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The transcription factor dHAND is a downstream effector of BMPs in sympathetic neuron specification

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SUMMARY

The dHAND basic helix-loop-helix transcription factor is expressed in neurons of sympathetic ganglia and has previously been shown to induce the differentiation of catecholaminergic neurons in avian neural crest cultures. We now demonstrate that dHAND expression is sufficient to elicit the generation of ectopic sympathetic neurons in vivo. The expression of the *dHAND* gene is controlled by bone morphogenetic proteins (BMPs), as suggested by BMP4 overexpression in vivo and in vitro, and by noggin-mediated inhibition of BMP function in vivo. The timing of *dHAND* expression in sympathetic ganglion primordia, together with the induction of *dHAND* expression in response to Phox2b implicate a role for dHAND as transcriptional regulator downstream of Phox2b in BMP-induced sympathetic neuron differentiation.

Key words: dHAND, Chick, BMP, Catecholaminergic neuron, Differentiation

INTRODUCTION

The development of nervous systems involves the generation of different neuronal cell types that are assembled into functional circuits. Neuronal identity is determined in a progressive process that integrates specific cell-cell interactions, positional information and general neurogenesis programs (Jessell and Lumsden, 1998). Extrinsic signals are responsible for the generation of regional identity in the nervous system and are required for the specification of particular types of neurons within these regions. Such extrinsic signals control, either directly or indirectly, the expression of cascades of transcriptional regulatory factors, in particular basic helix-loop-helix (bHLH) proteins, that promote the generation and differentiation of specific neuron subtypes (Anderson and Jan, 1998). Although different bHLH genes, for example, *Mash1* and the gene for neurogenin 1 (Lo et al., 1991; Ma et al., 1996), are expressed in different subsets of neuronal precursors and define two major sublineages within the mammalian PNS, they are used repeatedly in complementary domains in the CNS to control neuronal identity and differentiation (Ma et al., 1997; Cau et al., 1997; Casarosa et al., 1999). Thus, the same transcription factor is essential for the generation of different neuronal phenotypes in different lineages. This suggests that the lineage-specific cellular context, which may reflect the history of the cell (including positional information), determines the readout of the regulatory cascades, and also raises the question of the identity of these lineage-specific regulatory factors. It is therefore important that we understand how extrinsic signals lead to the lineage-specific expression of transcriptional regulators and how they control the expression of both lineage specific and pan-neuronal terminal differentiation genes.

To begin to address these issues, we have focussed on a neuronal lineage that has been very extensively characterized and that gives rise to peripheral sympathetic neurons (Francis and Landis, 1999). These neurons develop from neural crest precursor cells that aggregate after their migration at the dorsal aspect of the dorsal aorta. Expression of the lineage-specific noradrenergic marker genes tyrosine hydroxylase (*TH*) and dopamine-β-hydroxylase (*DBH*) is observed at stage 18 in the chick embryo (Ernsberger et al., 1995; Ernsberger et al., 2000) and E11 in the rat embryo (Cochard et al., 1979; Cochard et al., 1978; Teitelman et al., 1979). Important transcriptional regulators in this lineage are *Mash1* (Lo et al., 1991; Lo et al., 1994) and *Cash1* (Ernsberger et al., 1995), mouse and chick homologs of *Drosophila achaete scute* proneural genes, and the paired homeodomain genes *Phox2a* and *Phox2b* (Valarché et al., 1993; Morin et al., 1997; Pattyn et al., 1999). In the chick, *Cash1* mRNA is first detectable at stage 15 (Ernsberger et al., 1995), followed by the expression of *Phox2b* and *Phox2a* from stage 18 onwards, with an onset of *Phox2b* expression that precedes *Phox2a* expression (Ernsberger et al., 2000). The analysis of transgenic mice deficient for *Mash1*, *Phox2b* and *Phox2a* demonstrates an essential role of Mash1 and Phox2b for sympathetic ganglion development, whereas sympathetic ganglia are only marginally affected in *Phox2a*$^{-/-}$ animals (Guillemot et al., 1993; Morin et al., 1997; Pattyn et al., 1999). The expression of *Phox2b* is independent of *Mash1*, suggesting the presence of two interacting pathways, Mash-dependent and

Phox2b-dependent, that control autonomic neuron generation (Hirsch et al., 1998; Lo et al., 1998, 1999). Overexpression of *Phox2* genes in the chick PNS results in the generation of ectopic sympathetic neurons in peripheral nerve and the dorsal root ganglia (DRG), revealing that *Phox2* genes are sufficient for the specification of sympathetic neurons in trunk neural crest cells (Stanke et al., 1999).

Sympathetic neuron generation is controlled by signals from the axial structures, as revealed by results from experimental embryology (Teillet and Le Douarin, 1983; Stern et al., 1991; Groves et al., 1995). Recent evidence strongly implicates BMPs as factors that elicit sympathetic neuron generation. BMPs are expressed in the immediate vicinity of sympathetic ganglion primordia, in the dorsal aorta (Reissmann et al., 1996; Shah et al., 1996a). They induce the development of sympathetic neurons in cultures of neural crest (Varley et al., 1995; Reissmann et al., 1996; Shah et al., 1996a) and in vivo, upon overexpression (Reissmann et al., 1996). They also induce the expression of *Phox2a* and *Phox2b* in neural crest cultures (Reissmann et al., 1996). Recent loss-of-function experiments in the chick demonstrate an essential role of BMPs for sympathetic neuron development and implicate *Cash1* and *Phox2* genes in the BMP signal transduction in this lineage (Schneider et al., 1999).

