Development 119, 881-896 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

The mouse homeodomain protein Phox2 regulates *Ncam* promoter activity in concert with Cux/CDP and is a putative determinant of neurotransmitter phenotype

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SUMMARY

Transcriptional regulation of the gene encoding the cell adhesion receptor NCAM (neural cell adhesion molecule), a putative effector molecule of a variety of morphogenetic events, is likely to involve important regulators of morphogenesis. Here we identify two mouse homeodomain proteins that bind to an upstream regulatory element in the *Ncam* promoter: Cux, related to *Drosophila* cut and human CDP, and Phox2, a novel protein with a homeodomain related to that of the *Drosophila paired* gene. In transient transfection experiments, Cux was found to be a strong inhibitor of *Ncam* promoter activity, and this inhibition could be relieved by simultaneously overexpressing Phox2. These results suggest that the *Ncam* gene might be a direct target of homeodomain proteins and provide a striking example of regulatory cross-talk between homeodomain proteins of different classes. Whereas the expression pattern of *Cux*/*CDP* includes many NCAM-negative sites, *Phox2* expression was restricted to cells also expressing *Ncam* or their progenitors. The localisation data thus strongly reinforce the notion that *Phox2* plays a role in transcriptional activation of *Ncam* in *Phox2*-positive cell types.

In the peripheral nervous system, *Phox2* was strongly expressed in all ganglia of the autonomic nervous system and more weakly in some cranial sensory ganglia, but not in the sensory ganglia of the trunk. *Phox2* transcripts were detected in the primordia of sympathetic ganglia as soon as they form. *Phox2* expression in the brain was confined to spatially restricted domains in the hindbrain, which correspond to the noradrenergic and adrenergic nuclei once they are identifiable. All *Phox2*-expressing components of the peripheral nervous system are at least transiently adrenergic or noradrenergic. In the developing brain, *Phox2* was expressed at all known locations of (nor)adrenergic neurones and of their precursors. These results suggest that *Phox2*, in addition to regulating the NCAM gene, may be part of the regulatory cascade that controls the differentiation of neurons towards this neurotransmitter phenotype.

Key words: homeobox, NCAM, in situ hybridization, transcription, autonomic nervous system, noradrenergic neurons

INTRODUCTION

Homeobox-containing genes encode a class of transcription factors defined by an evolutionary conserved domain of 60 amino acids, termed the homeodomain (HD), which mediates sequence-specific DNA recognition (for review, see Gehring, 1990). An ever increasing number of homeobox genes have been isolated in mammals either through their homology to *Drosophila* genes (e.g. Kessel and Gruss, 1990; Price et al., 1991; Simeone et al., 1992) or serendipitously, through the identification of factors involved in transcriptional regulation of cell-specific genes (e.g. Adams et al., 1992; Karlsson et al., 1990; Ruvkun and Finney, 1991). Based on sequence homologies, their homeoboxes fall into several groups that are conserved across species (Scott et al., 1989; Kessel and Gruss, 1990). With the notable exception of some members of the *Pax* family, for which natural mouse mutants have been found (Gruss and Walther, 1992), and the few cases of knock-out experiments of *Hox* genes (Chisaka and Capecchi, 1991; Lufkin et al., 1991; Le Mouellic et al.,1992), their developmental role is inferred in most cases from their transient and restricted expression patterns during embryogenesis. Their expression domains are sometimes best defined by their

position along the main body axes. In other cases they correspond to regions that will give rise to a particular structure of the body, but expression may also be restricted to cell-types or differentiation stages rather than being spatially defined. This variety of expression patterns suggests the involvement of HD proteins in the whole spectrum of developmental processes, ranging from regional determination to cell differentiation.

The effector molecules of these developmental processes, which are thought to be regulated by HD transcription factors, are still mostly unknown. Good candidates are cell adhesion receptors, which appear early in development and are characterized by broad and complex expression patterns. Among them, the NCAMs (neural cell adhesion molecules), a group of closely related cell-cell adhesion receptors of the immunoglobulin superfamily, have been particularly well studied (for reviews, see Edelman and Crossin, 1991; Walsh and Doherty, 1991; Goridis and Brunet, 1992). Spatiotemporal changes in NCAM abundance, for the most part due to transcriptional regulation, correlate with, and may be essential to, a number of morphogenetic events. Also, *Ncam* expression seems to correlate, depending on the developmental stage, with position on the rostrocaudal axis, cell movements or cell differentiation (Goridis and Brunet, 1992). How these different modes of *Ncam* expression are controlled is not understood.

To reveal possible regulatory links between HD factors and *Ncam* transcription, we have begun to analyze the upstream region of the mouse *Ncam* gene (Hirsch et al., 1990). One of the regulatory elements, responsible for up-regulating promoter activity in the neuroblastoma line N2a and composed of three clustered binding sites for nuclear factors, named *a*, *b* and *c*, was found to contain ATTA motifs, known to be the core of most recognition sequences for HD proteins (Scott et al., 1989; Laughon, 1991). Site *b* was shown to actually bind *Drosophila* HDs in vitro, and its influence on the activity of the promoter to depend on the integrity of the ATTA motifs (Hirsch et al., 1991). Moreover, two *Xenopus* HD factors were shown to modulate the mouse *Ncam* promoter activity via site *c* (Jones et al.,1992).

In the present study, we identify two murine HD-containing factors expressed in the NCAM-positive neuroblastoma cell line N2a (a neural crest-derived tumor line), which bind to and transregulate the *Ncam* promoter. One, named Cux (cut-like homeobox) behaved as a repressor, whereas the other one, named Phox2 (paired-like homeobox 2), relieved this repression. Consistent with our functional data, we found that virtually all *Phox2*-expressing territories also expressed *Ncam*. *Phox2* expression was limited to the permanently or transiently (nor)adrenergic neural crest derivatives and to the (nor)adrenergic nuclei of the brainstem. Hence, Phox2, apart from modulating *Ncam* expression in certain cell types, may play a role in the determination of this neurotransmitter phenotype.

MATERIALS AND METHODS

Mice and cell lines

Embryos were obtained from timed matings of outbred NMRI mice (CERJ). The middle of the day following the detection of a vaginal plug was designated as embryonic day 0.5 (E0.5).

The C1300 mouse neuroblastoma-derived N2a cell line and the NG108 neuroblastoma × glioma hybrid were cultured in Dulbecco's modified Eagle's (DME) medium in the presence of 10% fetal bovine serum. PC12 rat pheochromocytoma cells were grown in the same medium on collagen-coated dishes and induced to differentiate by shifting to DME supplemented with 5% horse serum and 50 ng/ml of nerve growth factor (NGF) for 7 days.

Construction and screening of an expression library

A cDNA library in the expression vector lambda gt11 Sfi-Not (Promega) was constructed from N2a mRNA according to the manufacturer's instructions and screened with the following double-stranded oligonucleotides:

abx3: (aattCTTTGAAAATCGAACCGAATCTAAAATTCT-TTTTCCCCCTAATTATTAAAAACGTTCAAATTcgagct)x3;
a0b0x3: (aattCTTGAAACATCGTACCGATGCTAAGATGAG-TTTTCCCCCTAACTATCAAAAACGTTCAAATTcgagct)x3.

Successive screenings with the abx3 and a0b0x3 probes were done according to Vinson et al. (1988). Filters were cycled through an initial denaturation/renaturation round, then incubated with an abx3 probe, washed and exposed to film. The filters were then subjected to a new denaturation/renaturation round and exposed again. The whole procedure was repeated with the a0b0x3 probe, and then again with the abx3 probe. The clones that produced a signal after the first screening, which disappeared after the wash, failed to reappear with the a0b0x3 probe and proved reproducible in the last screening were kept for further study.

A full-length or near full-length *Phox2* cDNA was obtained by rescreening the same library with the original clone pab9. To clone the missing 5′ sequences of *Cux*, we first screened the same library with the originally isolated clone pab3, yielding clone T7KSEN and then an adult mouse brain library (Clontech) with a 5′ fragment of T7KSEN yielding clone T7EE07. The two nucleotide sequences, obtained by sequencing clones pab903 and pab907 for *Phox2* and the overlapping clones pab3, T7KSEN and T7EEO7 for *Cux*, have been deposited in the EMBL Data Library under the accession numbers X75014, and X75013, respectively.

Northern blot analysis

Total RNA was extracted from various mouse tissues and cell lines by the LiCl/urea method (Auffray and Rougeon, 1980), size fractionated on a denaturing 6% formaldehyde-1% agarose gel and transferred to Hybond C (Amersham). Hybridization was carried out overnight at 42°C with a [^{32}P]dATP-labeled, random-primed DNA probe in 50% formaldehyde, 5× SSC, 5× Denhardt's, 5 mM EDTA, 0.1% SDS, 7% dextran sulphate containing 200 μg/ml salmon sperm DNA. The probes were either the pab3 insert or the 3′ untranslated region of pab9 (nt 874-1475) subcloned into pGEM11-Z(f-) (Promega) (clone pab9SSE). Filters were washed once in 2× SSC, 0.1% SDS at room temperature, once in the same buffer at 65°C and twice in 0.2× SSC, 0.1% SDS at 65°C. They were exposed to X-ray film with intensifying screens at −80°C overnight.

In situ hybridization

Phox2, *Ncam* and *tyrosine hydroxylase* (*TH*) transcripts were detected by cRNA probes transcribed by SP6 or T7 RNA polymerase from templates cloned into pGEM (Promega) vectors: the *Phox2* cDNA clone pab9SSE (see above), the *Ncam* cDNA clone DW3-le used previously (Bally-Cuif et al., 1993) and the *TH* cDNA probe pTH-51 (Grima et al., 1985). *Phox2* expression was analyzed with the anti-sense probe on serial sections of mouse embryos between E8.5 and E17.5. Alternate sections were hybridized with an *Ncam* probe to compare the expression domains of the two mRNAs. The grain density on sections

hybridized with a *Phox2* sense probe never exceeded background levels. The RNA probes were labeled with ^{35}S-UTP (Amersham, 1000 Ci/mmol) and hydrolyzed to generate 150 nt fragments. Embryos were prefixed in 0.1 M sodium phosphate buffer (pH 7.3), 4% paraformaldehyde at 4°C overnight, cryoprotected in 15% sucrose, 0.1 M sodium phosphate buffer at 4°C overnight, and embedded in Tissue-Tek (Miles Laboratories). Cryosections, 8 μm thick, were transferred onto gelatin/chromium(III)potassium sulfate-subbed slides and dried at room temperature. Prior to hybridization, slides were post-fixed. The hybridization procedure was according to Wilkinson and Green (1990). Exposure time was 1-2 weeks.

