Expression of the c-ret proto-oncogene during mouse embryogenesis

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SUMMARY

The c-ret proto-oncogene encodes a receptor tyrosine kinase whose normal function has yet to be determined. To begin to investigate the potential role of this gene in vertebrate development, we have isolated cDNA clones representing the murine c-ret gene, and have analyzed the pattern of expression during mouse embryogenesis, using northern blotting, in situ hybridization to histological sections and whole-mount hybridization histochemistry. c-ret transcripts were detected beginning at day 8.5 of embryogenesis, and were observed in a number of cell lineages in the developing peripheral and central nervous systems, as well as in the excretory system. In the cranial region at day 8.5-9.5, c-ret mRNA was restricted to a population of neural crest cells migrating from rhombomere 4 and forming the anlage of the facioacoustic ganglion, and the layers of the postnatal retina containing ganglion, amacrine and horizontal cells. Outside the nervous system, c-ret was expressed in the nephric (Wolffian) duct at day 8.5-10.5, the ureteric bud epithelium (but not the surrounding metanephric mesenchyme) at day 11.0-11.5, and the growing tips of the renal collecting ducts (but not the previously formed, subcortical portions of the collecting ducts, or the mesenchyme-derived renal vesicles) at day 13.5-17.5. Our results suggest that the c-ret gene may encode the receptor for a factor involved in the proliferation, migration, differentiation or survival of a variety of neuronal cell lineages, as well as in inductive interactions during organogenesis of the kidney.

Key words: c-ret, tyrosine kinase receptor, mouse embryogenesis, nervous system, excretory system

INTRODUCTION

Normal development of the vertebrate embryo depends on the proper communication between diverse cell types. Several membrane-bound or diffusible molecules have been identified as components of such an intercellular communication system. These molecules mediate their effects by binding to specific cell surface receptors, a class of which has intrinsic and ligand-dependent tyrosine kinase activity. All receptor tyrosine kinases (RTKs) have a similar topology: they possess an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic segment containing the catalytic tyrosine kinase domain (Yarden and Ullrich, 1988). Binding of the ligand leads to dimerization of the receptor and activation of the kinase domain, manifested as autophosphorylation of the receptor and phosphorylation of specific substrates that mediate the intracellular signaling (Ullrich and Schlessinger, 1990).

The role of RTKs in cellular proliferation and oncogenic transformation has been studied extensively (Cantley et al., 1991), but their function during embryogenesis of multicellular organisms is less well understood. The best evidence that RTKs play a critical role in diverse developmental processes is derived from genetic studies in both invertebrates and vertebrates (Hafen and Basler, 1990; Pawson and Bernstein, 1990). In Drosophila melanogaster (D. melanogaster), several well-characterized developmental mutations have been shown to reside in genes encoding RTKs. One of the best studied examples is the sevenless locus, which encodes a tyrosine kinase receptor that interacts with a cell surface molecule encoded at the boss locus. Mutations at either of these loci prevent the differentiation of R7 photoreceptors and lead to abnormal patterning of the D. melanogaster eye. In mice, mutations in the Dominant White Spotting (W) and Steel (Sl) loci, which encode the kit RTK and its ligand respectively, cause similar defects in the haematopoietic, germ cell and melanocytic lineages (Green, 1989). Finally, RTKs of the trk subfamily...
have recently been shown to function as the high affinity receptors for the nerve growth factor (NGF)-related neurotrophins (Kaplan et al., 1991a,b; Klein et al., 1991a,b; Lamballe et al., 1991; Soppet et al., 1991). Although the phenotypic effects of mutations in members of the trk gene family have yet to be described, the established importance of NGF as a trophic factor for sympathetic neurons and neural-crest-derived sensory neurons of the peripheral nervous system (PNS), and certain cholinergic neurons in the central nervous system (CNS) (Levi-Montalcini, 1987; Martinez et al., 1985), strongly suggests a critical developmental role for the trk RTKs.

