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Ncx, a *Hox11* related gene, is expressed in a variety of tissues derived from neural crest cells

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Abstract We have isolated the murine homeobox gene (*Ncx*) that belong to a *Hox11* gene family. Expression of the *Ncx* gene was analyzed in total RNAs from embryos by reverse transcribed polymerase chain reaction (RT-PCR). The mRNA was detected in embryos after 9.5 days of embryogenesis (E9.5) and was maximal at E12.5. The RT-PCR also detected the message in total RNAs from adrenal glands and intestine in adult mice. The expression was further examined in various tissues from embryos by in situ hybridization. It was detected in dorsal root ganglia, cranial nerve ganglia (V, IX, X), enteric nerve ganglia and adrenal glands from embryos between E9.5 and E13.5. Since its expression is restricted to tissues derived from neural crest cells, Ncx may play a role in differentiation and proliferation of neural crest lineage cells.

Key words Homeobox · Adrenal glands · Sympathetic nerve · Dorsal root ganglia · Enteric ganglia

Introduction

Homeobox gene products have a conserved domain (homeodomain) of 60 amino acids that are known to bind to specific DNA sequences (Gehring 1994). These genes are classified into several gene families depending on their homology of homeobox sequences and their localizations on chromosomes in humans and mice (Burglin 1994). There are four clusters of homeobox genes that are located on four different chromosomes. Each homeobox gene rearranges in linear array in the cluster and the gene located on the more 3′ side of the cluster is expressed in the more anterior part of body segments in a mouse embryo (De Robertis 1994). Therefore, these clustered homeobox genes may play an important role in segmentation and determination of the anterior-posterior axis in embryos. Besides these clustered homeobox genes, there are some homeobox genes that do not locate in these four clusters. However, functional roles of these non-clustered homeobox genes are less understood.

The *HOX11* gene was cloned from the chromosomal breakpoint in T cell acute lymphoblastic leukemias with t(10;14) (Dubé et al. 1991; Hatano et al. 1991; Kennedy et al. 1991; Lu et al. 1991). It is mapped on the chromosome 10q24 in humans and does not belong to the clustered homeobox genes. *Hox11* null mutant mice demonstrated the asplenic phenotype, indicating that it is essential for the genesis of the spleen (Roberts et al. 1994; Dear et al. 1995). Although the *Hox11* was expressed not only in the primordium of the spleen but also in other tissues such as branchial arches, cranial ganglia and the motor neuron nucleus during embryogenesis (Raju et al. 1993; Roberts et al. 1994; Dear et al. 1995), the genesis of organs other than spleen is normal in the *Hox11*-deficient mice (Roberts et al. 1994; Dear et al. 1995). This functional redundancy of Hox11 suggests that some other gene products supplement the function. Several *Hox11* related genes have been cloned with the homeodomain sequence as a probe (Dear et al. 1993; Raju et al. 1993). Because of the homology of homeodomain sequences among these related genes, they may compose a *Hox11* gene family. However, the genomic organization, expression and function of these related genes are not yet known. We have cloned one of the *Hox11* related genes and have examined its expression by reverse transcribed polymerase chain reaction (RT-PCR) and in situ hybrid-

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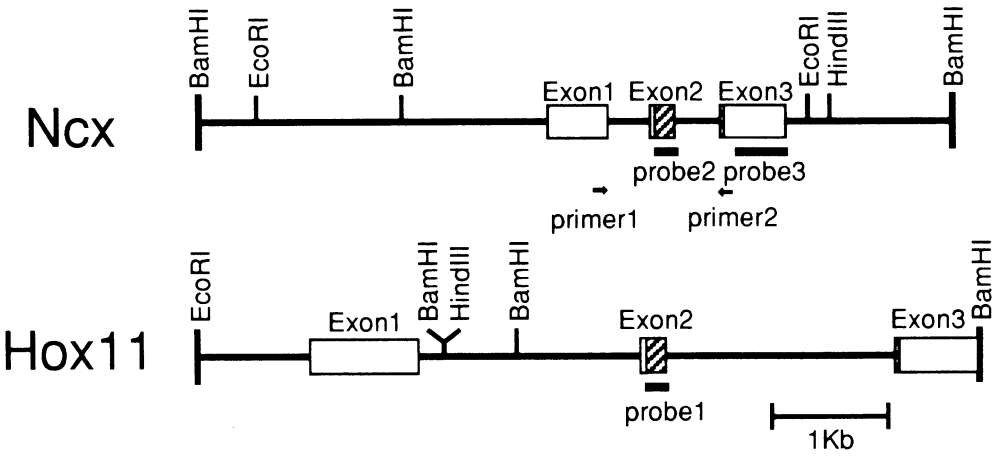