Constructs

In order to produce various domains of Phox2 and Cux in *E. coli*, fragments covering the HD of Phox (Gly84-Lys156), the 3′ repeat and HD of Cux (Pro994-Ileu1169), the Cux 3′ repeat alone (Pro994-Leu1068) and the Cux HD alone (Glu1113-Glu1183) were produced by polymerase chain reaction (PCR) with oligonucleotide primers containing the appropriate restriction sites and subcloned, between the *Nde*I and *Bam*HI sites of pET3a (for Phox2HD), or in the *Bam*HI site of pET3b (for Cux3′RHD, Cux3′R and CuxHD). For transient cotransfection experiments, pRC/Phox and pRC/Cux were constructed by subcloning the *Cux* cDNA (starting from position 442 and extending for 738 nucleotides past the stop codon) and the *Phox* cDNA (the full-length insert of clone pab 903) into the pRC/CMV (Invitrogen) expression vector. The reporter plasmid NSα-CAT is identical to the previously described PPSHS plasmid (Hirsch et al., 1990) except for a deletion from −495 to −462 (required for constructing the mutant version, see below). It contains the *Ncam* promoter from position −2600 to −37 relative to the translation start site fused with the CAT gene in the pconaCAT vector. The reporter plasmid NSαmut-CAT contains mutations in the *a* and *b* sites (Hirsch et al., 1990) introduced by PCR with the b0 and amut oligonucleotides (see below). The SRα-βgal plasmid contains the *lacZ* gene driven by the SRα promoter (Takebe et al., 1988).

Electrophoretic mobility shift and footprinting assays

The various domains of *Phox2* and *Cux* were expressed in BL21(pLysS) (Studier et al., 1990). Bacterial extracts were prepared according to Hoey et al. (1988) for Phox2 HD and according to Zappavigna et al. (1991) for Cux 3′RHD, except that the extract was kept in 1 M urea. Electrophoretic mobility assays (EMSA) were performed as described in Hirsch et al. (1990) except that electrophoresis was carried out in 0.5× Tris/borate/EDTA at room temperature on 5 or 8% polyacrylamide gels. Oligonucleotides used for competition are:

a: CTTTGAAAATCGAACCGAATCTAAAATTCT
b: CCCCTAATTATTAAAAACGTTCA
amut: CTTGAAACATCGTACCGATGCTAAGATGAG
b0: CCCCTAACTATCAAAAACGTTCA.

Footprinting assays were carried out with recombinant protein according to Hirsch et al. (1990).

CAT-assays

Transfections were done as described (Hirsch et al., 1990) by the calcium phosphate method, using 5 μg reporter plasmid along with 3 μg SRα-βgal (to correct for transfection efficiencies), expression plasmids at the concentrations indicated and pRC/CMV (to control for non-specific vector effects) up to a total of 20 μg DNA. 48 hours after transfection, cells were collected and promoter activity determined either by measuring chloramphenicol acetyltransferase (CAT) protein using the CAT-ELISA kit (Boehringer Mannheim) following the instructions of the manufacturer or by measuring CAT activity as described (Hirsch et al., 1990).

Measurement of NCAM cell surface expression in transfected cells

Cultures were cotransfected with 5 mg pRC/Cux and 3 μg SRα-βgal. 72 hours after transfection, cells were attached to poly-L-lysine-coated glass cover-slips and double-stained with anti-NCAM monoclonal antibody H28, that reacts with all NCAM isoforms (Hirn et al., 1983) and rabbit anti-βgalactosidase (βgal) antibodies (Tebu), which were revealed by rhodamine-coupled mouse anti-rat and fluorescein-coupled goat anti-rabbit secondary antibodies (Jackson), respectively. The results were analyzed with a Zeiss fluorescence photomicroscope using rhodamine and fluorescein optics. Control experiments showed the absence of cross-reaction with the inappropriate first antibody. To obtain a semi-quantitative assessment, all cells in randomly chosen fields were rated for NCAM expression, then assessed for βgal immunoreactivity, and the NCAM-high, -low and -negative cells were counted among transfected and non-transfected cells. All experiments were analyzed independently by two different observers.

RESULTS

Cloning and sequence analysis of *Phox2* and *Cux*

To identify the nuclear factors that bind to the HD-binding element in the *Ncam* promoter in N2a cells, we screened an expression library constructed from this cell line with a concatenated double-stranded probe, covering the *a* and *b* footprints (Hirsch et al., 1990). Nine clones, which gave a strong signal with the abx3 probe and reacted weakly or not at all with its mutated version, a0b0x3, were selected and found to fall into two classes.

The first type of clones encodes a novel protein with an HD of the *paired* (*prd*) type (Frigerio et al., 1986; Fig. 1A). Rescreening the library with one of the clones yielded pab 907, 1610 bp in length and containing an open reading frame (ORF) of 840 nt. The translation start site assignment is tentative, owing to the presence of two in-frame methionines at position +1 and +15, each surrounded by a perfect Kozak sequence (Kozak, 1987), and to the absence of an in-frame stop codon preceding Met+1. The amino-terminal region is rich in Pro's (17%) as described in the activation domain of other transcription factors (Williams and Tjian, 1991; Pei and Shih, 1991) and Gly's (20%). The HD contains a Leu-Lys-Ileu motif (positions 37-39) also found in the *Drosophila* HD protein orthodenticle and its mammalian homologues (Scott et al., 1989; Laughon, 1991; Simeone et al., 1992) (Fig. 1B). We call this new HD factor Phox2 since it has two features in common with the recently cloned Phox1 protein (Grueneberg et al., 1992; Cserjesi et al., 1992; Kern et al., 1992), which set them apart from most other factors with *prd*-like HDs: the absence of a *prd* domain and a Glu instead of a Ser at position 9 of the recognition helix (Scott et al., 1989; Laughon, 1991; Fig. 1B). These characteristics define a new sub-family of *prd*-like homeobox genes, which also includes *Mix1* (Rosa, 1989) and *S8* (Opstelten et al., 1991).

The second type of clones also contains a homeobox. The ORF of overlapping clones is 3993 bp long. Between position 3491 and 3673, we identified an HD which showed the highest degree of homology with that of the *Drosophila* protein cut (Blochlinger et al., 1988), particularly in the recognition helix around an His at position 51, not found in

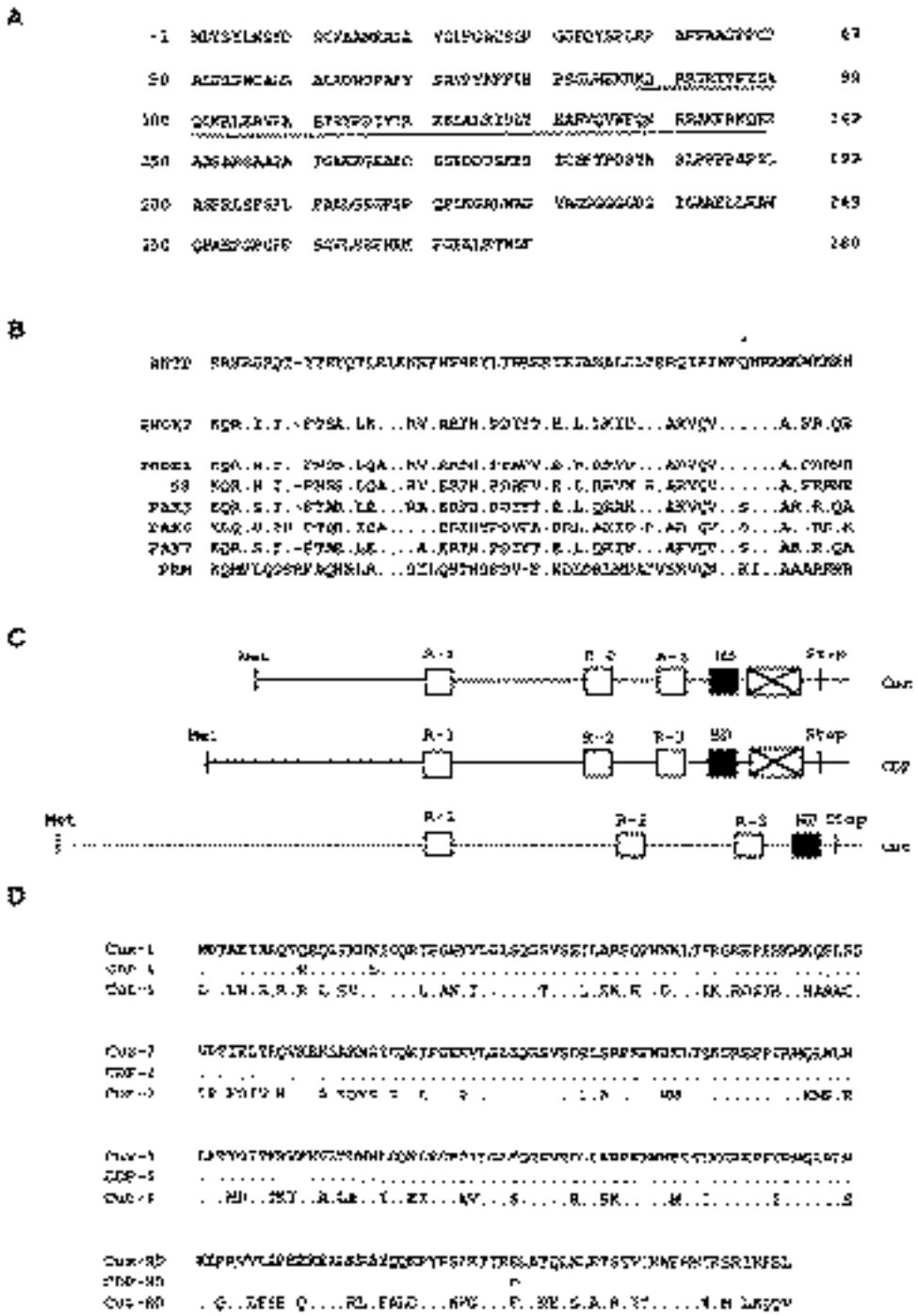

Fig. 1. Sequence analysis of *Phox2* and *Cux*. (A) Amino acid sequence (single letter code) of Phox2 deduced from the 1610 bp-long cDNA insert of clone pab907. The HD is underlined. (B) Alignment of the amino acid sequences of mammalian prd-like HDs (Scott et al., 1989; Laughon, 1991; Opstelten et al., 1991; Simeone et al., 1992). The sequence of the prototypical Antennapedia HD is given above. Phox2 shares with Phox1 (Grueneberg et al., 1992), S8 (Opstelten et al., 1991) and Mix1 (Rosa, 1989) a glutamine at position 50 (indicated by a *) which is crucial for DNA binding (Scott et al., 1989; Laughon, 1991) instead of the serine typical of *prd*-like HDs. There is no detectable sequence conservation between Phox2 and Phox1 outside the HD implying that they are not species homologues. (C) Schematic representation of the ORFs of *Drosophila* cut (Blochlinger et al., 1988), CDP (Neufeld et al., 1992) and Cux, showing the positions of the HDs and the three internal repeats (R1, R2 and R3). The crossed box represents a region of sequence divergence between CDP and Cux. (D) Alignment of the amino acid sequences of the HDs and the internal repeats of *Drosophila* Cut, CDP and Cux.