The c-ret proto-oncogene is a member of the RTK gene superfamily. It was originally identified as a transforming gene by transfection of T-cell lymphoma DNA into NIH3T3 cells (Takahashi et al., 1985). In vivo mutations of c-ret have also been implicated in tumorigenesis. The PTC (papillary thyroid carcinoma) oncogenes, detected frequently in thyroid papillary carcinomas (25%), result from somatic rearrangements that juxtapose the transmembrane and kinase domains of c-ret to unrelated 5′ sequences (Grieco et al., 1990; Jhiang et al., 1992; Bongarzone et al., 1993; Fabien et al., 1992). Recently, missense germ-line mutations in the extracellular domain of ret have been detected in a high proportion of cases of Multiple Endocrine Neoplasia type 2A (MEN2A; Mulligan et al., 1993), a dominantly inherited cancer syndrome consisting of thyroid medullary carcinoma, pheochromocytoma and parathyroid hyperplasia. These findings strongly suggest that mutations at the c-ret locus are the cause of dominantly inherited human neoplasia such as the MEN2A syndrome.

Despite the established role of c-ret in human tumorigenesis, the function of the normal locus is currently unknown. c-ret mRNA and/or protein have been found in tumours of neuroectodermal origin (neuroblastomas, pheochromocytomas and medullary thyroid carcinomas) and in human neuroblastoma cell lines (Tahira et al., 1991; Ikeda et al., 1990; Santoro et al., 1990; Takahashi et al., 1991). Reports on the expression of c-ret during mammalian embryogenesis are, however, inconclusive and conflicting (Tahira et al., 1988; Santoro et al., 1990). We report here the identification of cDNA clones of the murine homologue of the c-ret proto-oncogene and studies on the expression of c-ret mRNA during mouse embryogenesis. Our results demonstrate that c-ret is expressed predominantly in the developing nervous and excretory systems, and lead us to propose that the ret RTK is normally involved in several aspects of neurogenesis and kidney organogenesis.

MATERIALS AND METHODS

Embryos were derived from crosses between FVB/N inbred mice, or between (FVB/N × C57BL/6)F1, or (C57BL10 × CBA)F1 hybrid mice. The morning of vaginal plug was considered 0.5 days post coitum (dpc). The 8.5 dpc embryos were staged under the dissecting microscope by counting somites.

RNA was isolated from tissue culture cells or embryos according to published procedures (Auffray and Rougeon, 1980). The cDNA library was generated from poly(A)+ RNA isolated from Nb2a cells using the ZAP-cDNA synthesis kit (Stratagene) according to the manufacturer’s recommendations.

In situ hybridization analysis on sections and whole-mount hybridization histochemistry were performed essentially as described (Wilkinson and Green, 1990, Wilkinson, 1992).

RESULTS

Isolation of cDNA clones from the murine c-ret locus

To isolate cDNA clones of the murine homologue of the c-ret proto-oncogene, a cDNA library was constructed with poly(A)+ RNA from the murine neuroblastoma cell line Nb/2a (which, as shown below, expresses high levels of c-ret mRNA), and screened under stringent hybridization conditions with the insert of recombinant plasmid pret2.2, which contains human c-ret mRNA sequences (Takahashi and Cooper, 1987). Several positive clones were identified and a partial restriction enzyme map of two of them, pmcret7 and pmcret10, is shown in Fig. 1A. Partial sequenc-
Expression of c-ret proto-oncogene in mouse

Northern blot analysis of c-ret expression

The tissue distribution of c-ret mRNA in adult animals was examined by northern blot analysis of poly(A)+ RNA extracted from various organs. As shown in Fig. 2A, c-ret expression is tissue specific. Among the tissues analyzed, salivary gland and brain contained the highest levels of c-ret mRNA, while heart and spleen contained lower but detectable levels. No expression was detected in kidney, liver, ovary, thymus, lung or testis.

