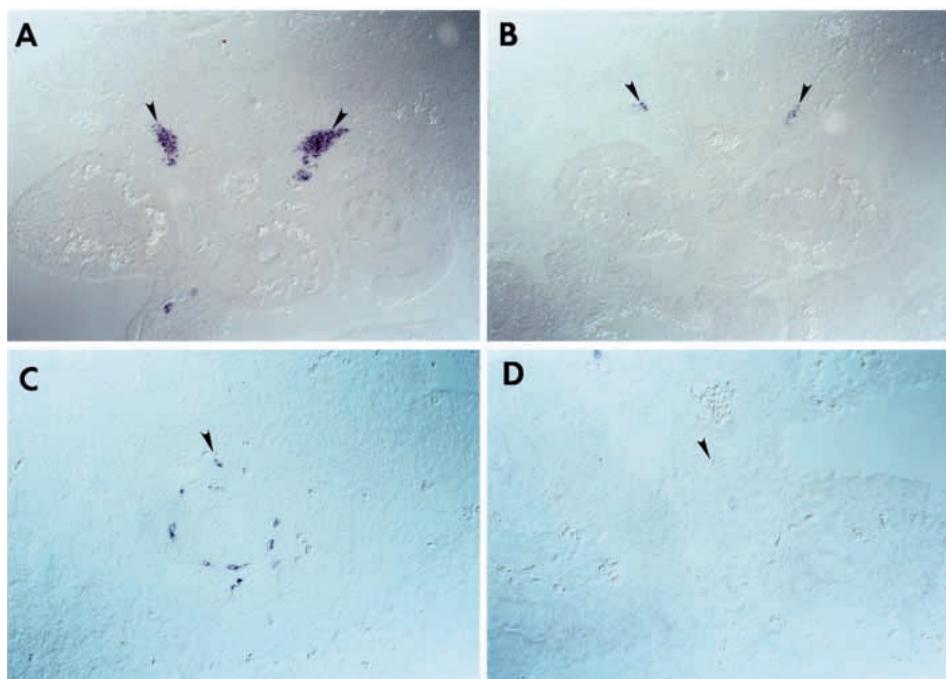
To determine whether expression of Phox2a in autonomic ganglia is dependent upon MASH1 function, we examined embryos from *Mash1*<sup>-/-</sup> mutant mice. At E12.5, there was a striking reduction in the number of Phox2a<sup>+</sup> cells in sympathetic ganglion anlagen (Fig. 1A,B). We have previously shown that these anlagen are populated in mutant embryos by partially differentiated neuronal precursors, which express markers such as neurofilament (NF) 160 and c-RET, but not peripherin or SCG10 (Sommer et al., 1995). Therefore, the reduction in the number of Phox2a<sup>+</sup> cells likely reflects a lack of Phox2a expression in these arrested precursors, consistent with the findings of Hirsch et al. (1998). The extent of the reduction in Phox2a<sup>+</sup> cells appeared

proportionally greater in superior cervical than in trunk sympathetic ganglia, consistent with the incomplete expressivity of the *Mash1*<sup>-/-</sup> phenotype at more caudal levels of the sympathetic chain, as previously reported (Guillemot et al., 1993).

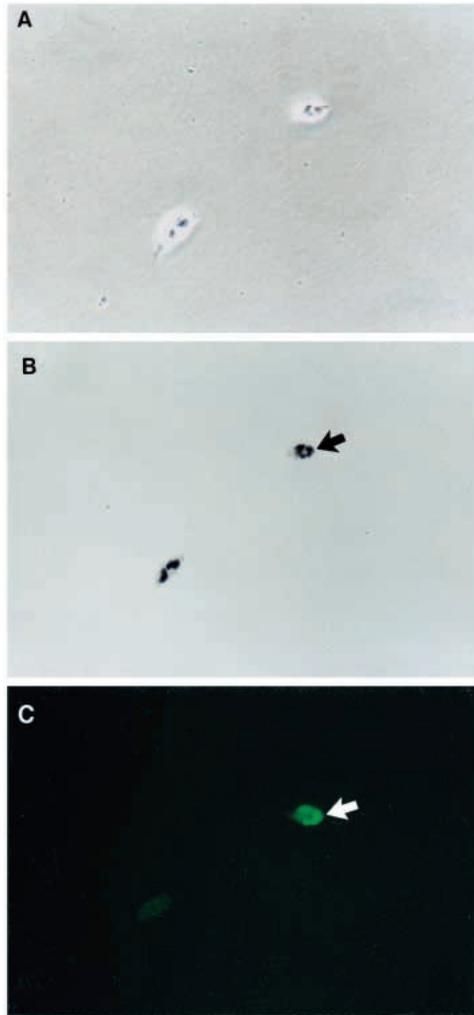
A striking reduction of Phox2a expression was also observed in the foregut (esophageal) enteric ganglia of *Mash1*<sup>-/-</sup> mice at E12.5 (Fig. 1C,D). Parasympathetic ganglia were not examined, but in the accompanying paper (Hirsch et al., 1998), a requirement of MASH1 for Phox2a expression is documented in rostral parasympathetic ganglia as well. Taken together, these data demonstrate that MASH1 is required for the proper expression of Phox2a in at least some ganglia of all three major branches of the autonomic sublineages of the PNS.

### MASH1 and Phox2a are co-expressed in the same cells

The foregoing data demonstrated a dependence of Phox2a expression on *Mash1* function, but did not distinguish whether this dependence reflects a cell-autonomous or non-autonomous function of the latter gene. To address this issue, we first asked whether expression of the two proteins could be detected in the same cells, in cultures of rat neural crest stem cells (NCSCs) undergoing autonomic neuronal differentiation. We have previously shown that exposure of NCSCs to BMP2 (or BMP4) leads to a rapid (6-12 hour) induction of MASH1, with consequent differentiation to autonomic neurons occurring in 3-4 days (Shah et al., 1996). In NCSC clonal cultures exposed to BMP2 (50 ng/ml) for 20 hours, 91.5% of colonies (*n*=59 colonies examined) expressed immunocytochemically detectable MASH1 (Fig. 2B), but did not yet exhibit a neuronal