HDs of any other class to date. The homology, which breaks down outside of the HD, resumes at the level of three internal repeats (Fig. 1C,D), even though they are differently spaced. In line with the recently proposed taxonomic guide lines (Scott, 1992), we called this gene *Cux*. Since then, human (Neufeld et al., 1992) and canine (Andres et al., 1992) homologues have been identified as *CDP* and *Clox*, respectively. The *CDP* and *Cux* sequences diverge at their 5′ ends, so that the first Met in the *Cux* ORF corresponds to Met + 184 in *CDP*. This observation together with the existence of an internal region of sequence divergence (crossed box in Fig. 1C) may be indicative of alternative splice variants (Neufeld et al., 1992). Starting with Met +1 in *Cux* and excluding the internal region of divergence, the CDP and Cux amino acid sequences are 91% identical. The overall identity between the (partial) amino acid sequence of Clox is 95% with that of Cux, excluding two regions of complete divergence: one from position 706 to 763 and one from position 1215 to 1353. The latter region differs among the three species homologues suggesting an extensive range of alternative splice variants.

Binding properties of Cux and Phox2

To assess the binding properties of Cux and Phox2, their

presumptive DNA-binding domains, the HD of Phox2 and the 3′ most repeat plus the HD of Cux (3′RHD), were tested by EMSA. When incubated with labeled α fragment centered around the *a*, *b* and *c* footprints (Hirsch et al., 1990), Phox2HD produced a retarded complex which was competed out by the *b* sequence, but not by *a* nor by *b0*, in which the ATTATTA motif has been mutated (Fig. 2A). Hence, Phox2 specifically binds through its HD to the *b* sequence, at the ATTATTA motif. The same type of competition experiments done with Cux 3′RHD revealed specific binding to the *a* sequence (Fig. 2B). Neither the Cux HD nor the 3′ repeat alone bound detectably to the promoter fragment (not shown). Hence, at least the 3rd repeat appears to participate in DNA binding. In footprinting experiments, Cux3′RHD was found to protect a 16-nt stretch in the *a* sequence (Fig. 2C). No obvious consensus emerges from a comparison between this sequence and other footprints, either obtained with nuclear extracts and ascribed to Cux/CDP (Barberis et al., 1987; Superti-Furga et al., 1988), or done with recombinant protein (Neufeld et al., 1992). We note, however, the repeated occurrence of a GATTNTNA motif in these footprints.

Transregulating activity of *Phox2* and *Cux* on the *Ncam* promoter

In order to assay the two candidate factors for transregulating activity, we transiently expressed them in N2a cells, together with the *CAT* gene placed under the control of a 2.6 kb fragment of the *Ncam* promoter. Transfecting increasing amounts of *Cux* led to a progressive decrease in promoter activity (Fig. 3A). This inhibition could be almost completely reversed by cotransfecting an equivalent amount of an *Phox2* expression vector (Fig. 3B). Cotransfection with *Phox2* alone did not detectably affect transcription (not shown). This is not surprising since *Phox2* mRNA is already present in vast excess over *Cux* mRNA in N2a cells. *Phox2* and *Cux* had no effect on *Ncam* promoter activity in three *Phox2*-negative cell lines (NIH 3T3, F9 and HeLa), pre-

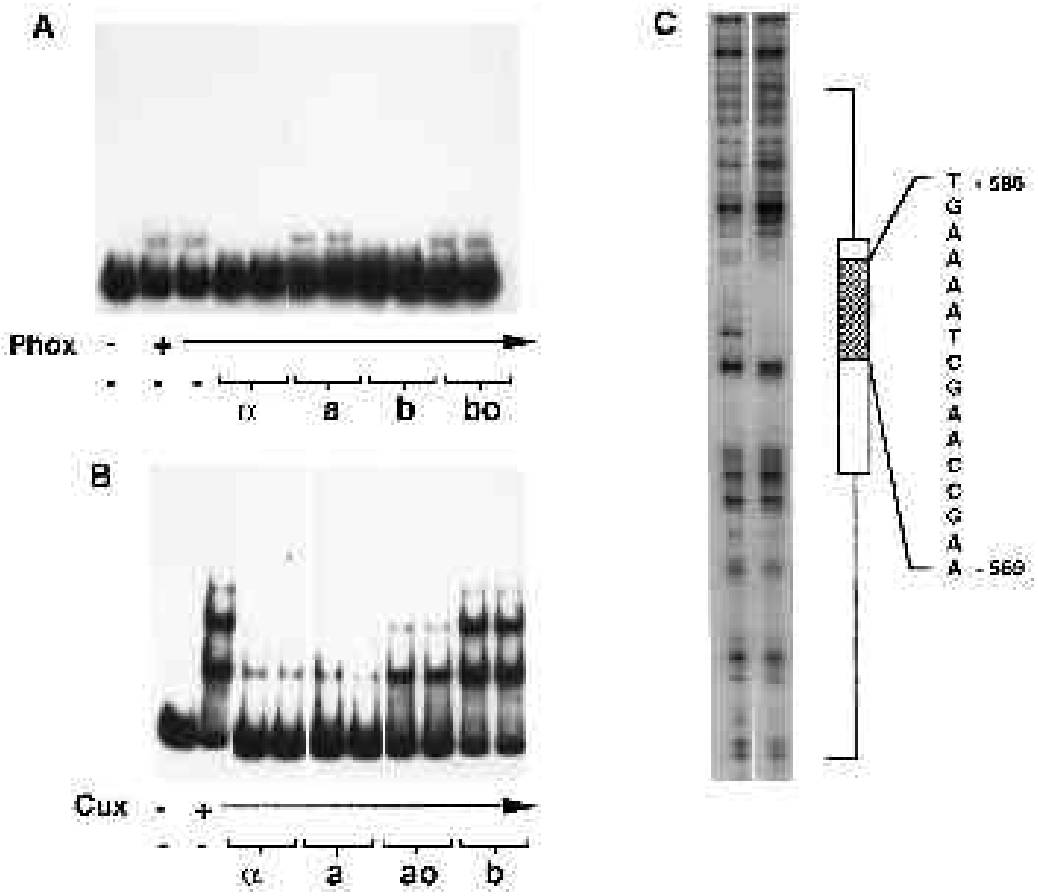

Fig. 2. Binding of Phox2 and Cux to the α fragment (−645/−497) of the *Ncam* promoter centered around the *a*, *b* and *c* footprints. (A) Specific binding of Phox2 to the *b* sequence. The probe was incubated without bacterial extract or with a bacterial extract containing approximately 40 ng of Phox2 HD in the absence or presence of a 100-fold molar excess of four different competitor DNAs as indicated: *a* itself, double stranded oligonucleotides *a* and *b*, and the mutated version of *b*, *b0* (see Material and methods). (B) Specific binding of Cux to the *a* footprint. The probe was incubated without bacterial extract or with a bacterial extract containing approximately 60 ng of a fragment of Cux covering the third repeat and the HD (Cux 3′RHD) (see Material and methods) in the absence or presence of a 100-fold molar excess of four competitor DNAs as indicated: *a* itself, the *a* and *b* oligonucleotides and the mutated *a0* version of *a*. (C) DNase I protection of the *a* fragment by recombinant Cux 3′RHD. The sense strand of the α fragment was end-labeled and incubated in the absence (left lane) or presence (right lane) of bacterial extracts containing Cux3′RHD. The *a* footprint obtained with N2a nuclear extracts (Hirsch et al., 1990) is indicated by an open box, and the extent of the sequence that is protected by Cux3′RHD is shaded.

sumably because they lack essential cofactors. Surprisingly, *Cux* was still able to inhibit transcription from a promoter, in which the *a* and *b* sites had been mutated, suggesting a functional redundancy between the *ab* footprint and other target sequences for Cux in the promoter fragment used. This possibility is supported by our observation that, when tested in EMSA, a longer promoter fragment (extending from position −245 to −941) carrying the *ab* site mutation still bound Cux3′RHD, albeit with lower affinity (not shown). However, in the absence of a functional *b* site, repression by *Cux* was not detectably relieved by *Phox2*, indicating that, at the concentrations used, the activity of *Phox2* is dependent on the integrity of its binding site (Fig. 3B).

Cux also repressed transcription of the resident *Ncam* gene in N2a cells. To show this, cultures cotransfected with *Cux* expression vector and a βgal-expression vector were double-stained with both anti-NCAM and anti-βgal antibodies. As expected, most N2a cells displayed strong NCAM cell surface fluorescence. However, in a substantial proportion of the cells that had been transfected (as shown by anti-βgal staining), NCAM immunoreactivity was reduced to low levels or undetectable (not shown). To obtain a semi-quantitative assessment, NCAM immunoreactivity was rated as high ($NCAM^{high}$), low (NAM^{low}) or undectable ($NCAM^{-}$) among transfected and non-transfected cells from 4 independent transfection experiments. Out of 518 anti-βgal-stained cells, 17.7% were scored as $NCAM^{-}$ and 40.9% as $NCAM^{low}$, whereas out of 2408 βgal-negative cells only 2.1% were scored as $NCAM^{-}$ and 5.2% as $NCAM^{low}$.