As Phox2 proteins are able to bind directly to the promoters of *TH* and *DBH* genes, and activate their transcription (Zellmer et al., 1995; Yang et al., 1998; Kim et al., 1998; Lo et al., 1999), the differentiation of sympathetic neurons can be explained, at least partially, by the BMP-dependent expression of *Phox2* genes. However, there is some evidence to suggest that a more complex network of transcriptional regulators is involved in the expression of terminal differentiation genes in sympathetic neurons. Although *Mash1* and *Phox2* genes are required for differentiation of noradrenergic neurons in the PNS and CNS, they are also expressed in lineages that do not express noradrenergic marker genes (Guillemot and Joyner, 1993; Tiveron et al., 1996; Pattyn et al., 1999). In addition, the bHLH proteins dHAND (Srivastava et al., 1995; Hollenberg et al., 1995) and eHAND (Cserjesi et al., 1995; Hollenberg et al., 1995) (also known as HAND2 and HAND1) are expressed in sympathetic neurons during normal development and were recently found to elicit the generation of sympathoadrenergic cells when expressed in quail neural crest cells (Howard et al., 1999). This raises the question of whether HAND- and Phox2-induced sympathetic neuron generation represent parallel or sequential pathways. In the present study we aimed to place the *HAND* genes in temporal and epistatic order with respect to the *Phox2* genes, and to relate *HAND* expression to precise stages of sympathetic neuron differentiation. In addition, we have investigated whether the expression of *HAND* genes is regulated by BMPs.

MATERIALS AND METHODS

In situ hybridization on sections

Nonradioactive in situ hybridization on cryosections and preparations of digoxigenin- or fluoresceine-labeled probes were carried out using a modification of the protocol of D. Henrique (IRFDBU, Oxford, UK), as previously described (Ernsberger et al., 1997). The following probes were used: *Cash1* (Jasoni et al., 1994), and the chick homologs of *dHAND* and *eHAND* (Howard et al., 1999), *Phox2a* (Ernsberger et al., 1995), *Phox2b* (Ernsberger et al., 2000), *Sox10* (Schneider et al., 1999), *DBH* (Ernsberger et al., 2000) and *NF160* (Zopf et al., 1987). For double-in situ hybridizations, Fast Red (Roche Diagnostics, Mannheim, Germany) was used as alkaline phosphatase substrate for staining of the first probe. Sections were photographed and the phosphatase was inactivated by incubating the slides in 0.1 M glycine (pH 1.8) for 10 minutes. Sections were then equilibrated in MABT (maleic acid buffer with Tween 20) and detection of the second probe by incubation with appropriate antibody, followed by staining procedure using NBT/BCIP (4-nitroblue tetrazolium chloride/5-Bromo-4chloro-3-indolyl-phosphate) as substrate was carried out as described above.

Analysis of embryonic *HAND* gene expression in vivo

Hamilton/Hamburger stage 18 chick embryos (Hamburger and Hamilton, 1951) were collected, specified according to somite number, fixed in 4% paraformaldehyde overnight and processed further, analogous to E8 specimen (see below). Consecutive 12 μm cryosections were analysed for expression of *Cash1*, *dHAND*, *eHAND*, *Phox2a*, *Phox2b* and *TH* by in situ hybridization.

Expression of transgenes in vivo using avian retroviral RCAS vectors

Fertilized virus-free chicken eggs were obtained from Charles River (Sulzfeld, Germany) and incubated for 2 days. After opening the eggshell and staging the embryos, aggregates of infected BMP4-RCASBP(A)- (Duprez et al., 1996), dHAND-RCASBP(B)- (Howard et al., 1999) or Phox2b-RCASBP(B)-producing (Stanke et al., 1999) chick embryo fibroblasts (CEF) were implanted into the embryos at brachial levels between the neural tube and the last somite formed (Reissmann et al., 1996). The eggs were sealed with tape and incubated until E8. Embryos were killed by decapitation. Trunk and cervical region of the embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) overnight, kept in 15% sucrose (in 0.1 M phosphate buffer, pH 7.0) overnight, embedded in Tissue-Tek (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands) and sectioned. Cryostat cross-sections (12 μm) were separately collected and analysed for expression of *Cash1, dHAND, eHAND, Phox2a, Phox2b, TH, DBH, SCG10* and *NF160* by in situ hybridization. At least three embryos were analysed for each of the genes investigated.

Implantation of agarose-beads loaded with noggin or BSA into chick embryos

The implantation technique used is described in detail in Schneider et al, 1999. Agarose beads were incubated in a small volume of loading buffer, containing either 1mg/ml noggin or BSA. Beads were implanted into 2-day-old chick embryos close to notochord and dorsal aorta. Two beads were implanted into 20-22 somite embryos, at the last somite formed and 2-3 somites more rostral. The eggs were further incubated. For the present analysis two embryonic stages, late stage 19 and stage 22 embryos were studied in detail. These stages were chosen because, in control embryos, *dHAND* is consistently expressed in the caudal implantation region at late stage 19, but not earlier. Trunk and cervical region of the embryos were fixed, embedded and sectioned. Consecutive 12 μm cryostat cross-sections were separately collected from a large region of the embryo, including the implantation area, and analysed for expression of *dHAND*, *TH*, *Phox2b* and *Sox10* by in situ hybridization. At least three embryos were analysed for each marker.