To determine whether c-ret is expressed during embryogenesis, poly(A)+ RNA was isolated from mouse embryos at different stages of development (8.5-16.5 dpc), and subjected to northern blot analysis. c-ret mRNA was detected in the embryo proper throughout the period examined (Fig. 2B), as well as in the extraembryonic membranes (Fig. 2B, lane Em). We also detected c-ret mRNA in the murine neuroblastoma cell line Nb/2a (Fig. 2C) and, at low levels, in the rat pheochromocytoma cell line PC12 (not shown), a finding consistent with previous reports of c-ret expression in other tumours and cell lines of neuroectodermal origin (Tahira et al., 1991; Ikeda et al., 1990; Santoro et al., 1990; Takahashi et al., 1991).

In all RNA samples analyzed so far, representing three vertebrate species, i.e. human (Tahira et al., 1990), rat (Tahira et al., 1988) and mouse (this report), a similar set of c-ret transcripts has been identified, migrating at 7.0, 6.0, 4.5 and 3.9 kb, with the 4.5 kb transcript being the most abundant. Analysis of human (Tahira et al., 1990), mouse (C. Marcos, M. Sukumaran and V. P.; unpublished data) and chicken (A. Schuchardt, V. P. and F. C., unpublished data) c-ret cDNA clones has demonstrated that the various transcripts are generated from alternative splicing and/or polyadenylation of the primary c-ret transcript. Such events are likely to be under tissue-specific regulation, as suggested by the reversed relative abundance of the 6.0 and 7.0 kb transcripts in mouse brain and salivary gland mRNA preparations (Fig. 2A).

In situ hybridization analysis of c-ret expression during mouse embryogenesis

Expression in cranial neural crest cells and their neurogenic derivatives

The expression of c-ret in tissues (e.g. brain) and cell lines of neuroectodermal origin (neuroblastomas and pheochromocytomas), and its expression in embryos during organogenesis, raised the possibility that the gene plays a role in the development of the vertebrate nervous system. Therefore, we sought to determine the spatial distribution of c-ret transcripts during mammalian embryogenesis. In situ hybridization analysis was performed on mouse embryos between 7.5 and 14.5 days of gestation, a period during which the most critical events of organogenesis are taking place. For these experiments, we used a riboprobe derived from pmcret7 which contains sequences for a small part of the extracellular domain, the transmembrane and intracellular domains as well as the entire 3' untranslated sequence of the 4.5 kb transcript (Fig. 1A and C. Marcos, M. Sukumaran and V. P., unpublished observations). At 7.5 days c-ret mRNA was absent from the embryo proper, but was detected in the trophoblast cells of the extraembryonic membranes (not shown). 24 hours later (8.5-9.0 dpc), as predicted by the northern blot analysis, c-ret transcripts were detected in

Fig. 2. Northern blot analysis of c-ret expression. (A) Samples (5 µg) of poly(A)+ RNA isolated from the designated tissues of 8- to 12-week-old mice were hybridized with antisense pmcret7 riboprobe. The sizes of the four c-ret transcripts are indicated. The apparent signal in the kidney lane was not observed in other experiments. (B) Similar analysis of poly(A)+ RNA samples (5 µg), isolated from embryos at the designated stages of development (8.5-16.5 dpc) and from extraembryonic membranes of 12.5 dpc embryos (lane Em). (C) 1 µg of poly(A)+ RNA from the murine neuroblastoma cell line Nb/2a hybridized with the pmcret7 riboprobe. The set of c-ret transcripts observed in the embryo, the extraembryonic membranes and the Nb/2a cell lines are identical to those observed in RNA preparations from the adult tissues.
specific groups of cells within the embryo. In the head, strong signal was localized to a cohesive group of cells immediately anterior to the otic vesicle and extending from the dorsal edge ventrally towards the forming second branchial arch (Fig. 3A-D). The position of these cells suggests that they are neural crest cells emigrating from rhombomere 4 (r4), and in a domain of surface ectoderm extending posterior to the c-ret-positive neural crest cells. (C,D) Parasagittal section through the head of a 9.25 dpc embryo. Expression of c-ret is restricted to a group of neural crest cells immediately anterior to the otic vesicle (ov), and extending from the dorsal edge of the embryo into the forming second branchial arch. (E,F) Frontal section through the head of a 9.5 dpc embryo. This section is oblique such that dorsal structures such as cranial ganglia (VII) are on the left while ventral structures such as branchial arches (ba 1,2,3,4) are on the right. c-ret-specific signal is localized in the anlage of the facioacoustic ganglion (VII) and over the posterior branchial arches. In the c-ret-positive branchial arch (ba4), expression is observed in the pharyngeal endoderm, the surface ectoderm and the mesenchymal cells lying in between. (G,H) Transverse section through the head of a 9.5 dpc embryo at the posterior edge of the otic vesicle (ov). Signal is observed in the pharyngeal (p) endoderm and the overlying ectoderm.