Northern blot analysis of *Phox2* and *Cux*

On RNA from N2a cells, a *Phox2* probe detected a major transcript of 1.7 kb (Fig. 4). In a small survey of cell lines and adult tissues, *Phox2* transcripts could be revealed only in the adrenal medulla and in two other cell lines of sympathoadrenal origin, PC12 and NG108 cells (Fig. 4), but not

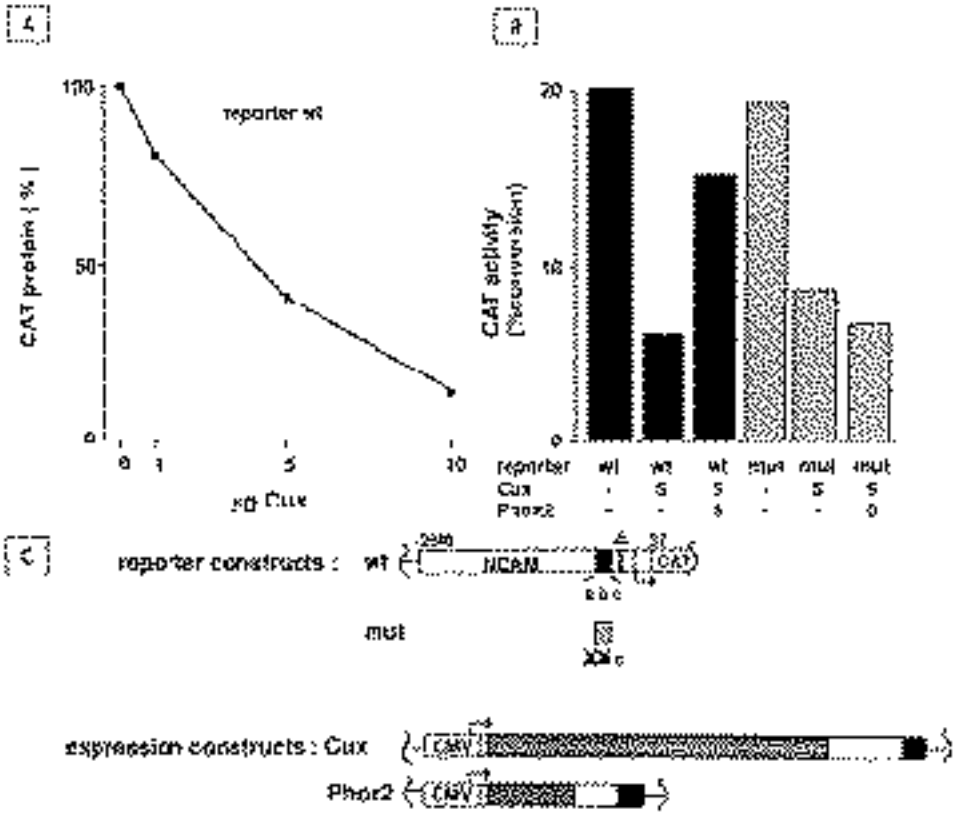

Fig. 3. Repression of *Ncam* promoter activity by *Cux* and relief of repression by overexpression of *Phox2*. N2a cells were transiently transfected with *Cux* and *Phox2* expression vectors together with 5 μg of reporter constructs containing a 2.6 kb fragment from the *Ncam* promoter fused to the CAT reporter gene (NSα-CAT) or with a mutated version of it (NSαmut-CAT). (A) Cells were transfected with NSα-CAT and different concentrations of the *Cux* expression vector. Results are expressed relative to the levels measured in the absence of *Cux* expression. (B) Cells were transfected with either the wild-type NSα-CAT reporter construct (wt) or its mutated version (mut) NSαmut-CAT together with 5 μg *Cux* expression vector plus the same amount of *Phox2* expression vector, or not, as indicated below. The values are expressed as percentage conversion of chloramphenicol into acetylated derivatives. (C) Schematic representation of the reporter and expression constructs. The reporter construct contains *Ncam* upstream sequences from −2600 to −37 linked to the bacterial *CAT* gene (Hirsch et al, 1990) with either the wild-type (wt) or mutated (mut) *a* and *b* sites. The position of the *a*, *b*, and *c* sites is indicated. Δ indicates the −495/−462 deletion in the promoter. The expression constructs contain either the *Cux* (position 442 to 5046) or the full-length *Phox2* cDNA inserted into the pRC/CMV vector. The ORFs and the bovine growth hormone-derived vector sequences are shaded lightly and darkly, respectively. In A and B, the values presented are the average of duplicate determinations from two representative transfection experiments. Variations between experiments did not exceed 15%.

in a variety of cells of different origin and in adult brain, liver, lung, kidney, thymus and the superior cervical ganglion (not shown). Additional, much weaker bands of 4.8 and 7.0 kb were also revealed. Their relationship to the major band has not been investigated. PC12 cells respond to NGF by differentiation towards a sympathetic neuron-like phenotype (Greene and Tischler, 1976). *Phox2* mRNA expression was up-regulated in differentiated cells, by around 10 fold as estimated by scanning of the autoradiograph (Fig. 4).

Cux has a much wider tissue distribution than *Phox2* in adult tissues. On RNA from N2a cells and from adrenal medulla, a *Cux* probe detected transcripts of approximately 6 and 8 kb. An 8 kb transcript was also detected in brain, lung, heart, skeletal muscle and thymus (not shown); by contrast, liver (Fig. 5), kidney or the teratocarcinoma cell line F9 were negative (not shown). During embryogenesis, *Cux* transcripts were detected in whole embryo RNA from E5.5 onwards (Fig. 5); yet, we could not detect any signal above background by in situ hybridization done on sections of E12 and E13 embryos. This could be due to a combination of low abundance and wide distribution of the message.

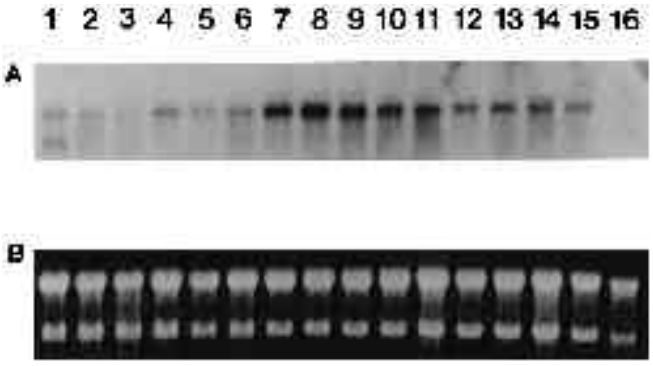

Fig. 5. Developmental profile of *Cux* expression during embryogenesis.(A) RNA (10 μg per lane) from total mouse embryos was probed with a *Cux*-specific probe. Lane 1, N2a cells as positive control; lanes 2-14, E5.5 to 17.5 mouse embryos (2: E5.5; 3: E6.5; 4: E.7.5; 5: E8.5; 6: E9.5; 7: E10.5; 8: E11.5; 9: E12.5; 10: E13.5; 11: E14.5; 12: E15.5; 13: E16.5; 14: E17.5); lane 15, neonatal mice; lane 16, liver as negative control. (B) Photograph of an ethidium bromide stain of the RNA gel prior to transfer showing that equivalent amounts of RNA were loaded per lane. Between E5.5 and E7.5, the embryos were analyzed with their extraembryonic membranes ; from E8.5 onwards, the embryos were freed of their extraembryonic membranes.

Phox2 expression in the embryo studied by in situ hybridization

Expression in the peripheral nervous system

The strongest expression of *Phox2* transcripts was observed in sympathetic ganglia (SG). At E13.5, we detected a strong signal in the superior cervical and stellate ganglia, in the thoracic sympathetic chain and in the preaortic sympathetic ganglia. A slightly weaker signal was observed in the adrenal medulla which contains chromaffin cells, endocrine equivalents of sympathetic neurons (Fig. 6). At this stage, *Phox2* transcripts were also detected in all parasympathetic ganglia, of which the sphenopalatine and submandibular ganglia are shown in Fig. 7B. *Phox2* transcripts are also present in the enteric nervous system as shown by the labeling of a ring of cells in the gastric and intestinal walls (Fig. 7D and data not shown). Hence, *Phox2* is expressed in every peripheral component of the autonomic nervous system.

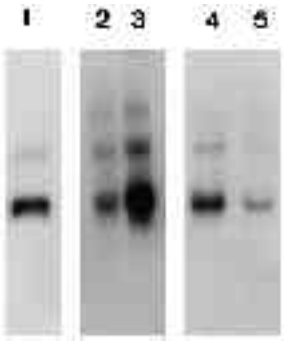

Fig. 4. Northern blot analysis of *Phox2* expression. Total RNA (1 μg in lane 1 and 10 μg in lanes 2-5) from the neuroblastoma N2a (lane 1), the pheochromocytoma cell line PC12, untreated (lane 2) or treated (lane 3) with NGF, the neuroblastoma × glioma hybrid cell line NG108 (lane 4) and adult adrenal medulla (lane 5) were hybridized to a *Phox2*-specific probe. The major transcript is 1.7 kb, minor transcripts at 4.8 and 7.0 kb can be detected in some cells.

The onset of expression of *Phox2* in the SG coincides with their formation (Rubin, 1985) and the appearance of TH and dopamine βhydroxylase immunoreactivities (Cochard et al., 1979), as demonstrated by the detection of *Phox2* transcripts in paraaortic clusters of cells as early as E10 (Fig. 8B). However, we never detected *Phox2* transcripts in the region of migrating neural crest cells lateral to the neural tube. Therefore, the onset of *Phox2* transcription in the SG coincides with the aggregation of the first sympathoblasts. The signal intensity in the SG remained at comparable levels until E17.5 (not shown). As we could not detect *Phox2* transcripts by northern blot hybridization in adult superior cervical ganglia, the data suggest downregulation of *Phox2* shortly before or after birth. On adjacent sections, we could not yet detect *Ncam* transcripts in the paraaortic *Phox2*-positive clusters at E10, but cells in this region had become *Ncam*-positive half a day later (not shown).