Fig. 1 Genomic maps of the *Ncx* and the *Hox11* gene. *Open* and *shaded* boxes indicate exons and the homeodomain, respectively

ization. We show here that the gene is specifically expressed in tissues derived from neural crest cells. We propose a name, *Ncx* (*N*eural *c*rest homeobo*x*), for this gene. We discuss a role of Ncx in embryogenesis.

Material and methods

Animals

(C57BL/6×DBA/2) F1 mice were purchased from Japan SLC (Hamamatsu, Japan). The midday following the plugging was defined as embryonic day 0.5 (E0.5).

Synthesis of probes

Digoxigenin (DIG)-labeled probes were made by PCR with the DIG DNA labeling mixture (Boehringer Mannheim, Mannheim, Germany). Probe 1 was a 199 bp of DNA fragment synthesized by PCR on the *Hox11* cDNA with the primers (ACCCCTATCAGAACCGGACG and CTCCATTTCGTCCTCCGGTT). Probe 2 was a 193 bp of DNA fragment synthesized by PCR on the *Ncx* cDNA with the primers (ATCCCTACCAAAACCAAACC and TTGGTGCGTCGGTTCTGGAA). Probe 3 was a riboprobe synthesized on the 3′ untranslated region of *NCx* cDNA by SP6 RNA polymerase using the DIG RNA labeling kit (Boehringer Mannheim).

RT-PCR and Southern blot analysis

Total RNA was extracted from various organs and embryos using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987). A cDNA was made from 100 ng of total RNA by reverse-transcribing the RNA with oligo(dT) as a primer at 42° C for 15 min in a volume of 20 µl. The cDNA (1 µl) was mixed with 2.5 U of Taq DNA polymerase, 2 mM $MgCl_2$, 5% formamide and primers in a vlume of 25 µl. The primers used for the *Ncx* cDNA were primer 1 (TTTGCAAAGGACAGGCTCACG) in exon 1 and primer 2 (GGTGCAGCAGCAGGCGACCA) in exon 3 of the *Ncx* DNA (Fig. 1). After 25 cycles of the PCR, the amount of *Ncx* cDNA in 10 µl of the PCR products was measured by Southern blot analysis as described previously (Yamamoto et al. 1995) with probe 2. Briefly, the PCR products were separated on a 1% agarose gel and transferred onto a nylon membrane (Amersham International, Buckinghamshire, England). The filter was hybridized with a DIG-labeled probe overnight at 42° C. Following hybridization, the filter was washed twice with 0.1×SSC at 68° C for 15 min. The DIG-labeled probe was detected by the en-