Planimetric analysis of in situ hybridisation signals

The technique used is described in detail in Schneider et al, 1999. Digital black/white images (grey value resolution 8 bit, i.e. 256 gray values, spatial resolution 512×512 pixel) of the sections were created using a Zeiss microscope connected to a VIDAS 2.1 image

analysis system (Kontron, Eching, Germany). The area of cells with hybridization signal was determined via the software (in pixels, later calibrated to μm^2). In this way, the program sums the areas of all positive cells. The areas of the hybridization signals were determined on serial sections rostral and caudal to the bead implanted at somites III-IV. For each marker, a total of eight sections were analysed per embryo, covering 400 μm along the anteroposterior axis, and the mean area per section was calculated for each embryo. The results are given as the mean area per section±s.e.m of at least four embryos analysed.

Neural crest cell culture

Neural crest cells were obtained from stage 13 (Zacchei, 1961) Japanese quail embryos *(Coturnix coturnix japonica*) for all culture experiments, as previously described (Howard and Bronner-Fraser, 1985; Howard et al., 1999). Fertilized quail eggs (GQH, GA, USA) were incubated at 38°C for 43-47 hours. The neural tube and associated somites were surgically removed from embryos using electrolytically sharpened tungsten needles and the neural tube fragments were incubated in 0.5% collagenase A (Boehringer Mannheim) for 12 minutes at room temperature. The neural tubes were released using gentle trituration with fire polished Pasteur pipettes and were collected in fresh growth medium, washed in one additional change of fresh growth medium and plated on 35 mm tissue culture plates coated with 24 mg/ml fibronectin (Gibco/BRL). The neural tube explants were removed from the dish using tungsten needles after 14-16 hours leaving behind the neural crest cells that had migrated out onto the dish. Culture plates were carefully screened on day 2 for the presence of motoneurons indicating that neural tube cells had been left on the dish. For these studies, no dishes containing motoneurons were used in the analysis. At the time the explants were removed, growth media was changed and every other day thereafter, for the 7 day culture period.

Growth media

Neural crest cell cultures were fed with medium containing Eagle's minimal essential medium (Gibco/BRL) supplemented with 15% horse serum and 2% 11-day chick embryo extract (CEE) prepared as previously described (Howard and Bronner-Fraser, 1985).

Catecholamine histofluorescence

Cells synthesizing and storing catecholamines were detected by the method of Falck et al., 1962 (Howard and Bronner-Fraser, 1985; Howard and Gershon, 1993; Howard et al., 1999). Catecholamines condense with formaldehyde in the absence of water at 80°C to form a specific fluorescent product with a characteristic excitation/emission of 420/480 nm, respectively. Briefly, cells are heat fixed and incubated for 60 minutes in the presence of paraformaldehyde at 80°C. To maintain paraformaldehyde free of water, powder is stored in an airtight chamber suspended over H_2SO_4. At the end of the incubation, the cells are mounted in mineral oil and noradrenaline is visualized using a specific catecholamine filter set.

RT-PCR

Whole-cell RNA was isolated from the outgrowth from 20 to 30 primary neural crest explants per condition using the Total RNA kit (Ambion) according to the manufacturers directions. Following DNAase treatment, total cellular RNA (1 μg from 20-30 primary explants) was used as template for first strand synthesis using 50 units Moloney murine leukemia virus reverse transcriptase (Gibco/BRL Superscript II), 1 mM each dNTP, 3 mM $MgCl_2$, and 5 mM random hexanucleotides in a final volume of 20 μl; the reaction was carried out for 60 minutes at 40°C. In pilot studies, we established that amplification of neither β-actin nor dHAND transcript was saturated, so our assays were carried out in the linear range of amplification.

RESULTS

dHAND elicits ectopic sympathetic neuron generation

Both *dHAND* and *eHAND* genes induce sympathoadrenergic differentiation when expressed in quail neural crest cultures (Howard et al., 1999). To investigate whether *HAND* genes are able to initiate this process also in a physiological context, *dHAND* was overexpressed in vivo, in chick neural crest cells using RCAS retroviral vectors. As *dHAND* seems to be expressed before *eHAND* in sympathetic neuron precursors (see below), we focussed on the overexpression of the potential upstream gene *dHAND*. Aggregates of cells producing dHAND-RCAS were implanted into the neural crest migration pathway of E2 embryos, resulting in largely unilateral infection of embryos, including peripheral ganglia and nerves. To analyse the effects of *dHAND* expression we chose to use the peripheral nerve, as this tissue contains no cells expressing neuronal marker genes during normal development (Stanke et al., 1999). In contrast, dHAND-RCAS-infected nerves contained a large number of cells expressing the pan-neuronal genes *SCG10* and *NF160* and the noradrenergic marker genes *TH* and *DBH* (Fig. 1). Double in situ hybridization for *SCG10* and *TH* demonstrated co-expression of noradrenergic and pan-neuronal marker genes. Also in the DRG, such cells were occasionally observed (not shown), as described previously in response to *Phox2* overexpression (Stanke et al., 1999). Infection of these tissues by control virus did not result in ectopic noradrenergic neurons (Stanke et al., 1999). These results demonstrate that dHAND is sufficient to induce sympathetic neuron development in vivo, in trunk neural crest cells.