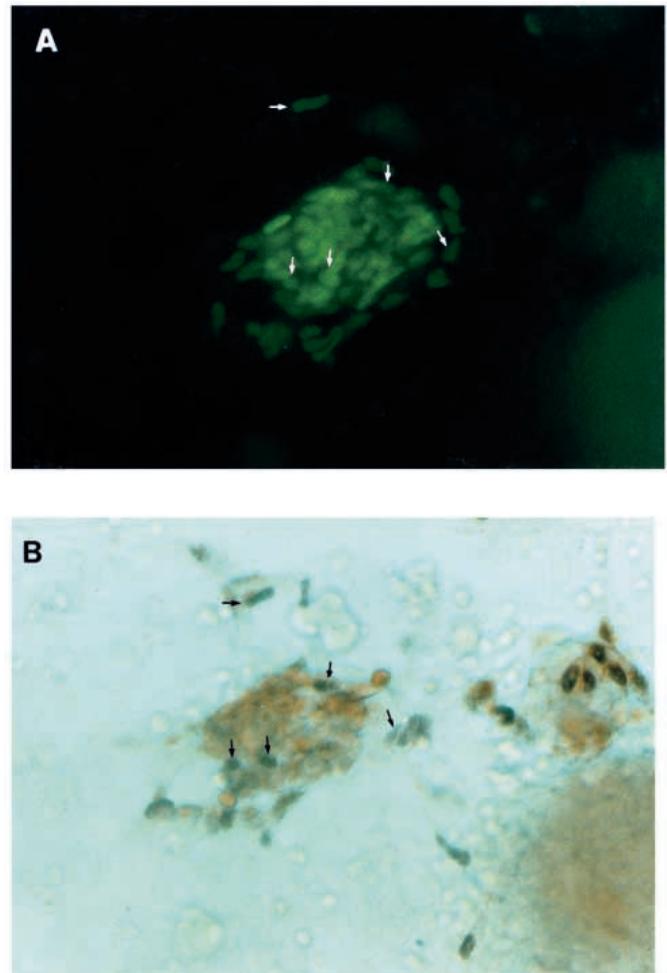
**Fig. 1.** Phox2a expression is dependent on MASH1 function in vivo. Sections through the mid-thoracic region of E12.5 wild-type (A, C) and *Mash1*<sup>-/-</sup> (B, D) littermate embryos, hybridized with a *Phox2a* cRNA probe. Note the striking reduction of Phox2a expression in the vicinity of the sympathetic ganglia (A, B, arrowheads) and the absence of Phox2a expression in the esophagus (C, D, arrowheads). Objective magnification  $\times 10$  (A, B);  $\times 20$  (C, D).



**Fig. 2.** Co-expression of endogenous MASH1 and Phox2a proteins in rat NCSCs exposed to BMP2. (A-C) Phase contrast, brightfield and epifluorescence views of the same microscopic field, containing neural crest cells fixed after 20 hours growth in BMP2 (50 ng/ml). (B) MASH1 immunohistochemistry; (C) Phox2a immunohistochemistry. Arrows indicate a double-labeled cell. Not all MASH1<sup>+</sup> cells co-expressed Phox2a (see Table 1 for quantification). Objective magnification  $\times 40$ .

morphology (Fig. 2A). Of these MASH1<sup>+</sup> colonies, 63% co-expressed Phox2a protein as detected by double-label immunocytochemistry (Fig. 2C and Table 1) (see Materials and Methods). Conversely, 91.9% of Phox2a<sup>+</sup> colonies co-expressed MASH1 (Table 1).

To confirm that the co-expression of MASH1 and Phox2a was not an artifact of growth in BMP2 in vitro, acutely dissociated E11.5 mouse gut cells plated for only 1 hour in the absence of BMP2 were triple-labeled for MASH1, Phox2a and c-RET, another autonomic lineage marker whose expression is correlated with that of MASH1 (Lo et al., 1994; Pachnis et al., 1993). Many clear examples of individual cells co-expressing MASH1 and Phox2a could be seen (Fig. 3, arrows) (although the brown cytoplasmic c-RET staining sometimes obscured the nuclear MASH1 staining in dense regions of undissociated cell aggregates). These data therefore indicate that at least some (although not necessarily all)



**Fig. 3.** Co-expression of Phox2a, MASH1 and c-RET in acutely dissociated enteric precursors. (A, B) Epifluorescent and brightfield views of the same microscopic field. A cluster of cells from E11.5 wild-type mouse gut was fixed 1 hour after plating and stained for Phox2a (A), MASH1 (B, purple nuclei) and c-RET (B, brown staining). Arrows in A and B indicate examples of Phox2a<sup>+</sup>, MASH1<sup>+</sup> cells. Some Phox2a<sup>+</sup> cells deep within the cluster appear MASH1<sup>-</sup> because the nuclear staining of the latter protein is obscured by the brown cytoplasmic c-RET staining. Objective magnification  $\times 40$ .

Phox2a<sup>+</sup> enteric precursors co-express MASH1. A similar co-expression of MASH1 and Phox2a was observed in neural crest-derived precursors freshly immuno-isolated from E14.5 rat gut using anti-c-RET antibodies (Lo and Anderson, 1995) (not shown). Taken together, these data indicate that the co-expression of the two transcription factors observed in cultured neural crest cells exposed to BMP2 is not an in vitro artifact, but occurs in vivo as well. These data in turn make it highly likely that the dependence of *Phox2a* expression on *Mash1* reflects a cell-autonomous requirement for the latter gene.

In chick embryos, expression of CASH1 (the chick homolog of MASH1 (Jasoni et al., 1994)) precedes that of cPhox2 in developing avian sympathetic ganglia (Ernsberger et al., 1995; Groves et al., 1995). In cultures grown for 20 hours in BMP2, approximately one-third of all MASH1<sup>+</sup> cells were Phox2a<sup>-</sup>. In contrast, only 8% of Phox2a<sup>+</sup> cells were MASH1<sup>-</sup>. This

**Table 1. Co-expression of MASH1 and Phox2a in BMP2-treated NCSCs**

BMP2 concentration (ng/ml)	MASH1 <sup>+</sup> (n)	Phox2a <sup>+</sup> (n)	MASH1 <sup>+</sup> Phox2a <sup>++</sup>	MASH1 <sup>+</sup> Phox2a <sup>-</sup> ‡	Phox2a <sup>+</sup> MASH1 <sup>+</sup> †	Phox2a <sup>+</sup> MASH1 <sup>-</sup>
0*	2% (52)	0%	0%	100% <sup>1</sup>	0%	0%
50*	91% (59)	63%	63%	37%	91.9%	8.1%
0 <sup>¶</sup>	4% (51)	0% (151)	na	na	na	na
0.001 <sup>¶</sup>	26% (73)	4% (78)	na	na	na	na
10 <sup>¶</sup>	84%(103)	19%(105)	na	na	na	na

\*The percentage of MASH1<sup>+</sup> or Phox2a<sup>+</sup> colonies after 20 hours in 0 or 50 ng/ml BMP2 is presented. *n* = number of colonies examined. Colonies were double-labeled with anti-MASH1 and anti-Phox2a antibodies.

†The percentage of MASH1<sup>+</sup> colonies that also expressed Phox2a was measured, as was the percentage of Phox2a<sup>+</sup> colonies that co-expressed MASH1.

‡Calculated by subtracting the measured value of double-positive colonies from 100%.