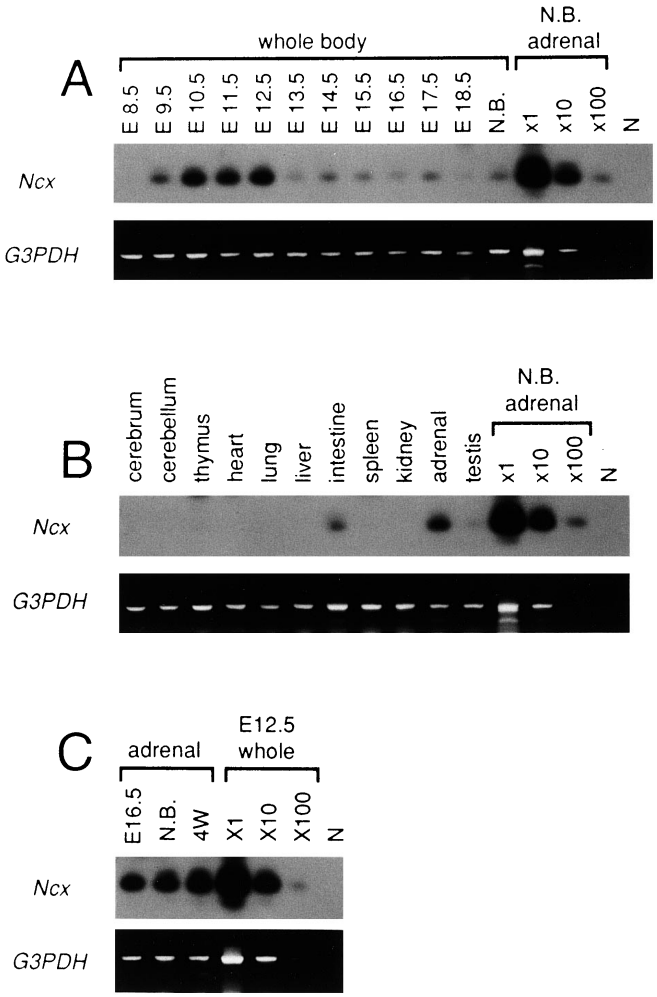

Fig. 2A–C Expression of *Ncx* mRNA. Expression of *Ncx* mRNA in total RNAs from mouse embryos and organs was examined by RT-PCR with Southern blot. **A** Expression of the *Ncx* mRNA in whole embryos. **B** Various organs from an adult mouse. **C** Adrenal glands from an embryo (*E16.5*), a newborn (*N.B.*) and a 4-week-old (4W) mouse. For quantitation of the message, amounts of *Ncx* mRNA in 1 µg (×*1*), 100 ng (×*10*) and 10 ng (×*100*) of total RNA from adrenal glands of newborn (*N.B.*) mice were examined by the RT-PCR. Expression of the *G3PDH* mRNA is shown as a control amount of the cDNA (*N* PCR without cDNA)