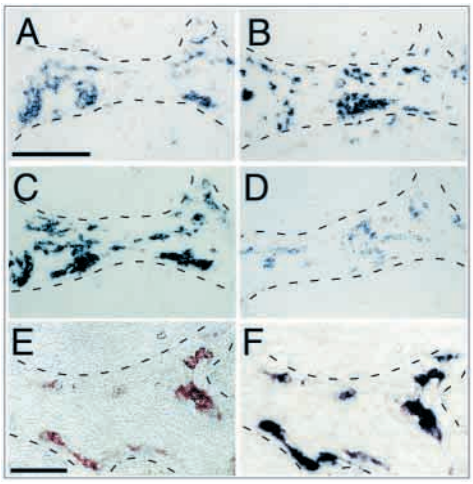

Fig. 1. dHAND overexpression in the developing chick embryo results in the generation of ectopic sympathetic neurons in the peripheral nerve. dHAND-RCASBP(B)-infected embryos were analysed at E8 for the expression of *TH* (A), *DBH* (B), *SCG10* (C) and *NF160* (D) in the brachial nerve by in situ hybridization. (E) and (F) show higher power magnifications of ectopic cells that express both SCG10 (E, red colour) and TH (F, dark-blue colour) as revealed by double in situ hybridization. Note that the staining patterns of the red and blue signals are identical. The outline of the nerves is indicated by broken lines. Scale bars: 100 μm for A-D; 50 μm for E,F.

The expression of *HAND* genes is controlled by BMPs in vitro and in vivo

HAND and *Phox2* genes display similar effects upon overexpression in neural crest cells both in vitro and in vivo. As *Phox2* gene expression is dependent on BMPs in vivo, it was of interest to analyse whether *HAND* gene expression could be controlled by BMPs. To address this issue, neural crest cultures were treated with BMPs and analysed for catecholaminergic differentiation and *HAND* expression. As described in previous studies (Varley et al., 1995; Varley and Maxwell, 1996; Reissmann et al., 1996; Shah et al., 1996), BMP treatment elicits the generation of a large number of catecholaminergic cells (Fig. 2A,B), which is paralleled by a 15-fold increase in *dHAND* mRNA, as demonstrated by RT-PCR analysis (Fig. 2C). This result suggests that *dHAND* expression is directly or indirectly controlled by BMPs in neural crest precursor cells.

To support these in vitro observations, BMP4 was overexpressed in vivo, using RCAS vectors. E2 chick embryos were infected by implanting virus-producing cells and analysed at E8 for the presence of ectopic neurons in peripheral nerve. Infected peripheral nerves contained a large number of *dHAND*-positive cells, noradrenergic cells and cells expressing a pan-neuronal marker gene (Fig. 3). *eHAND*-positive cells were also detectable, albeit in lower numbers and with a lower expression level (compared with *dHAND*-expressing cells; Fig. 3A,C). These results demonstrate that BMPs elicit the development of ectopic sympathetic neurons in peripheral nerve and suggest that dHAND is involved in the signal transduction cascade induced by BMP4.

To see whether BMPs are indeed required for *dHAND* expression, the action of BMPs during sympathetic neuron development was blocked by the application of the BMP antagonist noggin (Zimmerman et al., 1996). Noggin-loaded agarose beads were implanted into the trunk region of 2-day-old chick embryos (22-22 somites), positioned in the vicinity of notochord and dorsal aorta (Schneider et al., 1999). Noggin-treated and BSA-treated control embryos were analysed for *dHAND*, *Phox2* and *TH* (not shown) gene expression at stage 19 and stage 22. To analyse total ganglion cells the expression of *Sox10* (Kuhlbrodt et al., 1998) was investigated in parallel (Schneider et al., 1999). It was found that in noggin-treated embryos *dHAND*, *Phox2b* and *TH* expression is virtually absent at stage 19 (Fig. 4) and stage 22 (not shown). The general neural crest marker Sox10 is detectable in sympathetic ganglion primordia of noggin-treated embryos (Fig. 4), albeit Sox10-positive cells are present in reduced numbers. The late onset of *dHAND* expression is reflected by the much smaller area of *dHAND* positive cells, as compared with the area of *Phox2b*-positive cells in stage 19 controls, whereas at stage 22, *Phox2b* and *dHAND* are present in equal numbers (data not shown). The expression of *eHAND* was not detectable at the level of implantation in both control and noggin-treated embryos at these stages (see below).

These results demonstrate that the BMP-dependent development of sympathetic neurons involves the expression of *dHAND* as well as of *Phox* genes, and raises the question of whether these are parallel or sequential pathways.

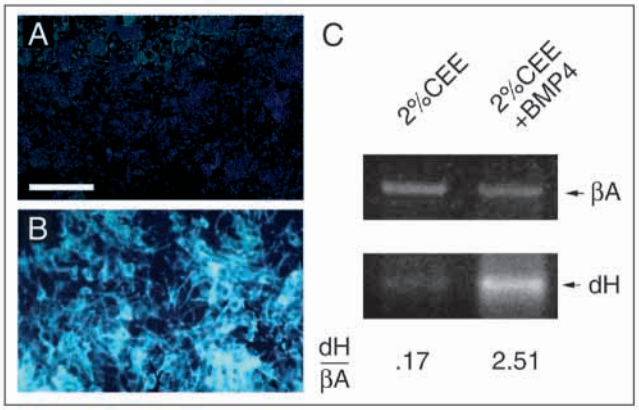

Fig. 2. BMP supports catecholaminergic (CA) differentiation of neural crest-derived cells and induces transcripts encoding *dHAND*. Formaldehyde-induced fluorescence (FIF) was used to assess CA differentiation in 2% (A,B) CEE medium in the absence (A) or presence (B) of 10 ng/ml BMP4. (C) RT-PCR was used to examine levels of transcript encoding *dHAND* (dH) and β-actin (βA) in sister cultures of those used for FIF. The ratio of intensity of ethidium bromide-labeled βA and *dHAND* bands (determined using NIH image) was used for semiquantitative assessment of *dHAND* transcript levels and is presented below each lane in the gels. Cells synthesizing and storing noradrenaline are not detected in cultures of neural crest cells maintained in 2% CEE medium (A). The addition of 10 ng/ml BMP4 significantly increases the number of cells differentiating as CA neurons in 2% CEE (B), as well as inducing the expression of *dHAND*(C). Scale bar: 50 μm.