Although no signal was ever detected in dorsal root ganglia, a hybridization signal was observed in some of the cranial sensory ganglia and their primordia. At E9, *Phox2* mRNA was present in a group of cells adjacent to the otic pit, that could correspond to the anlage of the VIIIth ganglion (not shown). At E10.5, there is a conspicuous region of *Phox2* positivity in the fourth branchial arch (Fig. 9B) which most likely corresponds to the area of migration of cells from the nodose placode to the primordium of the nodose ganglion (the distal ganglion of the Xth cranial nerve; Le Douarin et al., 1986; Kuratani and Wall, 1992). At E13, a weak signal was detected in the nodose (Fig. 7B) and petrose (IXth distal, not shown) ganglia, but not in the proximal ganglia of the same cranial nerves, which are entirely of neural crest origin (Le Douarin et al., 1986;

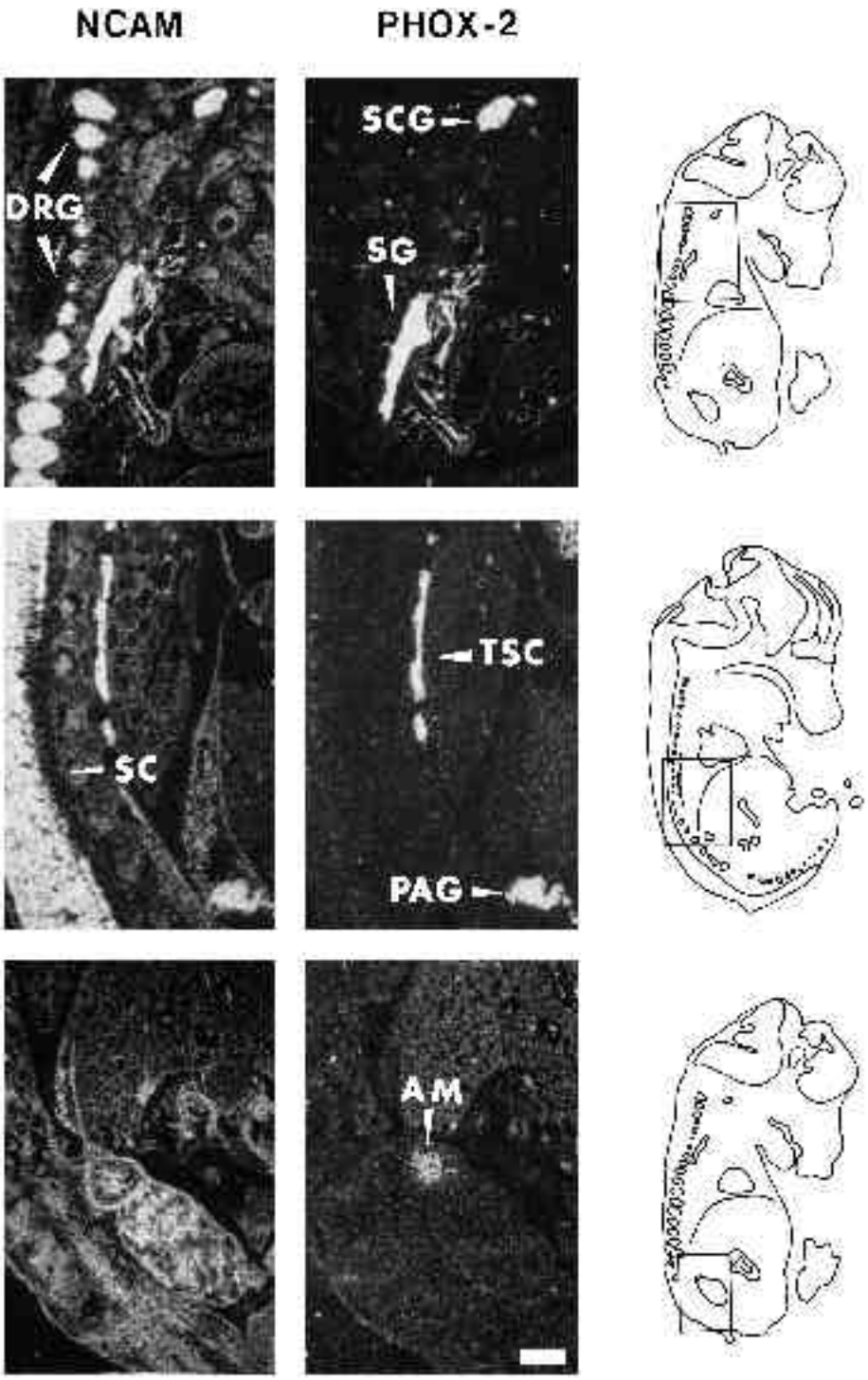

Fig. 6. *Phox2* and *Ncam* expression on sympathoadrenal derivatives in E13.5 embryos as detected by in situ hybridization. The areas of the embryo depicted are schematically indicated on the right panel. Adjacent parasagittal sections were hybridized with *Ncam*- (left panel) and *Phox2*- (middle panel) specific probes. AM, adrenal medulla; PAG, paraaortic ganglion; SC, spinal cord; SCG, superior cervical ganglion; SG, stellate ganglion; TSC, thoracic sympathetic chain. Scale bar, 0.1 mm.

Noden, 1991). Therefore, *Phox2* expression in the cranial sensory compartment of the PNS is unexpectedly restricted to ganglia whose neurones are of placodal origin (Le Douarin et al., 1986; Noden, 1991). *Phox2* expression in these ganglia appears to be transient: labeling of the VIIIth ganglion had disappeared by E10.5 (not shown), and the strong signal seen in the area of the forming nodose ganglion had become very weak by E13. All *Phox2*-positive cranial ganglia, as well as the area in the fourth branchial arch, also hybridized to the *Ncam* probe.

Expression in the CNS

The second class of *Phox2*-expressing territories is located in the CNS. We could first detect a faint *Phox2* signal in the CNS at E9 on a few cells in the ventrolateral hindbrain that correspond to the very first postmitotic neurons which also express *Ncam* (Bally-Cuif et al., 1993), but not at the level of the future spinal cord (not shown). At E10.5, a region extending from rhombomere 4 to 7 is now strongly *Phox2*-positive (Figs 9B, 10). The signal can be detected only down to the caudal border of the myelencephalon, whereas *Ncam*-

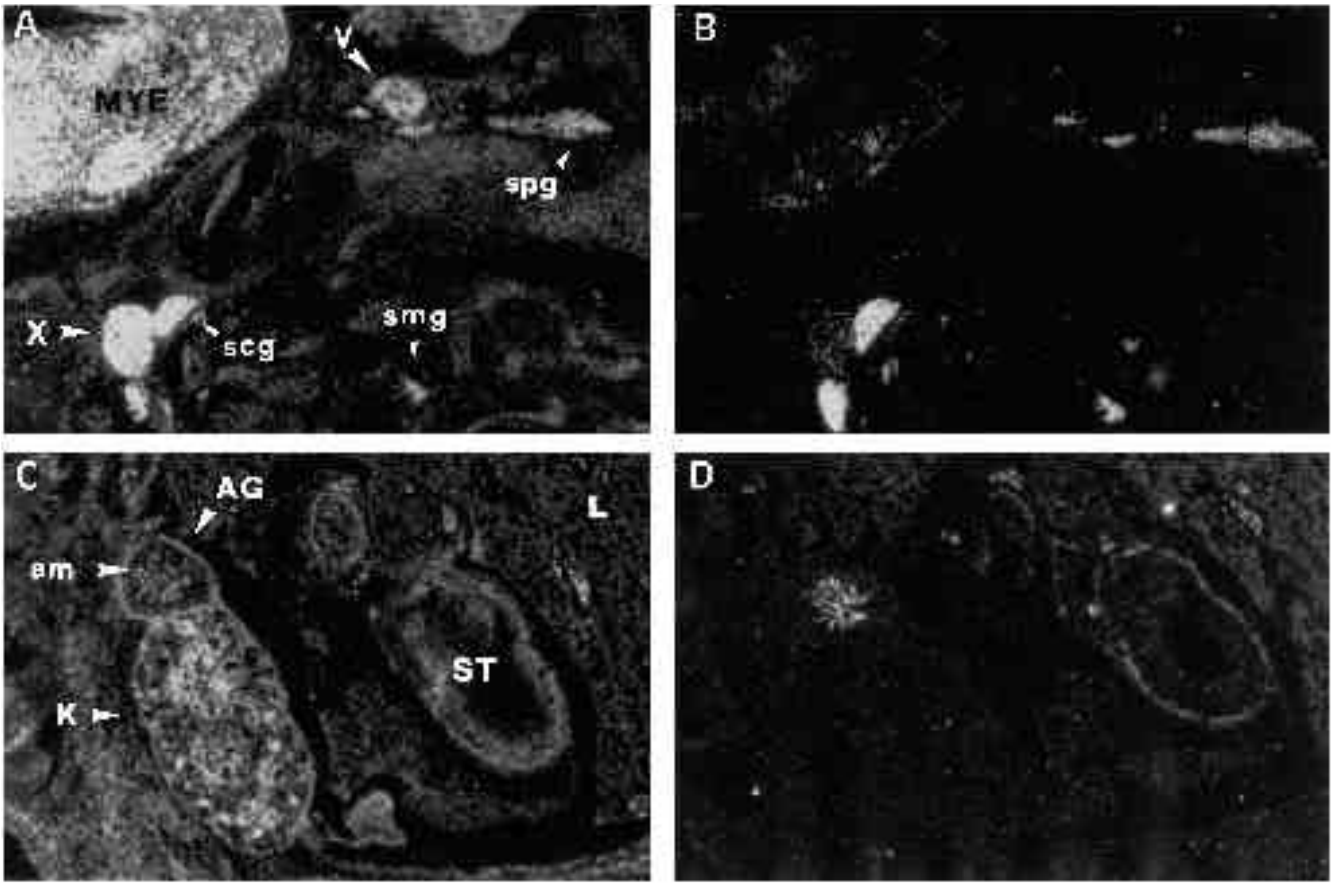

Fig. 7. *Phox2* and *Ncam* expression in ganglia of the autonomic nervous system and in cranial sensory ganglia from E13 mouse embryos. Pairs of consecutive parasagittal sections (A and B, C and D) were hybridized with *Ncam* (A,C) or *Phox2* (B,D) probes. Sympathetic (scg), parasympathetic (smg, spg) and enteric (in the wall of the stomach) ganglia as well as the anlage of the adrenal medulla are labeled with both probes. The nodose ganglion (X) gives a faint signal with the *Phox2* probe at this stage, whereas the trigeminal ganglion (V) is negative. AG, adrenal gland; am, adrenal medulla; K, kidney; scg, superior cervical ganglion; smg, submandibular ganglion; spg, sphenopalatine ganglion; ST, stomach; V, trigeminal ganglion; X, nodose ganglion. Bar, 0.5 mm.

positive differentiating neurons are present all along the future spinal cord. Transverse sections reveal two main territories of *Phox2* expression: a ventral column of cells on each side of the floor plate plus a dorsolateral column in the alar plate (Fig. 11B,D). Except for the fourth rhombomere, where the signal extends throughout the germinal layer of the neural tube (Fig. 10B), the signal is confined to the marginal zone, where the postmitotic neurons are known to accumulate. At this stage, NCAM is a general marker for all postmitotic neurons (Bally-Cuif et al., 1993), only a subpopulation of which also express *Phox2* (Fig. 11). As the ventricular zone is *Ncam*-negative at this stage, *Phox2* expression in the proliferative neuroepithelium of rhombomere four represents one instance of *Phox2*-positive cells which do not express *Ncam*. *Phox2* expression in the hindbrain fades out in the rostral metencephalon, but resumes at the met-mesencephalic border on each side of a narrow negative gap (Figs. 9B and 10A). This gap coincides with an interruption in the otherwise continuous expression of *Ncam* in the marginal zone of the brain (Fig. 9A; see also Bailly-Cuif et al., 1993). Transverse sections show the pattern of *Phox2* at the met-mesencephalic border as a faint ring interrupted on the dorsal midline and bounded ventrally by two patches of hybridizing cells (Fig. 11B and not shown).