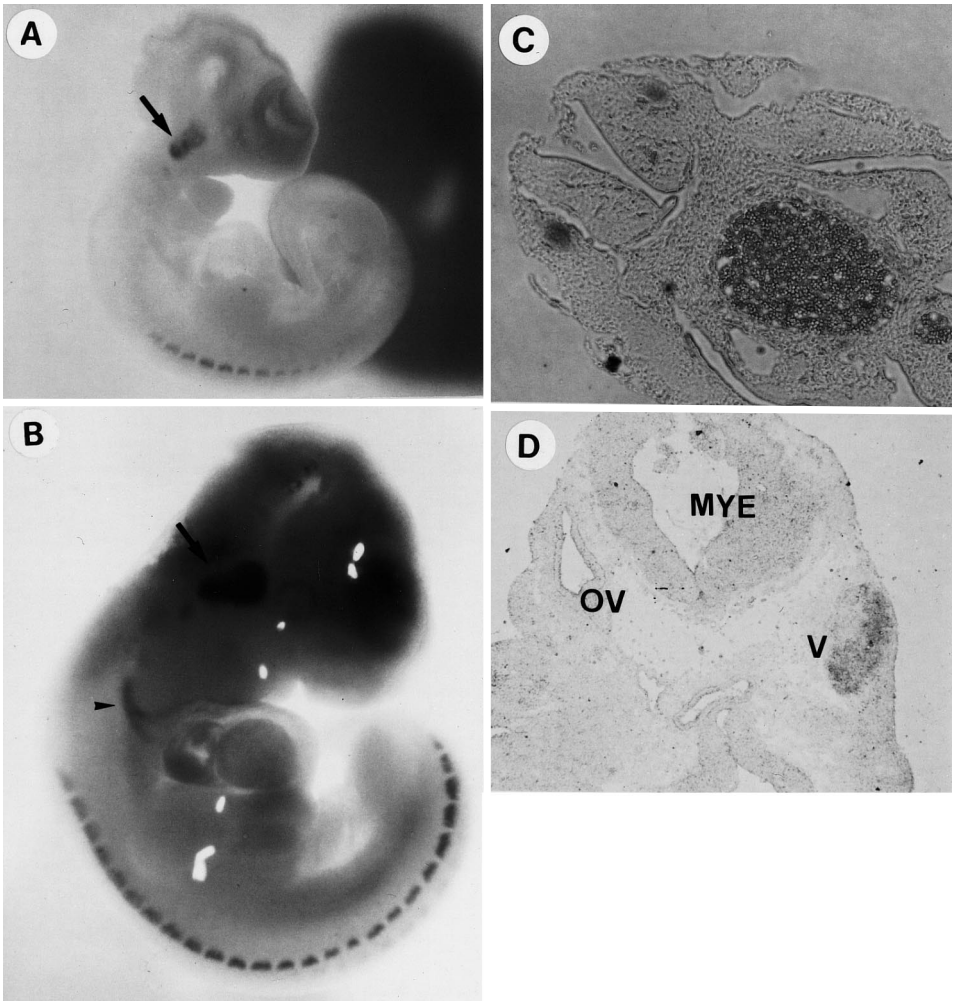

Fig. 3A–D Expression of the *Ncx* gene in embryos at E9.5 and E10.5. **A** Whole mount in situ hybridization of an embryo at E9.5. **B** Whole mount in situ hybridization of an embryo at E10.5 (in **A** and **B**: *arrow* an area of the fifth cranial ganglia, *arrowhead* an area of a group of cranial ganglia (IX, X). **C** A transverse section of the embryo shown in **B** at the level of the abdomen; positive signal was detected in dorsal root ganglia. **D** In situ hybridization of an embryo at E10.5; a transverse section of the embryo at the level of the myencephalon (*MYE* myencephalon, *OV* otic vesicle, *V* the fifth cranial ganglion)

hanced chemiluminescent detection system (Boehringer Mannheim). PCR for glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) cDNA was performed as described previously (Yamamoto et al. 1995). The amount of *G3PDH* cDNA in 10 µl of the PCR products was measured by gel electrophoresis with ethidium bromide.

In situ hybridization

Frozen sections fixed with 4% paraformaldehyde were incubated with proteinase K solution (10 µg/ml of proteinase K in 10 mM TRIS-HCl pH 8.0 and 1 mM EDTA) at 37° C for 10 min. The sections were hybridized with probe 3 at 55° C overnight, followed by RNase A treatment. Positive signals were detected by the Nucleic Acid Detection Kit (Boehringer Mannheim).

Whole mount in situ hybridization was performed essentially as described (Wilkinson 1992). Briefly, embryos fixed in 4% paraformaldehyde were hybridized with probe 3 overnight at 70° C, followed by incubation with RNase A. The embryos were further incubated with preabsorbed anti-DIG antibody overnight at 4° C. Positive signals were detected by the Nucleic Acid Detection Kit (Boehringer Mannheim).

Results

Genomic organization of the *Ncx* gene

In order to identify genes belong to a *Hox11* gene family, we screened the murine genomic DNA library with the *Hox11* homeodomain probe (probe 1) in low stringency. Several clones were picked up and their homeodomains were sequenced. The closest homologue of the *Hox11* gene (*Ncx*, also called *Hox11L1* (Dear et al. 1993)) was further characterized. The *Ncx* cDNA was cloned by the anchored PCR using mRNA from embryos at E16. A genomic map of the *Ncx* gene was determined using the cDNA as a probe and compared with that of the *Hox11* (Fig. 1). These two genes were quite similar in the organization of exons and the position of homeodomain sequence, suggesting that these two genes belong to the same gene family.