Expression of *dHAND* and *eHAND* during sympathetic ganglion development

To address the question of the temporal relationship of *HAND* and *Phox* genes, *HAND* gene expression was analysed during sympathetic ganglion development in the chick embryo. The previous description of *eHAND* and *dHAND* expression in the mouse (Hollenberg et al., 1995; Srivastava et al., 1995; Cserjesi et al., 1995) and chick (Howard et al., 1999) embryo suggested that the onset of HAND gene transcription coincides with adrenergic differentiation. The timing of sympathetic neuron differentiation and the sequential expression of different genes is difficult to study in the mouse and can be analysed with much higher resolution in the chick embryo. Extending

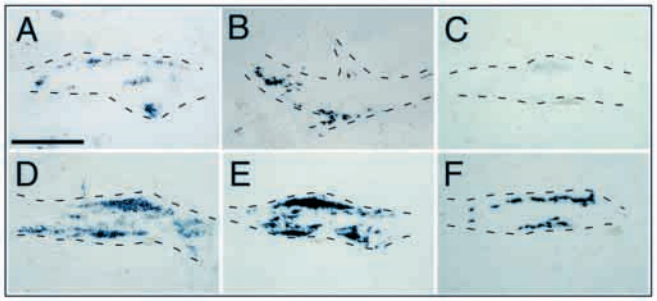

Fig. 3. Expression of BMP4-RCASBP in the chick embryo generates cells in the brachial nerve expressing transcription factors and terminal differentiation genes characteristic for sympathetic neurons. At E8, the brachial nerves of infected embryos show cells expressing mRNA for transcription factors *dHAND* (A), *Phox2b* (B) and *eHAND* (C), noradrenergic marker genes *TH* (D) and *DBH* (E), as well as the pan-neuronal gene *SCG10* (F). Broken lines indicate the outline of the nerve. Scale bar: 100 μm.

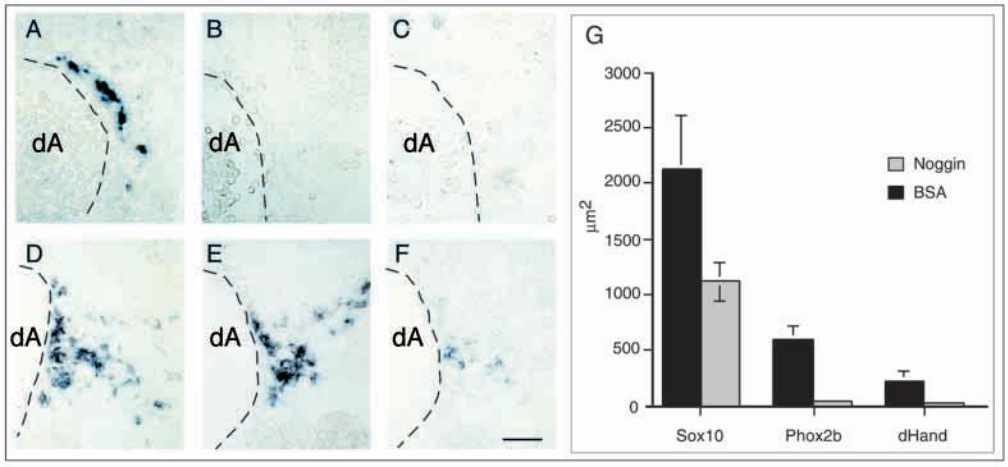

Fig. 4. BMPs are essential for the expression of the *dHAND* transcription factor in primary sympathetic ganglia. The expression of *Sox10* (A,D), *Phox2b* (B,E) and *dHAND* (C,F) was analysed at late stage 19 in noggin-treated (A-C) and BSA-treated (D-F) embryos by in situ hybridisation. The expression of *Sox10*, *Phox2b* and *dHAND* is quantified in (G) (see Materials and Methods). Noggin treatment reduces *Sox10* expression and prevents the expression of *dHAND* and *Phox2b*. dA, dorsal aorta. Scale bar: 100 µm.

previous studies on the expression of different marker genes in primordia of sympathetic ganglia at brachial levels (Ernsberger et al., 1995; Ernsberger et al., 2000), we have now analysed the expression of *dHAND* and *eHAND* by nonradioactive in situ hybridization. The analysis of consecutive sections revealed that *dHAND* expression became detectable after *Cash1* and *Phox2b* but before the general onset of expression of noradrenergic and neuronal marker genes (Fig. 5). The expression of *eHAND* was delayed when compared with *dHAND* and became detectable after the onset of *TH* and *DBH* expression (Fig. 5; not shown). The results are summarized for several embryos between early (29-30 somites) and late (35-36 somites) stage 18 in Table 1. The timing of *dHAND* expression is compatible with a role in the control of terminal differentiation, downstream of *Phox2b*. The expression of *eHAND* is not only delayed but also much weaker when compared with *dHAND* expression. The low intensity of the *eHAND* signal seems to be due to a low expression level in the sympathoadrenergic lineage, as a similar low expression level (compared with *dHAND*) is also observed at later stages of development (E8, not shown).