Fig. 3. Expression of c-ret in the head of 9.0-9.5 day mouse embryos. (A,C,E,G) Photomicrographs of sections under bright-field illumination; (B,D,F,H) photomicrographs of the same series of sections under dark-field illumination. (A,B) Frontal section through the hindbrain of a 9.25 dpc embryo. Signal is detected in the group of neural crest cells emerging from rhombomere 4 (r4), and in a domain of surface ectoderm extending posterior to the c-ret-positive neural crest cells. (C,D) Parasagittal section through the head of a 9.25 dpc embryo. Expression of c-ret is restricted to a group of neural crest cells immediately anterior to the otic vesicle (ov), and extending from the dorsal edge of the embryo into the forming second branchial arch. (E,F) Frontal section through the head of a 9.5 dpc embryo. This section is oblique such that dorsal structures such as cranial ganglia (VII) are on the left while ventral structures such as branchial arches (ba 1,2,3,4) are on the right. c-ret-specific signal is localized in the anlage of the facioacoustic ganglion (VII) and over the posterior branchial arches. In the c-ret-positive branchial arch (ba4), expression is observed in the pharyngeal endoderm, the surface ectoderm and the mesenchymal cells lying in between. (G,H) Transverse section through the head of a 9.5 dpc embryo at the posterior edge of the otic vesicle (ov). Signal is observed in the pharyngeal (p) endoderm and the overlying ectoderm.

mount preparations of 8.75-9.0 day embryos, a stage of embryogenesis at which the migration of all groups of cranial neural crest is well under way. In agreement with the data of Fig. 3A-D, strong c-ret-specific signal was restricted to a single group of cranial neural crest cells emerging from the neuroepithelium of the hindbrain at the level of r4 (Fig. 4A).

Two components of the hindbrain neural crest have been described: the early migrating cells, which populate the ventrally located branchial arches and adopt a mesenchymal fate, and the late emerging cells, which remain closer to the neural tube and adopt a neural fate (Nichols, 1981, 1986). The
detection of c-ret mRNA in both the dorsal and ventral neural crest of r4 (Fig. 3C,D) suggests that during this stage of embryogenesis, both the neurogenic and the mesenchymal components of the r4 crest express c-ret mRNA at comparable levels. Interestingly, during the embryonic period under consideration, the brain neuroepithelium including r4, was devoid of hybridization signal (Fig. 3A,B and data not shown).

In addition to the migrating neural crest cells, c-ret is expressed in the 8.5-9.0 day embryo in a well-defined domain of cranial surface ectoderm and pharyngeal endoderm associated with the posterior branchial arches (Fig. 3E-H). The c-ret-positive surface ectoderm includes the epibranchial placodes, which give rise to the sensory neurons of the inferior ganglia of the IXth and Xth cranial nerve ganglion complexes (Altman and Bayer, 1982). No signal was detected in the more anteriorly located epibranchial placodes.