At the next stage examined, E13.5, a restricted set of territories in the hindbrain express *Phox2* (Fig. 12). Expression is strongest in the immature locus coeruleus (area A6), identified by hybridization with a *TH* probe (Fig 13). The other areas correspond in number, extension and position to the remaining (nor)adrenergic and adrenergic nuclei of the hindbrain (Armstrong et al., 1982; Foster et al., 1985; Specht et al., 1981): areas A1 through 5, visible in Fig. 12, as well as areas C1 and C2 and the area postrema (not shown). By contrast, *Phox2* expression was never detected in the di- or telencephalon.

DISCUSSION

Cux and *Phox2*, two potential regulators of the *Ncam* promoter

We have cloned two HD proteins, Cux and Phox2, from a cell line of sympathoadrenal origin which bind to the *Ncam*

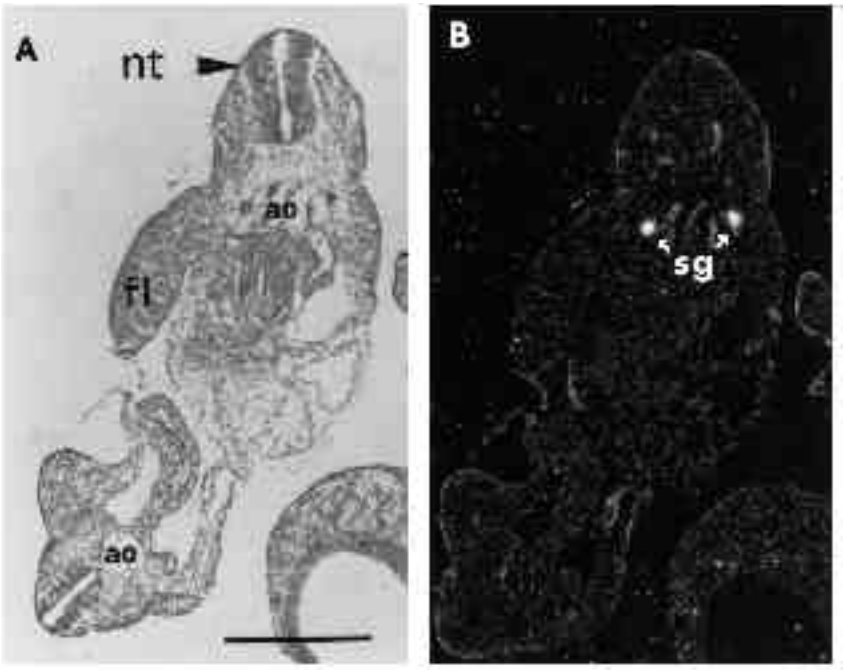

Fig. 8. Transverse sections of the E10 embryo showing *Phox2* expression in the forming sympathetic ganglion primordium. (A) Bright-field; (B) dark-field illumination. ao, dorsal aorta; fl, forelimb; nt, neural tube; sg, sympathetic ganglion anlage. Bar, 0.5 mm.

promoter. Cux is the murine homologue of human CDP (Neufeld et al., 1992) and canine Clox (Andres et al., 1992). Phox2, is a novel HD protein with an HD of the *prd* type (Frigerio et al., 1986).

When overexpressed in N2a cells, *Cux* was capable of down-regulating the activity of a reporter gene under control of the *Ncam* promoter, as well as the expression of the resident *Ncam* gene. Previously, *Clox* has been shown to repress the activity of an heterologuous promoter linked to a presumed Clox-binding element from the β-myosin heavy chain gene (Andres et al., 1992), and the down-regulation of CDP-like binding activity on the promoter of the macrophage-specific cytochrome β heavy chain gene was found to correlate with the onset of its transcription (Skalnik et al., 1991). In addition, CDP-like binding activity has been found to be strongly induced in committed, differentiating macrophages, chondroblasts and myoblasts, and to be down-regulated in the terminally differentiated corresponding cell types. This has led to the proposal that CDP may act as a general repressor of genes expressed in differentiated cells (Andres et al., 1992; Barberis et al., 1987; Neufeld et al., 1992). Since after E9, NCAM expression in the neuroecto-

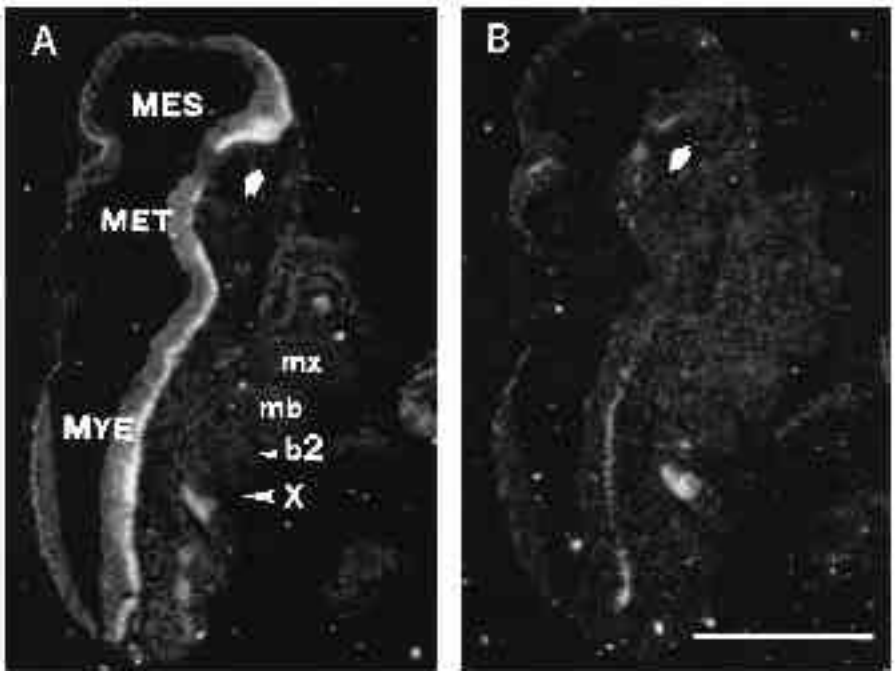

Fig. 9. *Phox2* and *Ncam* expression in the E10.5 embryo detected on parasagittal sections. Two consecutive sections were hybridized with *Ncam* (A) or *Phox2* (B) probes. In the neural tube, *Phox2* expression is restricted to the myelencephalon and the isthmic region, whereas *Ncam* transcripts are present along the mes- and rhombencephalon and extend caudally into the spinal cord region. Note the interruption of both *Phox2* and *Ncam* expression at the mes-metencephalic border. A strong signal is detected with both probes in the 4th branchial arch. b2: second branchial arch; mb: mandibular process; mx: maxillary process; MES: mesencephalic vesicle; MET: metencephalic vesicle ; MYE: myelencephalic vesicle; X: anlage of the Xth cranial ganglion. Bar, 1 mm.

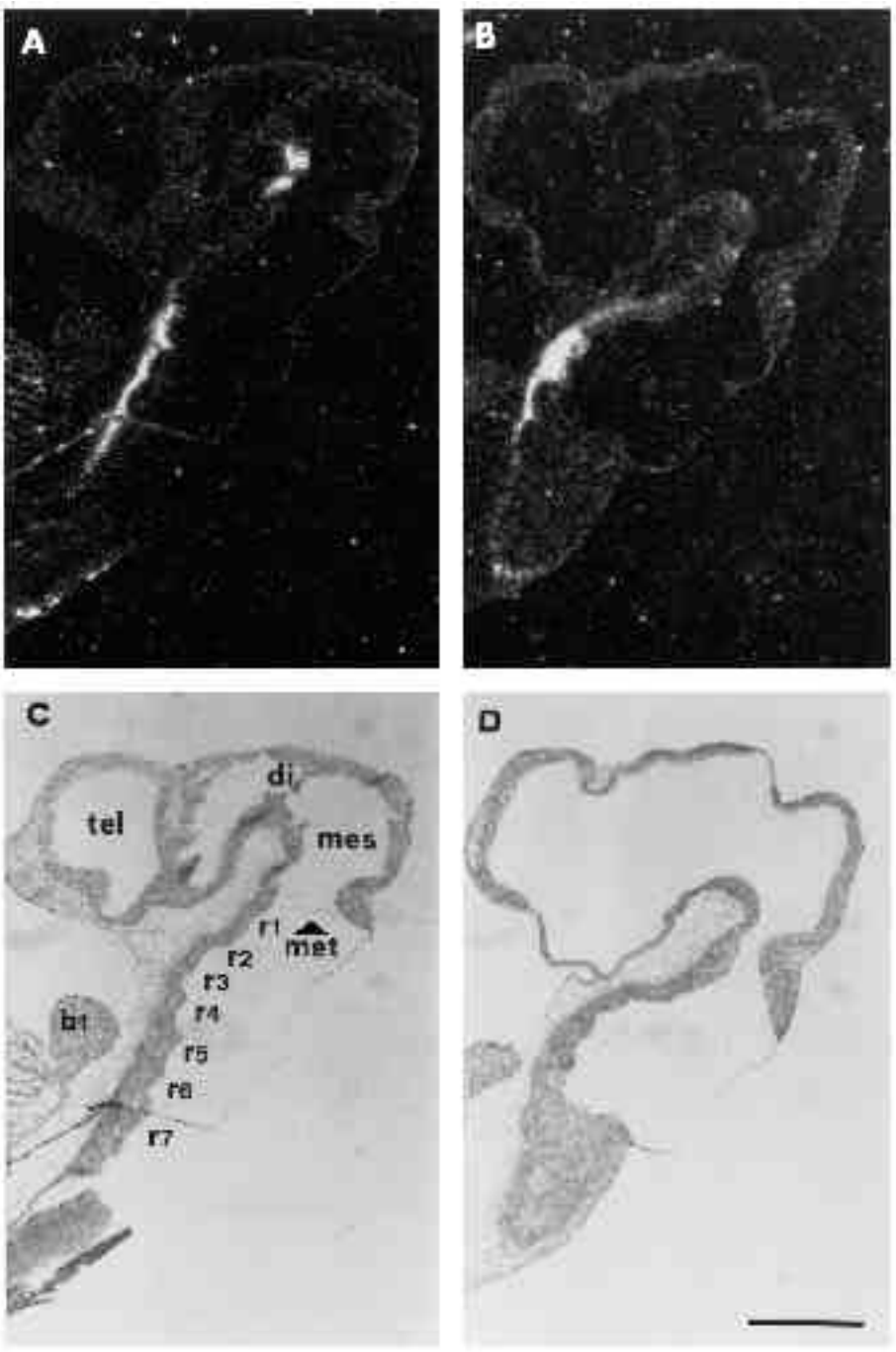

Fig. 10. *Phox2* expression in the rhombencephalon of the E10.5 embryo detected on parasagittal sections. (A,B) dark-field illumination, (C,D) corresponding bright-field illumination. In the myelencephalon, strong *Phox2* expression is seen in rhombomeres 4-7 that fades out rostrally and caudally to them; it resumes at both sides of the isthmus. Note that labeling of the ventricular zone is limited to rhombomere 4 (B). The line across r7 in A and C is the collapsed roof of the IVth ventricle. b1: first branchial arch; di: diencephalon; mes: mesencephalon; met: metencephalon; r1-7: rhombomeres 1-7; tel: telencephalon. Bar, 0.5 mm.

derm coincides with the differentiation of various classes of neurons, our results are compatible with this hypothesis. Even though the evidence remains patchy at present, the broad tissue distribution of *Cux*/*Clox* and of CDP-like activity, and the range of promoters that *Cux* seems able to repress (Andres et al., 1992, and our unpublished observations), all point to the possibility that Cux is one of the pleiotropic repressors that the continuously active control of gene expression in higher eukaryotes seems to call for (Blau, 1992).