Phox2 genes induce the expression of *dHAND* and *eHAND* genes

The expression pattern of the *dHAND* gene raised the possibility that its expression may be regulated by *Phox2* genes. To address the epistatic relationship between *Phox* and *HAND* genes, we overexpressed *Phox2* genes in the chick peripheral nervous system, using RCAS vectors. A previous study showed that Phox2 expression resulted in the generation of ectopic sympathetic neurons in the DRG and peripheral nerve (Stanke et al., 1999). Peripheral nerves of E8 embryos infected with Phox2b-RCAS or Phox2a-RCAS (not shown) contained a large number of cells that strongly expressed *dHAND* mRNA (Fig. 6). In contrast, *eHAND*-expressing cells were found in lower numbers, as described for sympathetic neurons during normal development (see above). These results demonstrate that *dHAND* and *eHAND* expression is induced by forced expression of Phox2a or Phox2b proteins.

We also investigated whether the overexpression of dHAND affected the expression of *Phox2* genes. Surprisingly, *Phox2b* and *Phox2a* expression was observed in dHAND-expressing peripheral nerves (Fig. 6) which indicates that Phox2b and dHAND display crossregulation, although during normal development *dHAND* is expressed after *Phox2b*.

DISCUSSION

The generation of sympathetic neurons is controlled by a network of transcriptional regulators, including the bHLH proteins Mash1 and Cash1 (Guillemot et al., 1993; Hirsch et al., 1998; Lo et al., 1998; Shah et al., 1996a; Lo et al., 1999), and the paired homeodomain proteins Phox2a and Phox2b

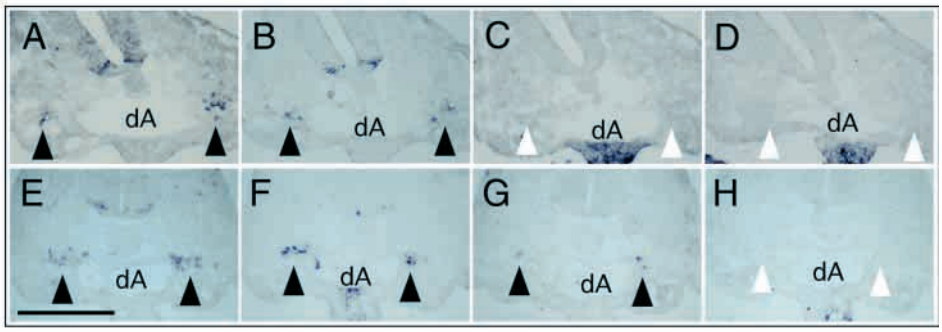

Fig. 5. The expression of *Phox2b* in the sympathetic chain at brachial level precedes the expression of HAND genes. Cross-sections of limb bud region of HH stage 18 embryos with 29 (A-D) or 36 (E-H) somites. In early stage 18 embryos, in situ hybridization reveals clusters of sympathetic precursors expressing *Cash 1* (A) and *Phox2b* (B) (black triangles) but no *dHAND* (C) or *eHAND* (D) (white triangles). Late stage 18 primary ganglia contain cells expressing *Phox2b* (E) and *dHAND* (F) (black triangles). A few cells start to express *TH* (G) (black triangles), whereas no *eHAND* message can be seen (H) (white triangles). *eHAND* expression is detectable at cranial levels in 36 somite embryos. dA, dorsal aorta. Scale bar: 500 µm.

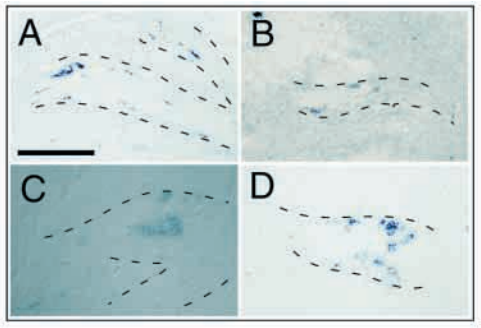

Fig. 6. Crossregulation of *Phox2b* and *dHAND* genes in ectopic cells in the brachial nerve of virus-infected embryos at E8 revealed by in situ hybridization. In Phox2b-RCAS(B) infected embryos (A,B), ectopic cells display expression of *dHAND* (A) and *eHAND (*B) genes. Conversely, both *Phox2b* (C) and *Phox2a* (D) genes are expressed in the ectopic cells generated by dHAND-RCAS(B) overexpression (C,D). The outline of the nerve is indicated by broken lines. Scale bar: 100 μm.

(Morin et al., 1997; Pattyn et al., 1999; Lo et al., 1999; Stanke et al., 1999). The expression of *Cash1* and *Phox2* genes and the further development of sympathetic precursors are dependent on extrinsic signals, i.e. BMPs released from the dorsal aorta in the immediate vicinity of sympathetic ganglion primordia (Schneider et al., 1999). Previous in vitro studies, showing that the *dHAND* and *eHAND* proteins induce catecholaminergic differentiation in neural crest cells, suggested a role for *HAND* genes in the sympathetic neuron development (Howard et al., 1999). We now demonstrate that *dHAND* expression is sufficient to elicit sympathetic neuron development in vivo. The expression of *HAND* genes is controlled in vivo and in vitro by BMPs as suggested by gain- and loss-of-function experiments. The onset of *dHAND* expression, observed after *Cash1* and *Phox2b*, but before *TH* and *DBH* expression suggests a sequential action of Phox2b and dHAND in the control of terminal differentiation genes in sympathetic neurons.