Expression of the *Ncx* gene

A RT-PCR system was established to examine expression of the *Ncx* gene during embryogenesis. A set of

primers that were complementary to the sequence of *Ncx* cDNA was selected with reference to the genomic map (Fig. 1). Two introns and one exon sequence were present between these primer sequences. Thus, these primers amplified a 332 bp on the *Ncx* cDNA and this product was distinguishable from the PCR product on the genomic DNA (1.1 Kbp) by size. By the RT-PCR, the expression was examined in total RNAs from whole embryos between E8.5 and newborn and from various organs of an adult mouse. As shown in Fig. 2A, the mRNA was detectable in embryos after E9.5 and was maximal at E12.5. Only a small amount of the message was detected until the newborn stage. In an adult mouse, a moderate amount of the mRNA was detected in total RNA from adrenal glands. Intestine also expressed a similar level (Fig. 2B). The amount in adult adrenal glands was compared with those in adrenal glands from an embryo at E16.5 and a newborn mouse (Fig. 2C). The same level was continuously expressed in these adrenal glands.

Expression of the *Ncx* gene in tissues derived from neural crest cells

Spatiotemporal expression pattern of the *Ncx* gene during embryogenesis was analyzed by in situ hybridization using the 3′ UT of *Ncx* as a probe (probe 3) and compared with that of the *Hox11* gene. Fig. 3A shows that the *Ncx* mRNA was detected in embryos at E9.5 by whole mount in situ hybridization. Positive signals were detected along the dorsal side of an embryo. The strong signal extended from the rostral part to the more caudal region of an embryo at E10.5 (Fig. 3B). In a transverse section of the embryo, these positive areas turned out to be dorsal root ganglia (Fig. 3C). Fig. 3A, B also show that areas of the fifth cranial ganglion beside the otic vesicles and a group of cranial ganglia (IX, X) were positively stained. The expression in the area of the fifth cranial ganglia was confirmed in transverse sections of embryos at E10.5 (Fig. 3D). These positive signals in cranial nerve ganglia were detected until E13.5 and became very weak after E16.5 (data not shown). In contrast, the *Hox11* gene was expressed in branchial arches, and positive areas of *Ncx* expression did not overlap those of *Hox11* at this stage of embryogenesis (data not shown).

Dorsal root ganglia and cranial ganglia contain sensory components of the peripheral nervous system derived from neural crest cells (Anderson 1993; Selleck et al. 1993; Stemple and Anderson 1993). Multipotent neural crest cells migrate from dorsal region of the neural tube to the periphery and give rise to sensory neuron progenitors, sympathoadrenal progenitors, melanocyte progenitors, and skeletal and connective tissue components of head. Therefore, the expression was further examined in these tissues derived from neural crest cells by in situ hybridization. In a section of an embryo at E13.5, positive signals were detected in areas of the fifth cranial ganglia and a group of cells adjacent to the fifth cranial ganglia where parasympathetic (sphinopalatine) ganglia were located (Fig. 4A). The expression in this area became very faint by E16.5 (data not shown). Strong signals were also observed at the ganglia of sympathetic trunks, cardiac ganglia, paraaortic sympathetic ganglia as well as dorsal root ganglia (Fig. 4B, C) and around intestinal walls that