Given the positionally restricted expression of c-ret in the r4 neural crest and the epibranchial placodes, it was of interest to examine its pattern of expression in cranial structures from later stage embryos. In the head of 9.5 day embryos, and consistent with the restricted expression of ret in r4 neural crest of earlier stage embryos, signal was localized in the condensing facioacoustic ganglion complex (VII-VIIIth) but not in the anlage of other cranial ganglia present anteriorly (Vth) or posteriorly (IXth, Xth) (Fig. 4B). Despite the expression of c-ret in the anlage of the facioacoustic ganglion, no signal was detected in the second branchial arch (Fig. 4B, ba2), which is populated to a large extent by the mesenchymal component of r4 crest. This indicates that by the time the r4 crest has completed its migration into the second branchial arch, the expression of c-ret is down-regulated. In 10.5 day embryos, in addition to the facioacoustic ganglion, the inferior ganglia of the IXth and Xth cranial nerve ganglion complexes are positive for c-ret transcripts, while the trigeminal ganglion (V) is still negative (Fig. 4C). Finally, in 13.5-14.5 day embryos, all cranial ganglia, including the trigeminal ganglion (Fig. 5A,B) and the superior ganglia of the IXth and Xth cranial nerve ganglion complexes (not shown) are positive for c-ret mRNA.

Overall, our data demonstrate that, in the head of the postimplantation mouse embryo, c-ret expression is associated with the development of cranial ganglia. Two distinct phases of c-ret expression can be discerned: an early, segment-specific, phase (8.5-9.5 day embryo), during which c-ret mRNA is restricted to neural crest cells derived from r4 and the anlage of the facioacoustic ganglion, and a later phase (10.5-14.5 days) during which signal appears gradually in all cranial ganglia irrespective of the origin of the contributing neural crest cells along the anteroposterior axis of the brain neuroepithelium. The positionally restricted localization of c-ret mRNA in the cranial neural crest and the surface ectoderm and pharyngeal endoderm of the branchial arches suggests that expression of c-ret is related to the intrinsic mechanisms of segmentation of the vertebrate head.

Expression in the developing sensory and autonomic ganglia of the trunk
c-ret mRNA was also present in the migrating neural crest cells of the trunk, albeit at lower levels compared to the r4 neural crest. In sections from 9.5 day embryos, a diffuse and relatively weak (but reproducible) signal was present dorsally between the neural tube and the surface ectoderm and ventrally in the sclerotome, areas of the somites in which the trunk neural crest cells first migrate and subsequently condense to form the dorsal root ganglia (DRG; not shown). In the DRG of later stage embryos (11.5-13.5 days), the hybridization pattern was modified. Intense punctate signal was observed in the ganglion itself, but was absent from the emerging peripheral spinal nerve (Fig. 5C). This suggests that expression of c-ret in the DRG is restricted to subsets of neurons and is absent from the glial cells of the ganglion or the spinal nerve.

Expression in the developing enteric nervous system
The expression of c-ret in the sensory ganglia raised the possibility that the gene is also expressed in the other major branches of the PNS, i.e. the enteric and the autonomic nervous system. The enteric nervous system (ENS) is composed of the myenteric and the submucosal ganglion complexes of the gut (Furness and Costa, 1987). The majority of the cells in the ganglia of the ENS are derived from the vagal crest originating from the postotic hindbrain (corresponding to somites 1-7) (Yntema and Hammond, 1954; Le Douarin and Teillet, 1973). The presumptive enteric ganglioblasts of the vagal crest migrate first ventrally through the posterior branchial arches into the foregut mesenchyme, and then rostrocaudally inside the gut wall mesenchyme, where they eventually coalesce and form the enteric ganglia along the entire length of the gut (Tucker et al., 1986; Baetge and Gershon, 1989; Gershon et al., 1993; Kapur et al., 1992). c-ret-positive cells have been identified in the migratory pathway of the presumptive enteric neuroroblasts of the vagal crest and in the myenteric ganglia of the gut. In the 9.0-9.5 day mouse embryo, strong expression was detected in mesenchymal cells of the third and fourth branchial arches, between the c-ret-positive pharyngeal endoderm and surface ectoderm (Fig. 3E,F). 12 hours later, a stream of c-ret-positive cells could be seen emerging from the posterior branchial arch mesenchyme migrating towards the foregut (Fig. 6A). At subsequent stages (10.5-11.5 days) c-ret-positive cells were found at increasingly more caudal levels of the embryonic gut (Fig. 6B,C). Finally, in the 13.5-14.5 day embryo, in which the rostrocaudal migration of the ENS precursors has been completed, strong c-ret expression was detected in the myenteric plexus along the entire axis of the gut (Fig. 6D). Although no independent molecular markers were used in these experiments, the overall spatial and temporal pattern of expression of c-ret in the developing gut is consistent with the gene being expressed in the majority of the migrating precursors and postmigratory cells of the ENS.