To analyse the effects of *HAND* gene expression on sympathetic neuron development in vivo, dHAND was ectopically expressed in chick embryos using RCAS vectors. As *dHAND* expression in sympathetic ganglia was observed earlier than *eHAND* expression, we focussed on the effects of *dHAND* overexpression. The infection of E2 chick embryos by implantation of virus-producing chick embryo fibroblast cells results in a widespread, but largely unilateral, infection of embryonic tissues, including the peripheral nervous system. As ectopic neurons can be most convincingly detected and analysed in the peripheral nerve that is completely devoid of neurons during normal development, dHAND-RCAS infected embryos were screened for *TH*-expressing cells in the brachial nerve. In dHAND-expressing nerve, a large number of cells was detected that co-expressed noradrenergic and pan-neuronal marker genes. In addition, some cells expressing *eHAND* were observed. Ectopic sympathetic neurons were previously observed in response to the expression of Phox2a or Phox2b in peripheral nerve (Stanke et al., 1999), suggesting that Phox2 and dHAND proteins are both sufficient to elicit sympathetic neuron development in vivo, acting either sequentially or in parallel. Neural crest precursor cells present in peripheral ganglia and nerve during development (Xue et al., 1985; Duff et al., 1991; Morrison et al., 1999; Stanke et al., 1999) are most likely the cells that differentiate into sympathetic neurons in response to forced expression of Phox2 and dHAND proteins.

Table 1. Expression of *Cash1, Phox2, dHAND* and adrenergic marker gene transcripts in the chick embryo

Hamburger/Hamilton stage 17/18				
Somites	29-30	31-32	33-34	35-36
Probe				
Cash1	+,+	+,+	+	+,+
Phox2b	+,+	+,+	+	+,+
dHAND	–,–	+,+	+	+,+
Phox2a	–,–	–,+	+	+,+
TH	–,–	–,–	–	+,+
eHAND	–,–	–,–	–	–,–*

All sections examined correspond to the wing bud region. Data from one embryo are listed in one row.

**eHAND* expression is detectable at cranial levels in stage 35-36 somite embryos.

+, sections with positive cells at the sites of the primary sympathetic ganglia dorsolateral to the dorsal aorta; –, sections devoid of positive cells in primary sympathetic ganglia.

Phox2 gene expression in vivo is dependent on the presence of BMPs (Schneider et al., 1999) and is induced by BMP4 in neural crest cultures (Reissmann et al., 1996; Shah et al., 1996; Lo et al., 1998; Lo et al., 1999) and upon overexpression in vivo (Reissmann et al., 1996). A sequential action of *Phox2b* and *dHAND* genes would implicate a common regulation by BMPs whereas a *Phox2b*-independent, parallel action of *dHAND* could be BMP independent. To address these issues, the effects of BMPs on *HAND* gene expression were analysed in vitro and in vivo. In neural crest cultures maintained in the presence of a low concentration (2%) of CEE, catecholaminergic differentiation is virtually completely blocked (Ziller et al., 1987; Howard and Bronner-Fraser, 1985). Under these conditions, BMP4 addition induces the generation of a large number of catecholaminergic cells. In parallel, the expression of *dHAND* is increased 15-fold. As the initiation of catecholaminergic differentiation may require a threshold level of dHAND expression, there is no requirement for a quantitative correlation between the extent of dHAND expression and catecholaminergic differentiation. A subthreshold expression level of dHAND in cultures without BMP4 is the most likely explanation for the lack of catecholaminergic neurons, although *dHAND* expression is detectable at low levels by RT-PCR.

To support these findings, BMP4 was expressed in vivo, using retroviral vectors. In BMP4-expressing peripheral nerve, a large number of cells expressing the transcriptional control genes *dHAND*, *eHAND* and *Phox2b*, noradrenergic marker genes *TH*, *DBH* and pan-neuronal genes *SCG10* and *NF160* was detected. These results demonstrate that *HAND* gene expression can be induced by BMPs in neural crest precursors in vitro and in vivo, in peripheral nerve. The results also support and extend previous findings that BMP overexpression causes increased numbers of *TH*-positive cells in vivo (Reissmann et al., 1996) by showing that regulatory genes (*HAND, Phox2*) and characteristic terminal differentiation genes (*DBH, SCG10, NF160*) are expressed in response to BMPs.

To investigate whether *HAND* gene expression is controlled

by BMPs during normal development in vivo, the function of BMPs was blocked, using the BMP antagonist noggin. We recently demonstrated that application of noggin-loaded agarose beads prevents the generation of sympathetic neurons. In noggin-treated embryos, the expression of *Phox2* genes, *TH*, *DBH*, *SCG10* and *NF160* is lacking (Schneider et al., 1999). The absence of dHAND- and Phox2b-positive cells now demonstrates that the expression of both genes is directly or indirectly controlled by BMPs. As the total number of ganglion cells, quantified by Sox10-staining, is significantly reduced at both stages investigated, the absence of *dHAND*-expressing cells may either be explained by the death of *Phox2b*-deficient neuron precursors (Schneider et al., 1999) or the inhibition of *dHAND* expression. It is difficult to decide between these possibilities, in particular as the analysis at earlier stages, when the number of *Sox10*-positive cells is not yet affected by noggin-treatment (Schneider et al., 1999), is not possible because of the late onset of *dHAND* expression. However, the induction of *dHAND* expression by BMP4 in overexpression experiments supports the notion of BMP-dependent *dHAND* expression.