Repression by *Cux* has been proposed to result from the displacement of the CCAAT box-binding protein CP1 (Barberis et al., 1987; Skalnik et al., 1991). In contrast, no CP1 site is obvious in the vicinity of the *Ncam* promoter element. Instead, the relief of *Cux*-mediated repression by *Phox2* identifies an HD protein as a functional partner of Cux. This is reminiscent of another case of a *prd*-like HD-containing protein acting via the functional modulation of other factors, i.e. the enhancement of SRF binding by Phox1 (Grueneberg et al., 1992). At this point it is not clear what mechanism accounts for the counter-repression exerted by *Phox2*, but the absence of overlap between the Cux and Phox2 binding sites makes simple competition for binding unlikely.

The developmental profile of *Phox2* expression in the embryo is fully compatible with a positive effect on *Ncam*

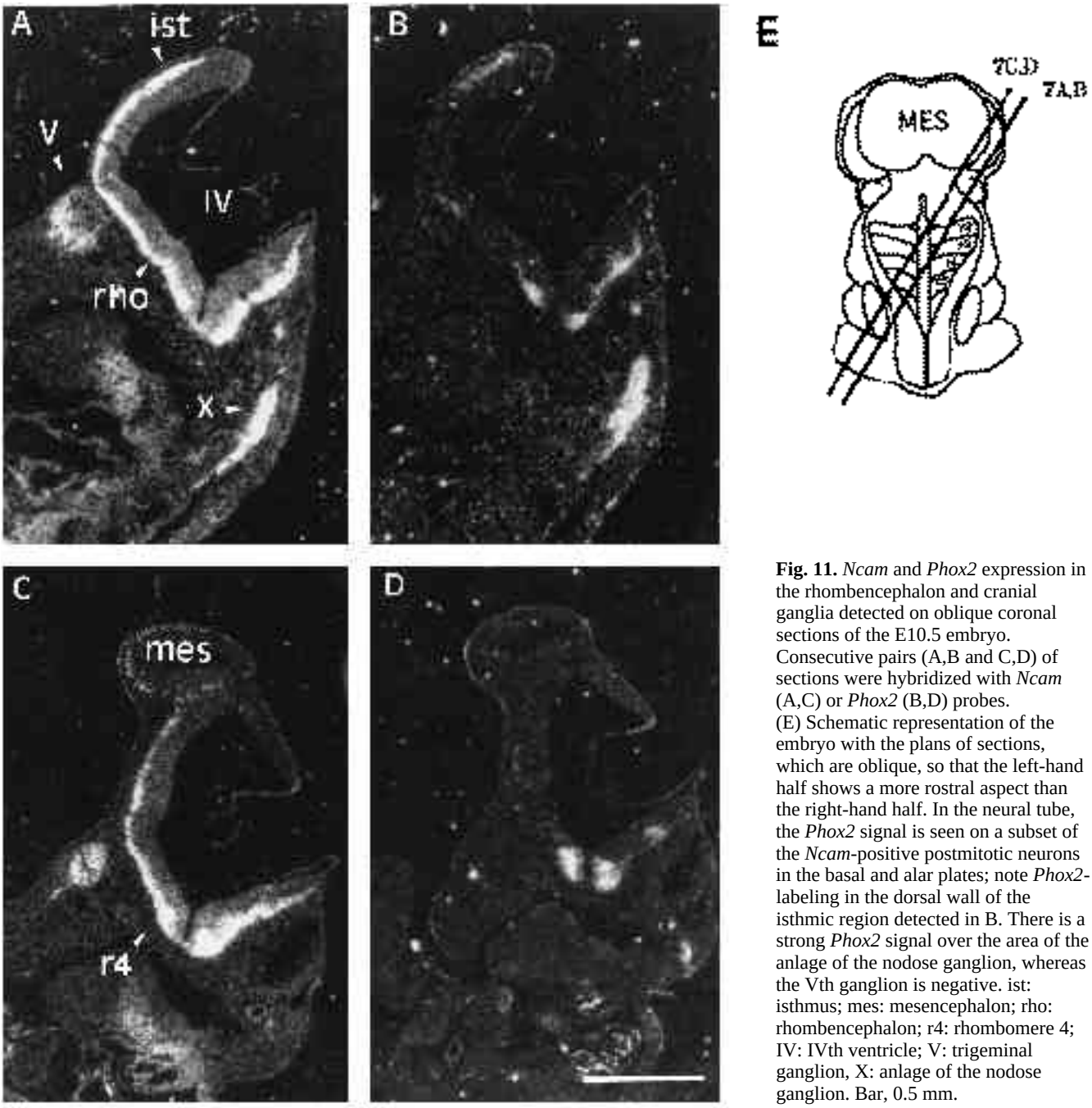

Fig. 11. *Ncam* and *Phox2* expression in the rhombencephalon and cranial ganglia detected on oblique coronal sections of the E10.5 embryo. Consecutive pairs (A,B and C,D) of sections were hybridized with *Ncam* (A,C) or *Phox2* (B,D) probes. (E) Schematic representation of the embryo with the plans of sections, which are oblique, so that the left-hand half shows a more rostral aspect than the right-hand half. In the neural tube, the *Phox2* signal is seen on a subset of the *Ncam*-positive postmitotic neurons in the basal and alar plates; note *Phox2*-labeling in the dorsal wall of the isthmic region detected in B. There is a strong *Phox2* signal over the area of the anlage of the nodose ganglion, whereas the Vth ganglion is negative. ist: isthmus; mes: mesencephalon; rho: rhombencephalon; r4: rhombomere 4; IV: IVth ventricle; V: trigeminal ganglion, X: anlage of the nodose ganglion. Bar, 0.5 mm.

transcription in vivo. With two exceptions, where *Phox2* expression on sympathoblasts and neuroepithelial progenitor cells was found to precede that of *Ncam*, all *Phox2*-positive territories also express *Ncam*: the ganglia of the autonomic nervous system, some of the cranial sensory ganglia (including the placodal anlage of the nodose ganglion) and a subset of postmitotic neurons in the rhombencephalon. Where this has been analyzed, the onset of *Phox2* expression always coincided with or slightly preceded that of *Ncam*. The involvement of *Phox2* in transcriptional regulation of *Ncam* is further supported by the spatial correlation between the expression of the two genes at the mes-metencephalic border. Both *Ncam* and *Phox2* transcripts are conspicuously absent in a narrow region corresponding to the ventral wall of the rhombencephalic isthmus whereas their expression resumes rostrally and caudally to it. A very speculative reading of these images is that a repressor accounts for the absence of *Ncam* transcripts in this region, and that *Phox2* is instrumental in defining the borders of the gap in *Ncam* expression.

In view of the expression profile of *Phox2* on neural crest derivatives, we would like to hypothesize that the functional interaction between Cux and Phox2 contributes to the known developmental profile of NCAM expression in the sympathoadrenal lineage of the neural crest. The SG and the adrenal medulla arise from the aggregation of neural crest