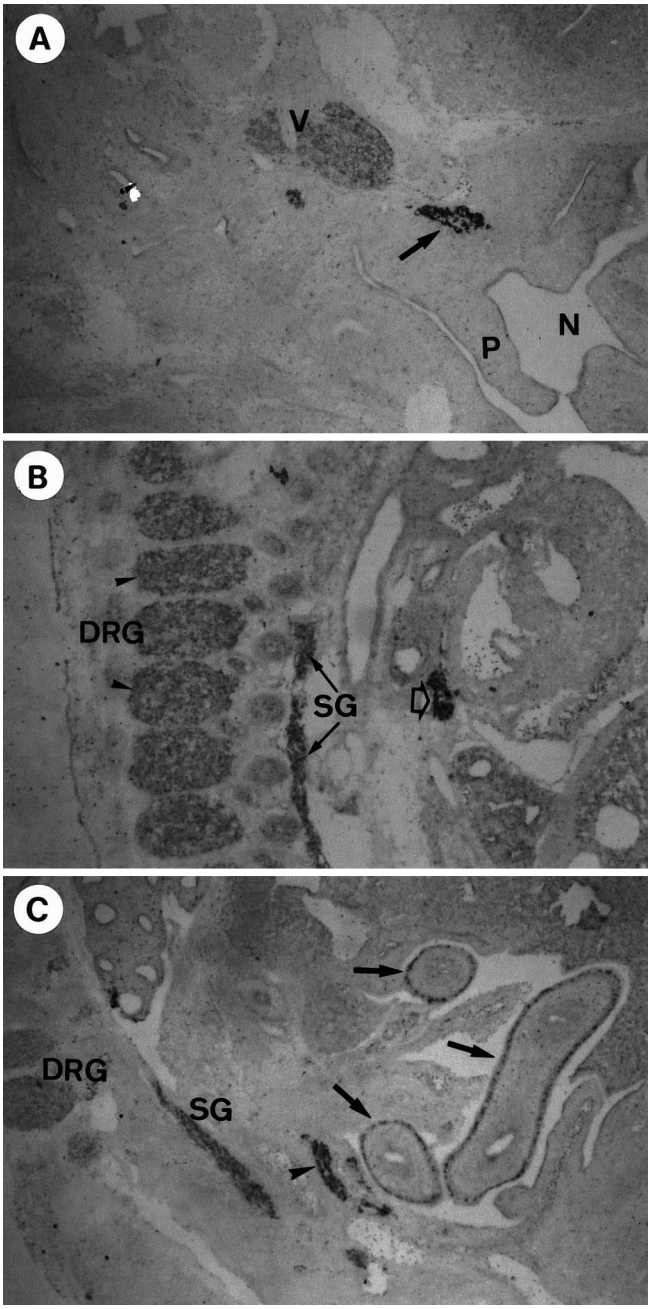

Fig. 4A–C Expression of the *Ncx* gene in neural crest lineage cells; in situ hybridization of an embryo at E13.5. **A** A parasagittal section of the embryo at the level of the head (*arrow* an area of the sphinopalatine ganglion, *N* nasal cavity, *P* palate). **B** A parasagittal section of the embryo at the level of the mid-trunk (*open arrow* cardiac ganglia, *DRG* dorsal root ganglia, *SG* sympathetic ganglia. **C** A parasagittal section of the embryo at the level of the lower abdomen (*arrows* walls of intestine, *arrowhead* retroperitoneal area where sacral ganglia were located). The right side of these figures is the cephalic side