The BMP-dependent expression of *Phox2* and *dHAND* genes in vitro and in vivo, together with the identical effects of Phox2a and dHAND-overexpression on sympathetic neuron development, implicates both transcription factors in the signal transduction pathway leading from the activation of BMP receptors to the expression of terminal differentiation genes. It remained unclear, however, whether *Phox2* and *HAND* genes act in parallel or sequentially. The onset of dHAND expression between the start of *Phox2b* expression and the expression of terminal differentiation genes, is compatible with the notion that dHAND is acting downstream of Phox2b in the control of sympathetic neuron differentiation. This conclusion is supported by two additional findings: (1) in the *Phox2b*$^{-/-}$ transgenic mice *dHAND* and *eHAND* expression is not detectable (C. Goridis, personal communication); (2) overexpression of Phox2b or Phox2a results in the expression of *dHAND* and *eHAND*. Although the latter result is compatible with a downstream function of dHAND, it is not conclusive, as dHAND is also able to induce *Phox2* gene expression. Thus, *dHAND*, although expressed later than *Phox2b* and dependent on *Phox2b*, is able to induce its upstream control gene in the overexpression experiment. A similar type of crossregulation has previously been observed between *Phox2b* and *Phox2a* (Stanke et al., 1999).

It should also be noted that migrating neural crest cells at trunk levels do not express detectable levels of *HAND* genes. The onset of *HAND* gene expression in the forming sympathetic ganglia implicates an action on differentiation of NC precursor cells rather than on migration of the cells. In the cardiac neural crest (Kirby et al., 1985), that migrates into branchial arches 3, 4 and 6, and contributes to the cardiovascular system, the expression of *dHAND* and *eHAND* is absent during the onset of migration and is first detectable at stage 15 in the chick embryo (Srivastava et al., 1995). The correlation between the onset of *HAND* expression and the condensation of neural crest cells at their terminal locations in the developing heart also suggests a role in differentiation or survival (Srivastava et al., 1997; Yamagishi et al., 1999; Srivastava, 1999), rather than on migration of neural crest cells. In the mouse, *dHAND* and *eHAND* genes are mainly expressed in complementary parts of the developing heart, whereas in the chick heart they display redundant expression and function (Srivastava et al., 1997; Srivastava 1999). The delayed expression of *eHAND* compared with *dHAND*, *TH* and *DBH* in chick sympathetic ganglia implicates a different, later role of eHAND in sympathetic neuron development. The effects of forced expression of eHAND on the generation of catecholaminergic cells in avian neural crest cultures (Howard et al., 1999) would then reflect a potential that is not realized during normal development.

The development of sympathetic neurons is controlled by two interacting pathways of BMP-dependent transcription factors, involving Phox2b, Mash1 and Cash1 (Guillemot et al., 1993; Hirsch et al., 1998; Lo et al., 1998; Morin et al., 1997; Pattyn et al., 1999). Although both are essential for sympathetic neuron development, the initial expression of *Phox2b* is independent of *Mash1* and vice versa. In *Mash1*-deficient mice, *eHAND* expression is absent at E10.5 (Ma et al., 1997), suggesting that *eHAND* belongs to the genes that are controlled by both *Mash1* and *Phox2b*. As *dHAND* expression in *Mash1*-deficient mice has not been studied, it remains unclear whether dHAND is expressed independently of *Mash1*, or dependent on both *Mash1* and *Phox2b*, like Phox2a. The absence of sympathetic neurons in *Mash1*$^{-/-}$ animals in spite of Phox2b expression implies the existence of a *Mash1*-dependent transcription factor that interacts with Phox2 signaling and is essential for sympathetic neuron development (Hirsch et al., 1998; Lo et al., 1998). If *dHAND* expression does depend on *Mash1*, dHAND would represent a promising candidate for this postulated factor.

What is the molecular mechanism of action of HAND proteins in sympathetic neuron precursor cells? Phox2a and Phox2b bind to and activate the promoter of *TH* and *DBH* genes (Zellmer et al., 1995; Yang et al., 1998; Kim et al., 1998; Lo et al., 1999). As *Phox2* and *dHAND* genes crossregulate each others expression and thus are present simultaneously in sympathetic neuron precursors, it seems likely that these factors act in a cooperative manner to activate noradrenergic marker genes, a hypothesis that can be tested experimentally. It should be noted that BMP-induced noradrenergic differentiation of sympathetic neurons may also involve a collaboration between Phox2 and cAMP-dependent transcription factors (Lo et al., 1999; Swanson et al., 1997).

Phox2-dependent generation of noradrenergic neurons is observed not only in neural crest precursors of sympathetic ganglia but also in the major noradrenergic cell population in the CNS, the locus coeruleus (Morin et al., 1997; Hirsch et al., 1998; Pattyn et al., 1999). However, as additional neurotransmitter phenotypes are generated in the PNS and CNS from precursor cells that express and depend on *Phox2* genes, noradrenergic development seems to represent only one out of several possible differentiation pathways for *Phox2*-expressing precursors. The choice between these differentiation pathways is most probably determined by additional regulatory factors. The present findings establish the dHAND transcription factor as an important member of the network of transcriptional regulators involved in sympathetic neuron development. The restricted expression in sympathetic and enteric neurons suggests that *dHAND* genes may be involved in the generation of a subset of neuronal phenotypes within the autonomic nervous system.

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