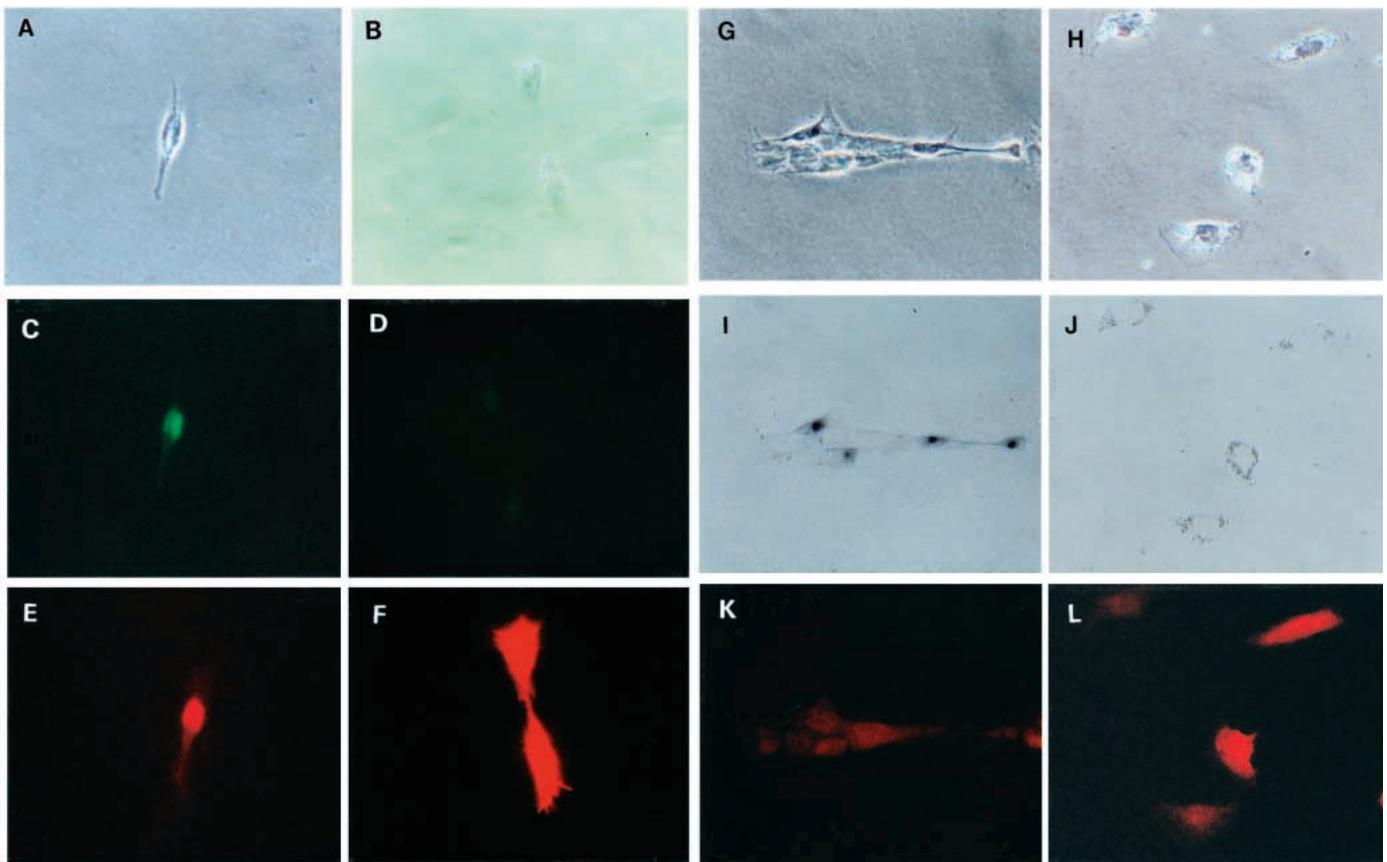
<sup>1</sup>This number represents a single colony (out of 52 scored) that expressed MASH1 under this condition; since no colonies expressed Phox2a, 100% of MASH1<sup>+</sup> colonies were Phox2a<sup>-</sup>.

<sup>¶</sup>The percentage of MASH1<sup>+</sup> or Phox2a<sup>+</sup> colonies was determined by single labeling in a different experiment. *n* = number of colonies examined under each condition. na, not applicable (double-label immunohistochemistry was not performed in this experiment).

trend was observed both at lower doses of BMP2 (Table 1) and at earlier time points following BMP2 exposure (data not shown). These data are consistent with the idea that expression of MASH1 may precede, and be a prerequisite for, initial expression of Phox2a in most (but not all) NCSCs in vitro as in vivo.

#### Forced expression of MASH1 is sufficient to promote induction of Phox2a in the absence of exogenous BMP2

The foregoing data demonstrated a strong correlation between the expression of MASH1 and Phox2a in NCSC cultures grown in BMP2. These data raised the question of whether expression



**Fig. 4.** Constitutive expression of MASH1 in NCSCs is sufficient to induce expression of endogenous Phox2a and c-RET. (A-F) NCSCs infected with either a MASH1 retrovirus (A, C, E) or control E12 retrovirus (B, D, F), and analyzed for expression of Phox2a 40 hours post-infection. (A, B) Phase-contrast views of cells expressing myc-epitope-tagged MASH1 (E) or E12 (F). The MASH1-expressing cells express Phox2a (C), whereas the E12-expressing cells do not (D). E12 is expressed in both nucleus and cytoplasm. (G-L) Analysis of endogenous c-RET expression in infected cells. MASH1-infected cells (G, K) express c-RET (I), while E12-infected cells (H, L) do not (J). For quantitative data, see Table 2. Objective magnification  $\times 40$ .

of MASH1 would be sufficient to induce expression of endogenous Phox2a, in the absence of exogenously added BMP2. To address this question, we infected NCSCs with a replication-incompetent retroviral vector encoding a myc epitope-tagged *Mash1* cDNA (Lo et al., 1997), and examined cells double-labeled with antibodies to the myc epitope and Phox2a at 40 hours post-infection (see Materials and Methods). As a control, the cells were infected with a retrovirus encoding E12, a more broadly expressed bHLH protein (Murre et al., 1989a) that is a dimerization partner of MASH1 (Johnson et al., 1992a).

In cultures examined 40 hours post-infection, constitutive expression of retrovirally encoded MASH1 clearly induced expression of endogenous Phox2a (Fig. 4C,E) while E12 had little or no effect (Fig. 4D,F). 46% of myc-tag<sup>+</sup> colonies co-expressed Phox2a (Table 2; this value likely represents an underestimate, as the anti-Phox2a antibody gave a weak background and therefore only very strongly labeled cells were scored as Phox2a<sup>+</sup>). In contrast, endogenous Phox2a was expressed by only 1% of uninfected colonies in the same plate, or 1.5% of E12-infected colonies in sister cultures (Table 2). The lack of induction of Phox2a by E12 strongly suggests that the effect of MASH1 reflects its positive transcriptional activation activity (Johnson et al., 1992a) (direct or indirect), rather than a sequestration of negative regulators such as Id (Benezra et al., 1990), since E12 should also be capable of heterodimerization with such negatively acting bHLH factors (Lassar et al., 1991; Murre et al., 1989b).