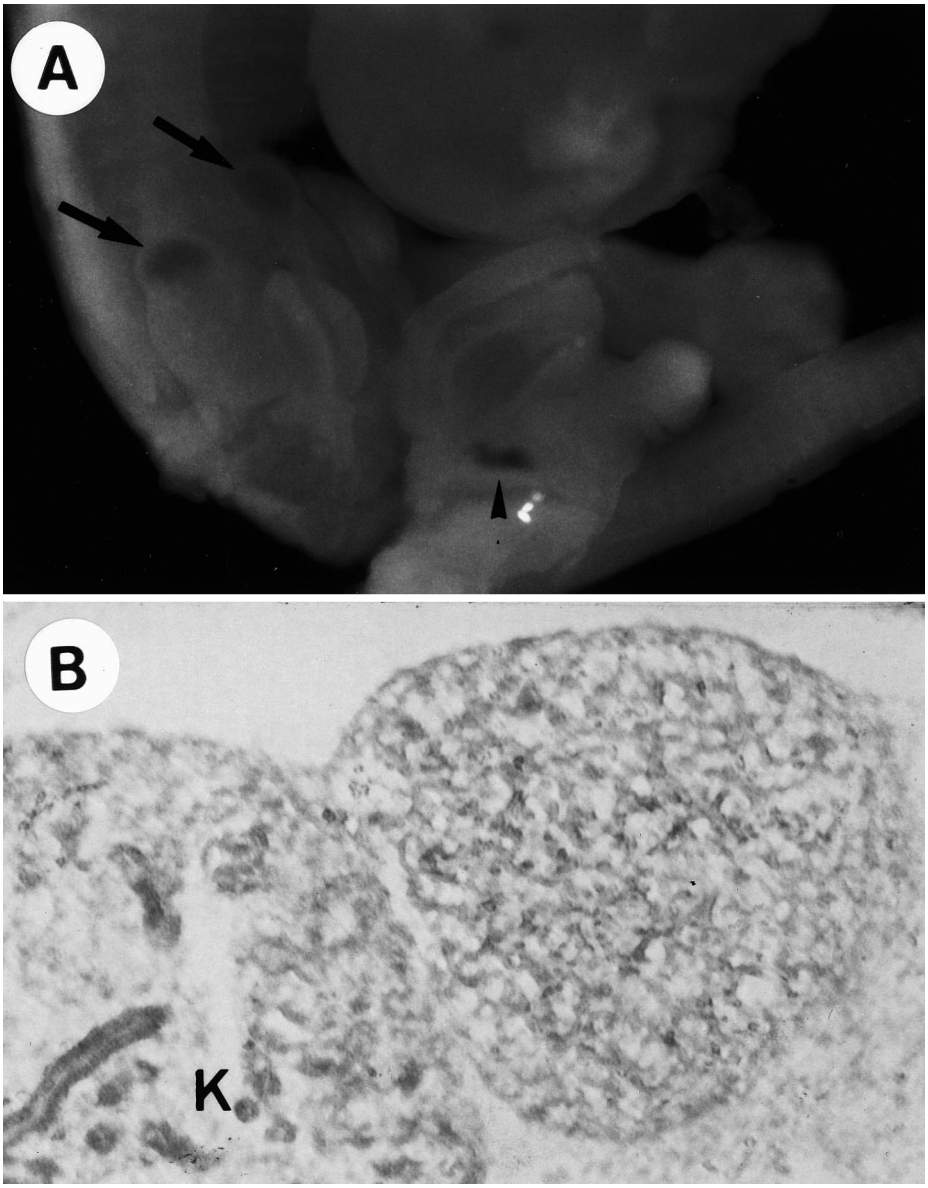

Fig. 5A–B Expression of the *Ncx* gene in adrenal glands from an embryo at E13.5. **A** Whole mount in situ hybridization; intestine and liver were removed from an embryo to see the posterior abdominal wall (*arrows* adrenal glands, *arrowhead* posterior part of the urinary bladder where sacral ganglia were located). **B** In situ hybridization of an adrenal gland (*K* kidney). The right side of these figures is the cephalic side

contained ganglia of the enteric nervous system (Fig. 4C). No positive signal was detected in skin melanocytes, cartilage and connective tissue components of the head (data not shown).

The results shown in Fig. 2C demonstrated that expression of the *Ncx* gene was detected in adrenal glands by the RT-PCR. The expression in adrenal glands was further examined in an embryo at E13.5 by whole mount in situ hybridization. The mRNA was observed in adrenal glands and in the posterior wall of the urinary bladder (Fig. 5A). In parasagittal sections of the embryo, the expression was detected in the adrenal medulla, which contains chromaffin cells, the endocrine equivalents of sympathetic neurons (Fig. 5B). Overall, the *Ncx* gene was expressed in the adrenal medulla, and in sympathoadrenal and sensory ganglia derived from neural crest cells.

Discussion

The *Ncx* mRNA was detected in sensory ganglia (dorsal root ganglia, the fifth cranial ganglia and superior-jugular ganglion), sympathetic ganglia (paraaortic ganglia) and the adrenal medulla in embryos between E10.5 and E13.5 (Figs. 3, 4). The expression was also detected in the nerve plexus of the gut wall. These data indicate that the expression is specific for neural cells of the autonomic, sensory and enteric nervous systems derived from neural crest cells. Although melanocytes and cephalic mesenchyme are also derived from neural crest cells (Anderson 1993), we could not identify the expression in these cells. Since in situ hybridization does not permit resolution at a single cell level, further experiments with a sensitive method are necessary to confirm the expression in these cell types.