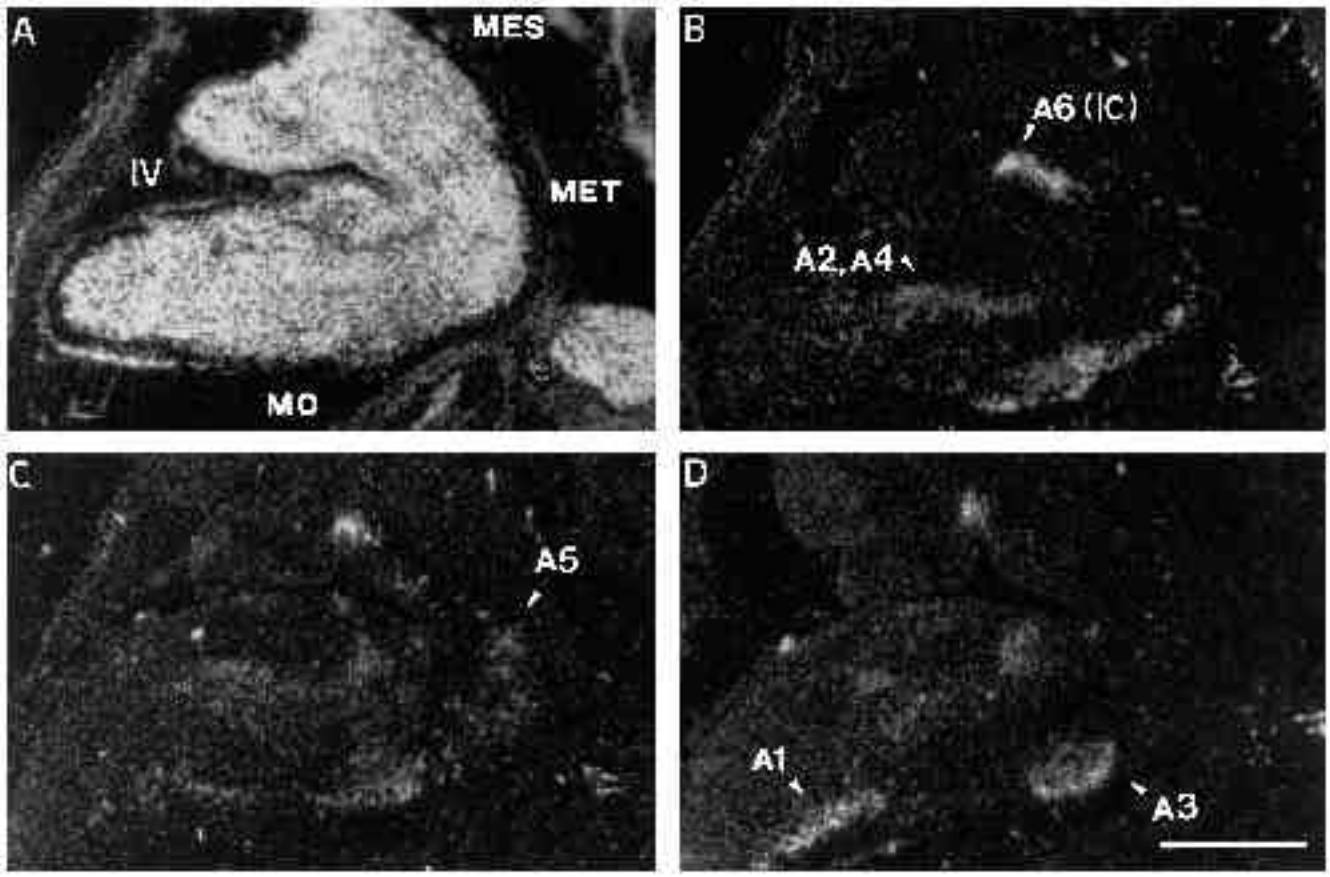

Fig. 12. *Ncam* (A) and *Phox2* (B,C,D) expression in the E13.5 embryo detected on parasagittal sections of the hindbrain. A1-5: areas 1-5 representing the noradrenergic nuclei of the metencephalon; A6 (lc): locus coeruleus; MES: mesencephalon; MET: metencephalon; MO: medulla oblongata; IV: fourth ventricle. Bar, 0.5 mm.

progenitors, after their ventral migration from the neural tube. NCAM expression is shut off as the cells emigrate from the crest and is reinduced as they settle in the autonomic ganglia (Thiery et al., 1982; Lallier and Bonner-Fraser, 1988) and the adrenal primordium. Thus we would like to propose that repression of *Ncam* transcription by Cux might be terminated during ganglion formation by the appearance of Phox2, acting as a counterrepressor. Though we cannot back our hypothesis by the expression pattern of *Cux* in the embryo, it is expressed in the two cell lines of sympathoadrenal origin tested, N2a and PC12, as well as in the adult adrenal medulla.

Together with the localisation data, the results of functional assays in cultured cells greatly strengthen the case for a regulation of *Ncam* transcription by homeobox genes (Hirsch et al., 1991, Jones et al., 1992). Other HD factors may be involved in *Ncam* regulation in *Phox2*-negative cell types, as already suggested by the observation that the products of two *Xenopus Hox* genes modulate the activity of the mouse *Ncam* promoter (Jones et al., 1992).

Phox2 expression in relation to the noradrenergic phenotype

A survey of the tissue distribution of *Phox2* during embryogenesis shows that the classic locations of adrenaline and noradrenaline synthesis in the adult are *Phox2*-positive as soon as they are recognizable as distinct structures: the SG, the adrenal medulla and the (nor)adrenergic nuclei of the future brainstem and pons. The alar myelencephalic column of *Phox2*-positive cells is in a location appropriate to give birth to the lateroventral and dorsomedial groups of noradrenergic neurons. The locus coeruleus and locus subcoeruleus originate from the isthmic region (TaberPierce, 1973), which is circled by *Phox2*-positive cells. Thus, and in spite of the paucity of published data on the location of origin of brain catecholaminergic neurons, our data are compatible with *Phox2* being a very early marker of these cells. These observations raise the possibility that *Phox2* is part of the regulatory cascade which determines the noradrenergic phenotype.

At first sight, this hypothesis is called into question by the fact that *Phox2* expression extends to sites that are not classically described as catecholaminergic: parasympathetic, enteric and cranial sensory ganglia. However, noradrenergic traits in the form of TH and/or DBH immunoreactivity are transiently expressed during embryogenesis, and sometimes persist into adulthood, in rat parasympathetic (Grzanna and Coyle, 1978; Landis et al., 1987; Leblanc et al., 1989) and enteric ganglia (Jonakait et al., 1979; Baetge et al., 1990), as well as in some cranial sensory ganglia (Jonakait et al., 1984). In line with this, a *lacZ* transgene driven by the *DBH* promoter was found to be expressed at these locations

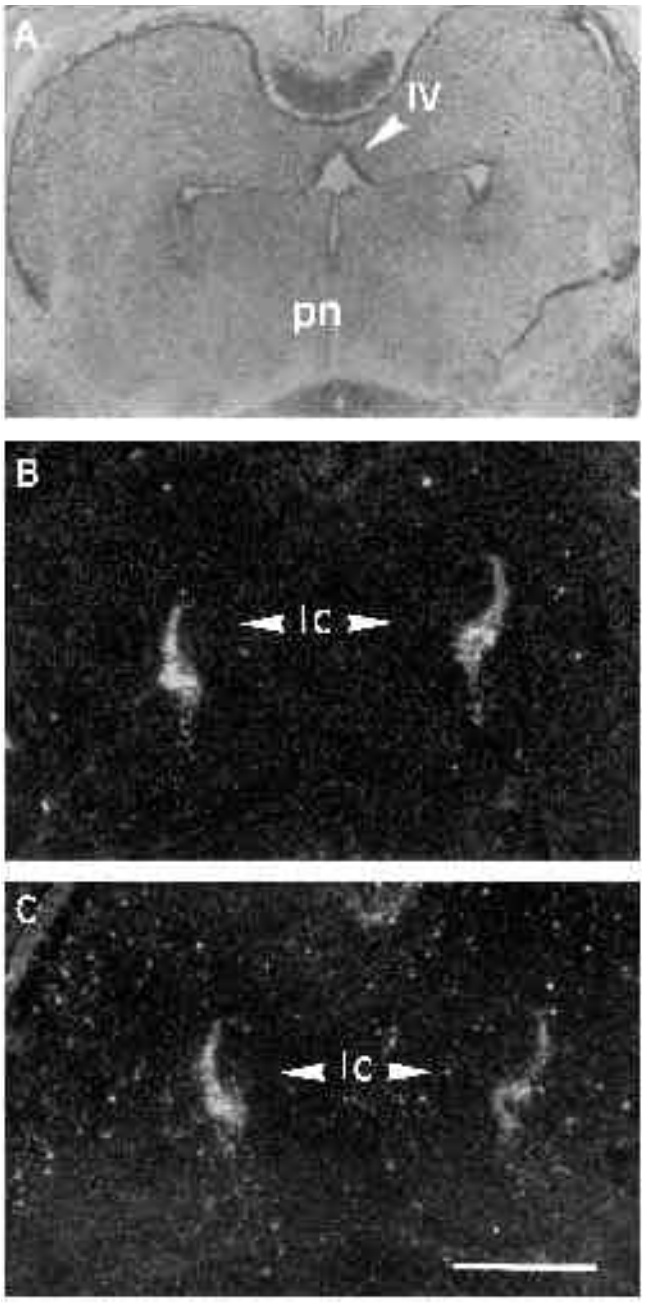

Fig. 13. *Phox2* and *TH* expression in the E13.5 day embryo detected on transverse sections of the metencephalon. Serial sections of the metencephalon (A, bright-field illumination) were hybridized with a *Phox2* (B) or *TH*-specific probe (C). lc: locus coeruleus; pn: pons; IV: IVth ventricle. Bar, 0.5 cm.

(Kapur et al., 1991; Mercer et al., 1991). On the basis of this data, it has been proposed that all postganglionic neurons of the autonomic nervous system are initially noradrenergic before some of them shift to other transmitter phenotypes in response to envirommental cues (Black, 1982; Kapur et al, 1991). The absence of *Phox2* expression in the proximal ganglia of the IXth and Xth cranial nerves parallels the reported absence (Jonakait et al., 1984) or extreme paucity (Katz, 1991) of TH cells in these ganglia. This parallel does not extend to the *Phox2*-negative dorsal root ganglia, which also transiently express TH during ontogeny (Jonakait et al., 1984). However, the reported lack of expression of the *DBH* promoter-*lacZ* transgene (Kapur et al., 1991) may be taken as evidence that *DBH* is not expressed and that these neurons display a dopaminergic rather than a noradrenergic phenotype. Another potential discrepancy concerns *Phox2* expression on postmitotic neurons in the basal plate of the rhombencephalon, since the (nor)adrenergic neurons are supposed to originate from the alar plate. Additional work is required to test whether there might be other sites of origin of some of the adrenergic neurons and/or another location of transiently catecholaminergic cells.

Although in situ hybridization does not permit resolution at the cellular level, two lines of evidence suggest that *Phox2* expression is, at least in part, neuronal: the presence of the transcripts in the neural tube before the emergence of glial cells and the high-level expression of the gene in two cell lines with neuronal characteristics, N2a cells and PC12 cells, which upregulate *Phox2* transcripts during differentiation towards a neuronal phenotype.

To our knowledge, Phox2 is the first transcription factor expressed on a subset of neurones defined by its neurotransmitter phenotype. Islet-1 has been reported to be expressed in sympathetic neurons and chromaffin cells of the adrenal medulla, the locus subcoeruleus and a number of structures in the brainstem some of which could correspond to noradrenergic nuclei, but also in many other locations (Thor et al., 1991). MASH1, which belongs to a different class of transcription factors, has also been suggested to function in the determination of the sympathoadrenal lineage (Lo et al., 1991). However, this gene is much more widely expressed in the CNS than *Phox2*, and has not been reported to be present in the noradrenergic areas of the brainstem. One way *Phox2* could be involved in the determination of noradrenergic neurons, is by controlling the expression of enzymes along the catecholamine synthesis pathway. In this respect, we noted some striking coincidences between the expression patterns of *Phox2* and a *DBH* promoter-driven *lacZ* transgene (Kapur et al, 1991), including, in addition to bona fide noradrenergic structures, cranial sensory, parasympathetic and enteric ganglia and even more intriguingly, cells in the basal plate of the fourth ventricle. Following this lead, we actually found a high affinity Phox2 binding site in the DBH gene promoter (I. V., unpublished results). Further work aimed at the identification of upstream and downstream genes as well as functional assessment of *Phox2* in transgenic animals will help define the regulatory cascades in which this gene participates.

We thank H. Deagostini-Bazin for preparing Phox2 fusion protein and help with the gel shift experiments. Pascal Weber is thanked for excellent artwork. We thank J. Mallet for the kind gift of the TH probe. This work was supported by institutional grants from CNRS and INSERM and by a specific grant from Association pour la Recherche contre le Cancer (to C. G.), and grant DGICYT-PB87/688-COL (to S. M.).

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(Accepted 21 July 1993)