Induction of endogenous MASH1 and Phox2a expression in NCSCs by BMP2 is also accompanied by induction of endogenous c-RET expression (see Fig. 6, below), and many MASH1<sup>+</sup> cells (70.7%;  $n=41$  colonies examined) in BMP2-treated cultures co-express c-RET. We therefore asked whether exogenous MASH1 could also induce endogenous c-RET expression. In cultures infected with the MASH1 retrovirus, 60% of myc-tag<sup>+</sup> colonies were c-RET<sup>+</sup>, while only 8% of uninfected colonies in the same plate expressed the receptor (Fig. 4I,K; Table 2). Only 1% of E12-infected colonies expressed endogenous c-RET, a number similar to the percentage of uninfected c-RET<sup>+</sup> colonies (2.5%) in the same

**Table 2. Constitutive expression of MASH1 induces expression of Phox2a and c-RET in NCSCs**

	MASH1 virus ( $n$ )		E12 virus ( $n$ )	
	myc-tag <sup>+</sup>	myc-tag <sup>-</sup>	myc-tag <sup>+</sup>	myc-tag <sup>-</sup>
Phox2a <sup>+</sup>	46±1.5%* (96)	1±1% (68)	1.5±1.5% (46)	0% (85)
c-RET <sup>+</sup>	60±3% (154)	8±1% (67)	1±1% (87)	2.5±2.5% (107)

\*Retrovirally infected clones were analyzed 40 hours after plating by double-label immunocytochemistry with anti-myc epitope tag antibody plus anti-Phox2a or anti-c-RET. The percentage of myc-tag<sup>+</sup> colonies that contained any cells positive for Phox2a or c-RET is given. Most or all cells in colonies scored as marker<sup>+</sup> expressed the marker indicated. The results represent the mean ± s.d. for two independent experiments.  $n$  = total number of infected or uninfected colonies scored in the combined experiments. Most or all colonies in each dish were circled under phase microscopy, without knowing whether they were retrovirally infected (myc-tag<sup>+</sup>) or not. The number of myc-tag<sup>+</sup> or myc-tag<sup>-</sup> colonies within these cohorts of circled cells was determined subsequently.

plate (Fig. 4J,L and Table 2). Taken together, these data indicate that forced expression of MASH1 in neural crest cells can bypass the ability of BMP2 to induce two pan-autonomic markers, Phox2a and c-RET. These data in turn suggest that the induction of Phox2a and c-RET observed in response to BMP2 is a consequence, at least in part, of the induction of MASH1.

### Forced expression of MASH1 promotes neurogenesis in NCSCs

We also asked whether MASH1 is sufficient to promote overt neuronal differentiation in NCSCs. 40 hours after infection (a time when approx. 50-70% of infected colonies expressed Phox2a or c-RET) only 16% ( $n=121$  colonies examined) of MASH1-infected colonies contained any NF160<sup>+</sup> neuronal cells. However 5 days after infection, 68% of myc-tag<sup>+</sup> colonies contained NF160<sup>+</sup> neurons in MASH1 retrovirus-infected cultures (Fig. 5A-C; Table 3). A similar result was obtained using peripherin as a pan-neuronal marker (data not shown). In contrast, at this time point only 4% of uninfected colonies in the same cultures, or 3% of E12-infected colonies in sister cultures, contained any neurons (Table 3). Not every cell within MASH1-infected colonies was a neuron, however: 16% of the cells in myc-tag<sup>+</sup> colonies ( $n=1318$  cells counted) expressed neurofilament and had a neuronal morphology; the rest expressed MASH1 but were NF160<sup>-</sup> and had a non-neuronal morphology (Fig. 5D-F). Nevertheless, in uninfected colonies, only 1.1% ( $n=3404$  cells counted) of the cells was a neuron. These data indicate that forced expression of MASH1 accelerates neurogenesis in at least some NCSCs. Despite this acceleration, the induction of such pan-neuronal markers by exogenous MASH1 appears to occur more slowly than that of Phox2a and c-RET.

### Forced expression of Phox2a is sufficient to promote expression of c-RET but not neurogenesis

As induction of Phox2a accompanies and precedes neurogenesis induced by MASH1, we sought to determine whether Phox2a, in turn, could bypass the neurogenic activity

**Table 3. Constitutive expression of MASH1 induces neuronal differentiation in NCSCs**

	MASH1 retrovirus ( $n$ )		E12 retrovirus ( $n$ )	
	myc-tag <sup>+</sup>	myc-tag <sup>-</sup>	myc-tag <sup>+</sup>	myc-tag <sup>-</sup>
Neuronal colonies	68±21%* (59)	4±6% (162)	3±7% (29)	2±3% (185)
Non-neuronal colonies	33±21%	96±6%	97±7%	98±3%
Neuronal cells	16%† (1318)	1.1% (3404)	0.7% (291)	1% (3964)

\*Retrovirally infected colonies were analyzed 5 days after replating by double-label immunocytochemistry with anti-myc-tag plus anti-NF160 antibodies (see Materials and methods). The percentage of colonies containing NF160-positive cells with a process-bearing neuronal morphology is given. Similar results were obtained using peripherin as a neuronal marker (not shown). Value represents the mean ± s.d. of three independent experiments.  $n$  indicates the total number of colonies scored in the combined three experiments.

†All of the cells were counted in all of the circled colonies in the cohort analyzed, and the percentage of NF160-positive cells was measured.  $n$  indicates the total number of cells scored combined from three independent experiments.

**Table 4. Constitutive expression of Phox2a induces expression of c-RET but not neurofilament 160 in NCSCs**

	Phox2a retrovirus ( <i>n</i> )		MASH1 retrovirus ( <i>n</i> )		E12 retrovirus ( <i>n</i> )	
	myc-tag <sup>+</sup>	myc-tag <sup>-</sup>	myc-tag <sup>+</sup>	myc-tag <sup>-</sup>	myc-tag <sup>+</sup>	myc-tag <sup>-</sup>
c-RET <sup>+</sup> * (40 hours)	70.5±1.5% (81)	5.5±2.5%	64±1% (173)	6.5±0.5% (96)	4±2% (86)	5±0% (119)
NF160 <sup>+</sup> † (5 days)	1.5±1.5% (58)	0±0% (78)	68.5±9.5% (97)	1.5±1.5% (42)	0±0% (64)	0±0% (66)

\*NCSC colonies were fixed 40 hours post-infection with the indicated retroviruses, and double-labeled with antibodies to the myc epitope tag and c-RET. The percentage of positive colonies from two independent experiments (average ± range) is presented. Numbers in parentheses indicate the total number of infected (myc-tag<sup>+</sup>) or uninfected (myc-tag<sup>-</sup>) colonies examined in the two experiments.

†The percentage of NF160<sup>+</sup> colonies was measured in dishes fixed 5 days after infection, in the same experiments as were assayed for c-RET expression after 40 hours. The average ± range of two independent experiments is shown. In one experiment, the percentage of peripherin<sup>+</sup> colonies was measured and was similar to the percentage of NF160<sup>+</sup> colonies, for each of the three viruses tested. The infections with MASH1 and E12 retroviruses were performed in parallel with the Phox2a infections, as positive and negative controls, respectively. The values presented therefore differ slightly from those in similar, but independent experiments, with these viruses, shown in Tables 2 and 3.

of MASH1. To this end we constructed a retroviral vector encoding a myc epitope-tagged Phox2a cDNA, and again infected NCSC cultures. Although abundant nuclear expression of the epitope-tagged Phox2a transgene was detected at 5 days post-infection, neither morphological neuronal differentiation nor induction of NF160 or peripherin was detected (Table 4).