Neural crest cells develop at the dorsal part of the neural tube in embryos at E8.5 (Rugh 1990) and migrate

to peripheral regions at E9.5 (Kaufman 1992). Although the mRNA was not detected in embryos at E8.5 by RT-PCR (Fig. 2) or by whole mount in situ hybridization (data not shown), the message became detectable in embryos at E9.5 (Figs. 2, 3). The mRNA was observed in the area of the fifth cranial ganglion in embyos at E9.5 (Fig. 3D) where neural crest cells were differentiating to cranial ganglion (Rugh 1990). The amount was maximal in embryos at E12.5 (Fig. 2) when neural crest derived cells actively proliferate (Kaufman 1992). However, the expression was not detected in migrating neural crest cells. These results suggest that Ncx plays an important role in differentiation and proliferation of neural crest lineage cells. Since the message was continuously detected in adrenal glands until the adult stage (Fig. 2C), it is also possible that Ncx is required for maintaining a function of adrenal glands after birth.

Several known genes are expressed in neural crest lineage cells. The c-*ret* proto-oncogene, which encodes a receptor tyrosine kinase, is expressed in cranial ganglia, the autonomic nervous system, dorsal root ganglia and the enteric nervous system (Pachnis et al. 1993). Since the c-*ret* expression is detectable in migrating neural crest cells, a role of c-*ret* may be different from that of *Ncx* in the differentiation of neural crest cells. *MASH1*, which encodes a helix-loop-helix transcription factor, is expressed in precursors of sympathetic and enteric neurons but not in those of sensory neurons (Lo et al. 1991), suggesting its function in determination of the sympathoadrenal lineage. *Phox2*, which encodes a homeobox protein, is expressed in ganglia of the autonomic nervous system, adrenal medulla and some cranial sensory ganglia, but not in spinal dorsal root ganglia (Valarché et al. 1993). These genes are also expressed in the developing central nervous system of neuroepithelial origin (Lo et al. 1991; Guillemot and Joyner 1993; Valarché et al. 1993). However, we could not detect *Ncx* expression in the central nervous system. Therefore, the expression seems to be restricted to tissues derived from neural crest cells. Furthermore, the expression is observed in a broader subpopulation of tissues derived from neural crest cells. In that sense, *Ncx* could be a marker gene for the neural crest lineage cells.

There are four *HOX* gene clusters in humans and mice (Scott 1992; Krumlauf 1994). These clustered genes are expressed in embryos in a spatial-specific way during embryogenesis and play important roles in establishing the general body plan, including the determination of the anterior-posterior axis in embryos (Krumlauf 1994). The *Hox11* gene locates outside of these gene clusters and is expressed in the primordium of the spleen, branchial arches and cranial ganglia in embryos (Raju et al. 1993; Roberts et al. 1994; Dear et al. 1995). The *Ncx* gene also locates outside of the *Hox* gene clusters (Wen et al. 1994) and its expression is restricted to tissues derived from neural crest cells, suggesting that *Hox11* and *Ncx* are expressed in embryos in a cell lineage-specific way. Since Hox11 controls the genesis of the spleen (Roberts et al. 1994), Ncx may play a role in the genesis of organs derived from neural crest cells.

Cloning the *Ncx* gene was based on the homology of the *Hox11* homeodomain sequence. Homology of the homeodomain amino acid sequences between them is 87% (Dear et al. 1993). The helix 3 sequences that are important for determining the DNA binding specificity is identical between them (Dear et al. 1993). These facts raise the possibility that Hox11 and Ncx share some functional roles. Both genes are expressed in cranial ganglia in the embryo at E13.5 (Raju et al. 1993). *Hox11* null mutant mice did not demonstrate any structural abnormality in the nervous system (Roberts et al. 1994), suggesting that the functional of Hox11 in cranial ganglia is supplemented by that of Ncx in the mutant mice. Since the *HOX11* gene acted as an oncogene in transgenic mice (Hatano et al. 1992), it is also possible that the *Ncx* gene is responsible for oncogenesis. Further analysis is necessary to elucidate the functions of the *Hox11* gene family.

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