The inability of Phox2a to promote neurogenesis could simply reflect the possibility that the retrovirally encoded transgene is functionally inactive in NCSCs, despite its nuclear localization. To address this question, we sought to determine whether retroviral Phox2a was capable of inducing expression of any endogenous marker genes in NCSCs. We therefore focused on c-RET. We first examined the correlation between expression of endogenous Phox2a and c-RET. As mentioned earlier, induction of endogenous MASH1 and Phox2a by BMP2 is also accompanied by induction of c-RET expression. Moreover, many cells in (uninfected) BMP2-treated cultures co-expressed endogenous Phox2a and c-RET (Fig. 6). 83.3% of Phox2a<sup>+</sup> cells were also c-RET<sup>+</sup> (*n*=30 clones examined), and 62.5% of c-RET<sup>+</sup> cells were Phox2a<sup>+</sup> (*n*=40 clones examined). Thus, there is a strong correlation between expression of endogenous Phox2a and endogenous c-RET. Furthermore, there is a loss of c-RET expression in some cranial sensory ganglia of *Phox2a*<sup>-/-</sup> mice (Morin et al., 1997). These latter data suggest that Phox2a is necessary for c-RET expression, at least in some neuronal populations.

Constitutive expression of retroviral Phox2a in NCSCs indeed promoted induction of endogenous c-RET, in the absence of BMP2 (Fig. 7). In cultures analyzed 40 hours post-infection, 70.5% of myc-tag<sup>+</sup> (i.e. exogenous Phox2a<sup>+</sup>) colonies co-expressed c-RET (Table 4). By contrast only 5.5% of uninfected colonies in the same dish expressed c-RET (Table 4). Expression of c-RET in Phox2a-infected cells was maintained even after 5 days, although there was no morphological neuronal differentiation nor expression of NF160 or peripherin (data not shown). We also examined Phox2a retrovirus-infected cultures for expression of endogenous dopamine-β-hydroxylase (DBH), a neurotransmitter-synthesizing enzyme that contains binding sites for Phox2a in its promoter, and whose expression in vivo is strongly correlated with that of DBH (Tissier-Seta et al., 1993; Zellmer et al., 1995). However no DBH immunoreactivity was detected above background in Phox2a-

infected cells, using a monoclonal antibody that detected abundant expression in E14.5 chromaffin cell precursors (data not shown). Taken together, these data indicate that the retrovirally encoded *Phox2a* gene is indeed functionally active in NCSCs, but is sufficient to promote expression of only some components of the autonomic neuronal differentiation program under these conditions.

## DISCUSSION

The determination of neuronal identity involves the coordinated function of different transcription factors, whose expression and activity are in turn regulated by extracellular signals (Tanabe and Jessell, 1996). Relatively little is known about the components of neuronal phenotype, which these transcription factors in turn regulate. By using retroviral vectors to introduce exogenous transcription factor-encoding genes into primary cultures of neural crest stem cells, we have begun to dissect the genetic circuitry that controls autonomic neuronal differentiation in response to environmental signals. BMP2, an instructive signal for autonomic neurogenesis, leads to a rapid induction of endogenous MASH1 (Shah et al., 1996). We have now shown that exogenous MASH1 is sufficient to promote the expression of autonomic subtype markers such as *Phox2a* and *c-ret*, in the absence of exogenous BMP2. In addition, MASH1 promotes overt neuronal differentiation and expression of pan-neuronal markers in a majority of infected colonies. Exogenous Phox2a, in turn, is insufficient to promote the expression of pan-neuronal properties, but is sufficient to promote expression of endogenous c-RET. Taken together, these data suggest a model in which MASH1, acting in response to BMP2, couples the expression of both pan-neuronal and subtype-specific components of the autonomic neuronal identity (Fig. 8A). Such a model supports the idea that different components of neuronal identity are specified by different genetic subprograms (Anderson and Jan, 1997), but that these subprograms are coupled.

### Independent but coordinated specification of different components of neuronal identity

Our results extend previous studies which have suggested that pan-neuronal and certain subtype-specific components of PNS neuronal identity can be disassociated. A targeted mutation in *Phox2a* prevents expression of DBH and c-RET in some cranial

sensory ganglia, but does not affect expression of pan-neuronal markers (Morin et al., 1997). Furthermore, in chick embryos notochord ablation prevents expression of catecholamine fluorescence (Stern et al., 1991), TH, GATA-2 and cPhox2a in developing sympathetic ganglia, but allows expression of CASH1 and SCG10 (Groves et al., 1995). These data suggested that expression of Phox2a and GATA-2 is dispensable for expression of pan-neuronal genes, and is positively correlated with expression of the autonomic subtype-specific catecholamine biosynthetic enzyme genes.

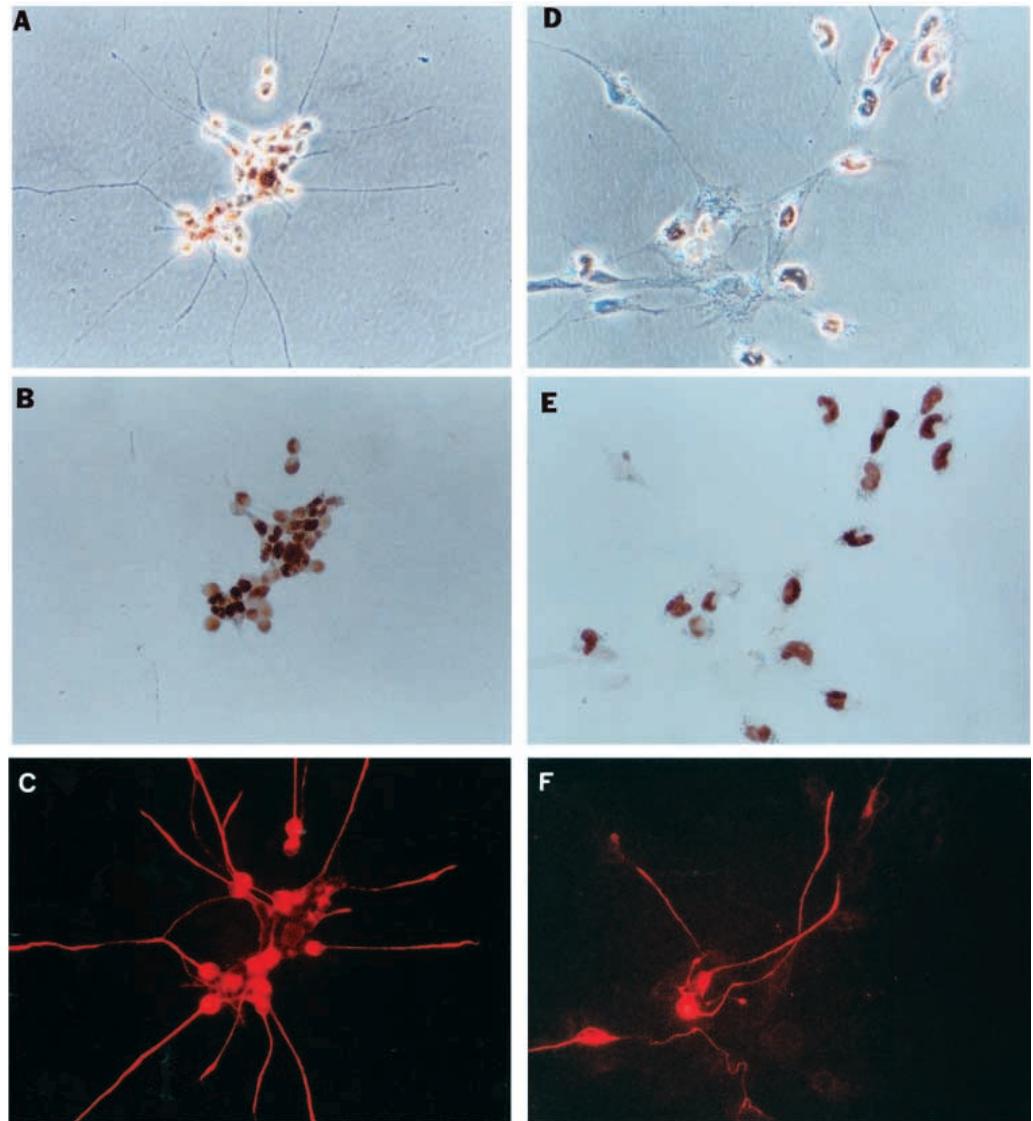
The present data indicate that Phox2a is sufficient to promote expression of a subtype gene, *c-ret*, but not of pan-neuronal genes such as neurofilament or peripherin. These data are consistent with the general idea that subtype and pan-neuronal genes are under separable control. However, Phox2a was not sufficient to drive expression of DBH in NCSCs grown under our present culture conditions. This may indicate that expression of subtype properties is further subdivided between programs controlling neurotransmitter expression and those controlling other aspects of autonomic identity, such as trophic factor receptor expression. It is important to note, however, that expression of neither DBH nor TH is induced by BMP2 in NCSCs grown at clonal density (Shah et al., 1996) (although TH, at least, is induced by BMP4 (or BMP7) in mass-cultured avian neural crest cells; Reissman et al., 1996; Varley and Maxwell, 1996; Varley et al., 1995). Thus, Phox2a might prove sufficient to promote *DBH* expression when tested in neural crest cells cultured under conditions that allow expression of the latter gene in response to environmental signals.

As mentioned earlier, notochord ablation in chick embryos completely prevents expression of Phox2a, but allows expression of CASH1 (albeit at reduced levels) in sympathetic ganglia primordia (Groves et al., 1995). These data might suggest that expression of CASH1 is insufficient for induction of Phox2a, in contrast to the results of the present experiments. It is possible, however, that Phox2a induction requires a certain

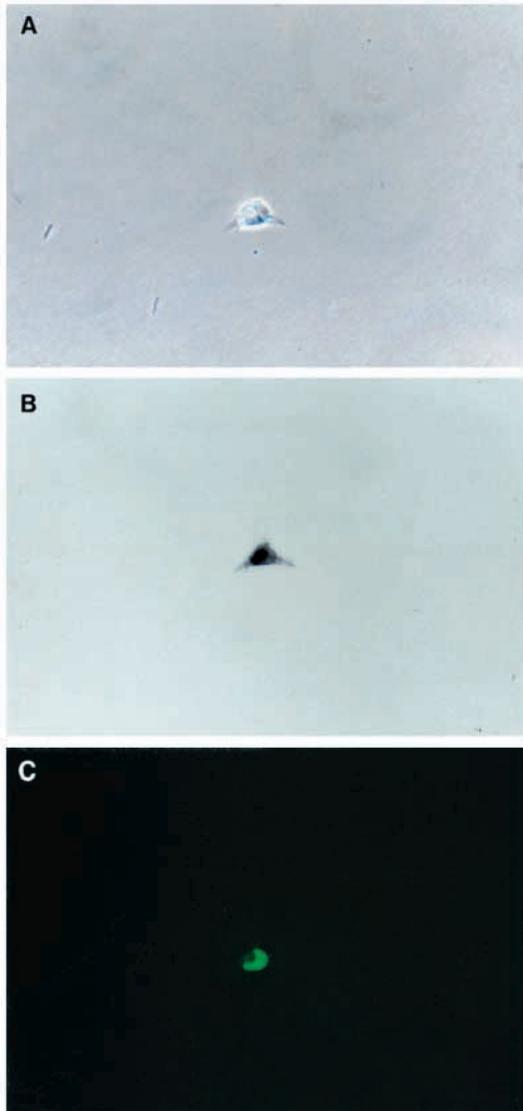
threshold level of MASH1 expression or activity, and that the reduced levels of CASH1 expression in notochord-ablated embryos (Groves et al., 1995) are below this threshold. In support of this idea, low concentrations of BMP2 induced detectable MASH1 expression in many cells, but only a subset of these cells expressed Phox2a (Table 1). Alternatively, induction of Phox2a may require both MASH1 and the action of another extracellular signal, that is dependent on the notochord *in vivo* and is supplied by our culture medium *in vitro*.

### Function of MASH1 in neurogenesis

The present data provide the first evidence that expression of MASH1 is able to promote neurogenesis in an uncommitted cell: 68% of colonies derived from MASH1-infected founder



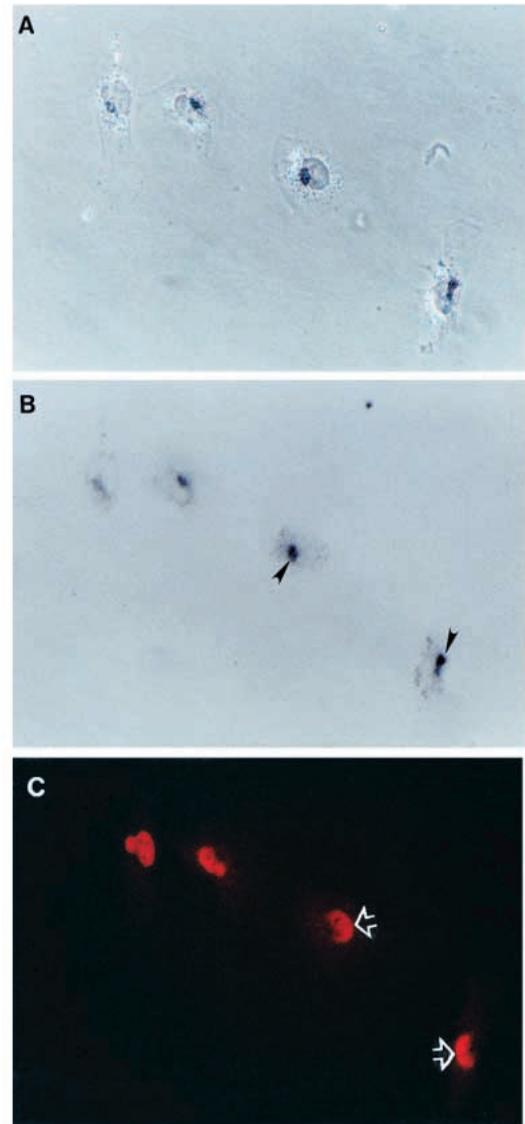
**Fig. 5.** Constitutive expression of MASH1 in NCSCs is sufficient to induce neuronal differentiation in some cells at 5 days post-infection. (A-C) Example of a colony expressing retroviral MASH1 (B) in which many cells express NF160 (C) and have a process-bearing morphology (A). (D-F) Example of a colony in which many of the retroviral MASH1-expressing cells (E) have a non-neuronal morphology (D) and do not express neurofilament (F). For quantification see Table 3. MASH1-infected neuronal cells also expressed peripherin, another neuronal marker, and E12-infected cells did not differentiate to neurons (data not shown and Table 3). Objective magnification  $\times 40$ .



**Fig. 6.** Co-expression of endogenous Phox2a and c-RET in BMP2-treated uninfected NCSCs. A single cell after 20 hours in 50 ng/ml BMP2 (A) is seen to express both c-RET (B) and Phox2a (C). 83% of Phox2a<sup>+</sup> colonies co-expressed c-RET and 62.5% of c-RET<sup>+</sup> colonies co-expressed Phox2a. Objective magnification  $\times 40$ .

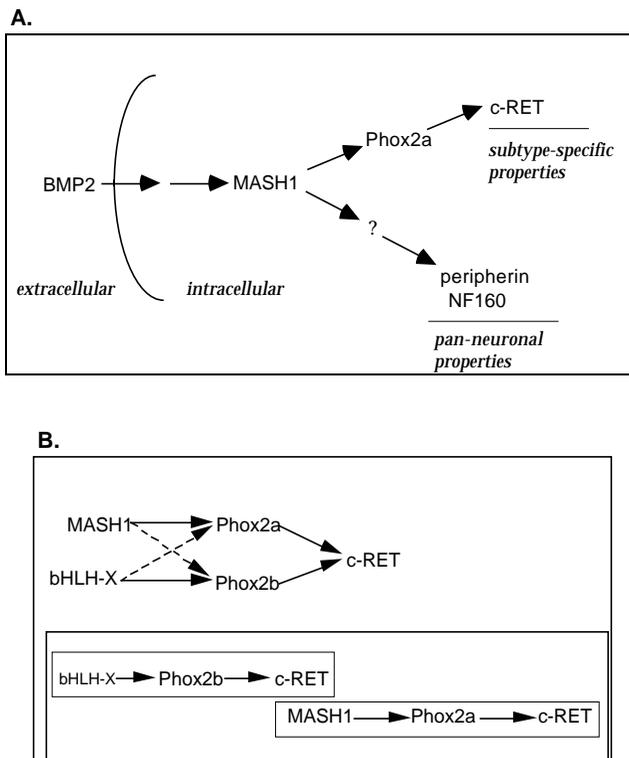
cells contained at least some neurons at 5 days post-infection, whereas only 4% of uninfected colonies in the same plate contained any neurons at this time point. By this criterion, the activity of MASH1 in NCSCs appears analogous to that of the myogenic bHLH proteins, such as MyoD (Davis et al., 1987), which can induce muscle differentiation when overexpressed in non-muscle cells (reviewed in Olson and Klein, 1994). The failure to observe a similar phenotype when MASH1 is transfected into other, non-crest-derived cell types, such as P19 cells (Johnson et al., 1992b), may reflect a requirement for cofactors that are crest lineage-specific, and/or the use of continuous cell lines rather than primary cells.

Although the majority of MASH1-infected NCSC colonies contained neurons, only about 16% of the cells within these colonies were neuronal. The reason for this incomplete penetrance is not yet clear, but could reflect cellular



**Fig. 7.** Constitutive expression of Phox2a induces expression of endogenous c-RET at 40 hours post-infection. Cells expressing myc-epitope-tagged Phox2a (arrows, C) have a non-neuronal morphology (A). All of the cells express detectable endogenous c-RET, which is detectable as a perinuclear dot of staining (arrowheads, B). Control infections using the E12 retrovirus did not yield any significant induction of c-RET expression (Table 4). In separate dishes in which cultures were analyzed after 5 days instead of 40 hours, c-RET expression persisted in Phox2a-infected cells, but the cells retained a non-neuronal morphology and did not express either NF160 or peripherin (Table 4 and data not shown), in contrast to the results obtained with the MASH1 retrovirus. Objective magnification  $\times 40$ .

heterogeneity in competence to respond to MASH1, a threshold requirement for the level of MASH1 required to induce neurogenesis, or intracolony cell-cell interactions that inhibit MASH1 function in some of the cells. Whatever the case, neurogenesis was not promoted by E12, another more broadly expressed bHLH protein (Murre et al., 1989a). Therefore the effect of MASH1 to promote neurogenesis in NCSCs is unlikely to reflect simply the sequestration of negative regulators such as Id (Benezra et al., 1990; Duncan et al., 1992), as E12 should be capable of such sequestration as well.



**Fig. 8.** Regulatory interactions between BMP2, MASH1 and Phox2a during autonomic neurogenesis. (A) Summary of results obtained in the present studies. BMP2 induces MASH1, which in turn is sufficient to induce expression of both pan-neuronal markers, Phox2a and c-RET. The arrow should not be taken to imply that MASH1 is necessarily a direct activator of Phox2a. Phox2a is in turn sufficient to induce (directly or indirectly) expression of c-RET, but not of pan-neuronal markers. ? indicates that the effect of MASH1 to activate pan-neuronal markers is probably indirect. (B) Summary of regulatory interactions implied by loss-of-function, gain-of-function and timing of expression data. The upper diagram indicates potential genetic interactions without regard to cell lineage or timing. Dashed arrows indicate that MASH1 may be sufficient to activate Phox2b in vitro, although it is not necessary for Phox2b expression in vivo (Hirsch et al., 1998). The lower arrow indicates that there exists an as-yet unidentified gene(s) (bHLH-X), which regulates Phox2a in a manner functionally redundant with MASH1, as seen in the enteric nervous system. The lower diagram accounts for the fact that c-RET expression is detected before that of MASH1 in some enteric precursors; this could reflect either sequential activation within a single lineage (large, outer box) or expression in two distinct but temporally overlapping lineages (small inner boxes).

In addition to its ability to promote morphologic neuronal differentiation, MASH1 promoted expression of two pan-neuronal markers, peripherin and NF160. Previously, we showed that in *Mash1*<sup>-/-</sup> mutants, expression of peripherin is lost, but that of NF160 (as well as some other neuronal markers such as neuronal  $\beta$ -tubulin) is unaffected (Sommer et al., 1995). Taken together, these data indicate that MASH1 is both necessary and sufficient for the expression of some pan-neuronal markers (e.g. peripherin), and sufficient but not necessary for the expression of others (eg. NF160). This suggests that MASH1, when overexpressed, can either substitute for, or promote the expression of, other transcription factors normally required for expression of a subset of pan-

neuronal markers. Interestingly, the bHLH factor N-myc was recently shown to be sufficient to promote expression of NF160, but not morphologic neuronal differentiation, in avian neural crest cells (Wakamatsu et al., 1997). It will be of interest in the future to determine the relative roles of MASH1 and N-myc in controlling the expression of pan-neuronal genes in NCSCs.

Recently, we reported that retrovirus-mediated constitutive expression of MASH1 in non-neurogenic, post-migratory crest cells (Lo and Anderson, 1995) was insufficient to promote neurogenesis on its own, but maintained competence for neuronal differentiation induced by BMP2 (Lo et al., 1997). Why is MASH1 sufficient to promote neurogenesis in the absence of exogenous BMP2 in NCSCs, but not in the post-migratory cells? The fact that NCSCs cultured on their own will eventually generate neurons (Stemple and Anderson, 1992), while the non-neurogenic subset of the post-migratory cells do not (Lo et al., 1997; Lo and Anderson, 1995), suggests that the former cells are poised for neurogenesis in a way that the latter are not. This difference could make NCSCs more susceptible than the post-migratory cells to the neurogenic influence of MASH1. For example, the post-migratory cells might lack necessary co-factors for MASH1, which can be induced by BMP2 and which are present constitutively in NCSCs. Alternatively, forced expression of MASH1 in NCSCs might promote expression of BMP-family members, leading to an autocrine induction of neuronal properties. Further work will clearly be necessary to resolve this paradox.

### Regulatory relationships between MASH1, Phox2a and c-RET

The present data, taken together with those in the accompanying paper (Hirsch et al., 1998), indicate that MASH1 is both necessary and sufficient for proper expression of Phox2a in the peripheral autonomic nervous system. Therefore, MASH1 positively regulates Phox2a in the autonomic lineage, although whether this relationship is direct or indirect is not yet established. While other bHLH proteins have been shown to function downstream of MASH1 in the olfactory (Cau et al., 1997) and sympathetic (Ma et al., 1997) lineages, Phox2a is the first non-bHLH transcription factor identified for which MASH1 is both necessary and sufficient. Interestingly, expression of another paired homeodomain protein, PHD1, follows and is dependent on that of MASH1 in the olfactory epithelium (Saito et al., 1996). Moreover, in *Drosophila* the paired box-containing gene *pox-neuro* regulates some aspects of chemosensory neuron identity and is under the control of *achaete-scute* (Vervoort et al., 1995). Thus paired and paired-homeodomain transcription factors may represent a general class of downstream effectors of bHLH genes such as MASH1. Whether any of these transcription factor-encoding genes is directly activated by MASH1 remains to be determined, and will require a detailed genetic and biochemical analysis of their respective promoters.

The present data also suggest a cascade in which MASH1 activates c-RET indirectly, via induction of Phox2a (Fig. 8A). Consistent with this model, there is some reduction in c-RET<sup>+</sup> expression in sympathetic ganglia of *Mash1*<sup>-/-</sup> embryos (L. L. and D. J. A., unpublished). However, c-RET expression appears normal in esophageal ganglia in *Mash1*<sup>-/-</sup> embryos (Guillemot et al., 1993; Lo et al., 1994), despite the absence of Phox2a<sup>+</sup> cells (Fig. 1D). Similarly, expression of c-RET in autonomic ganglia is unaffected in *Phox2a*<sup>-/-</sup> mice (Morin et al., 1997).

This is likely due to compensation by Phox2b, which has a homeodomain identical to that of Phox2a (Pattyn et al., 1997) and which is expressed normally in *Mash1*<sup>-/-</sup> embryos (Hirsch et al., 1998). Thus, Phox2a is sufficient (Fig. 8A) but probably not necessary (Fig. 8B, upper) for induction of c-RET by MASH1. We can also not formally exclude the possibility that MASH1 is required in addition to Phox2a for expression of c-RET, although this seems unlikely since abundant endogenous MASH1 expression does not occur in Phox2a-infected cells (L. L. and D. J. A., unpublished data).

A further complexity arises when considering the relative timing of MASH1 and c-RET expression in vivo. In vitro, MASH1 expression precedes or accompanies that of c-RET; the same sequence is seen for some sympathetic ganglia in vivo. By contrast, however, in the enteric nervous system c-RET expression precedes that of MASH1 (Lo et al., 1994). This reversed order of expression may be explained by the fact that expression of Phox2b normally precedes that of Phox2a in enteric precursors (Pattyn et al., 1997), and that Phox2b is likely to activate c-RET expression as well (Fig. 8B, lower). It is not yet clear whether this successive expression occurs within the same lineage (Fig. 8B, lower, large outer box), or rather in distinct sublineages (Blaugrund et al., 1996; Durbec et al., 1996), which temporally overlap (Fig. 8B, lower, small inner boxes). If the former were true, it would imply that MASH1 is involved in the maintenance or upregulation, rather than in the initiation, of c-RET expression.

The role, in turn, of c-RET in autonomic neurogenesis is not yet clear. c-RET is required for development of enteric (Schuchardt et al., 1994) and rostral sympathetic ganglia (Durbec et al., 1996). Preliminary data suggest that two ligands that activate c-RET, GDNF (Robertson and Mason, 1997) and neurturin (Kotzbauer et al., 1996), can act as survival factors and mitogens for E14.5 c-RET<sup>+</sup> enteric progenitors (L. L. and D. J. A., unpublished), but other data suggest that GDNF may promote differentiation to particular phenotypes as well (Maxwell et al., 1996).

In summary, our data begin to provide an outline of how instructive environmental signals interact with the transcriptional regulatory machinery of neural stem cells to promote the specification of a particular neuronal identity. Extracellular signals, such as BMP2, induce a cascade of transcription factor gene expression. Some of these transcription factors, such as Phox2a, control expression of neuronal subtype-specific genes, such as those encoding trophic factor receptors. Others, such as MASH1, appear to couple the expression of both pan-neuronal and subtype-specific genes. The fact that expression of endogenous structural and regulatory genes can be elicited by retroviral transduction of exogenous transcription factor genes in cultured neural crest cells, where the extracellular environment can be easily manipulated, should facilitate the further dissection of the interplay between cell-extrinsic and cell-intrinsic determinants of neuronal identity.

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### Note added in proof

We have recently observed that *Phox2a* and *Phox2b* can induce expression in some NCSCs of tyrosine hydroxylase. Thus, *Phox2a* is able to activate expression of *some* neurotransmitter synthetic enzyme genes.