c-ret mRNA is also expressed in the autonomic ganglia of the PNS. At embryonic day 9.5, c-ret transcripts were detected in groups of cells by the dorsal aorta, where derivatives of the trunk neural crest coalesce to form the sympathetic ganglia anlage (Fig. 6A). Expression is maintained at later stages, e.g. 14.5 days, when sympathetic gangliogenesis has been essentially completed (Fig. 6D). At this stage, no expression of c-ret was observed in the endocrine derivatives of the sympathoadrenal lineage, the adrenal chromaf-
**Fig. 4.** Expression of c-ret in neural crest and cranial ganglia of 8.5-10.5 dpc embryos detected by whole-mount hybridization histochemistry. (A) In the head of the 8.5 dpc embryo, staining is restricted to the neural crest (nc) cells emerging from rhombomere 4 (r4) of the hindbrain and to a well-defined domain of surface ectoderm (se) located ventrocaudally to the c-ret-positive r4 neural crest. (B) In the 9.5 dpc embryo, staining is observed in the anlage of the facioacoustic ganglion (VII) present immediately anterior to the otic vesicle (ov), and in cells of the pharyngeal clefts corresponding to the posterior branchial arches (open triangle). (C) In the 10.5 dpc embryo, c-ret-specific signal is observed in the facioacoustic ganglion (VII) and the inferior complex of the IXth and Xth cranial ganglia. No signal is present at this stage in the trigeminal ganglion, present anteriorly to the VIIth ganglion, and in the superior complex of the IXth and Xth ganglia.

**Fig. 5.** Expression of c-ret mRNA in the trigeminal and the dorsal root ganglia of the 13.5-14.5 dpc embryo. (A,B) Bright-field and dark-field photomicrograph of a frontal section through the head of a 14.5 dpc embryo. The two ovoid symmetrical structures expressing high levels of c-ret mRNA are the trigeminal ganglia (tg). (C) Bright-field photomicrograph of a section through thoracic dorsal root ganglia (drg) of a 13.5 dpc embryo. Signal (dark grains) is observed over a subset of cells of the dorsal root ganglia and is absent from the emerging spinal nerve (sn).
Fig. 6. Expression of *c-ret* in the vagal neural crest and the derivative myenteric plexus of embryonic gut. (A) Frontal section through the branchial arch region of a 9.75 dpc mouse embryo (p, pharynx). Strong signal is observed in single mesenchymal cells migrating from the posterior branchial arches towards the foregut, as well as in the sympathetic ganglia (sg) forming on either side of the dorsal aorta (ao).

(B) At a slightly later stage (10.0 dpc embryo), *c-ret*-positive cells are migrating in a rostrocaudal direction within the mesenchyme of the midgut (mg) wall. (C) Section through the gut of 11.5 dpc embryo. *c-ret*-positive cells have migrated into the distal portion of the developing small intestine (i), and are starting to coalesce to form the enteric ganglia. (D) Section through the gut of a 14.5 dpc embryo. At this stage only the outer myenteric plexus has formed. Signal is observed in a ring-like fashion in the myenteric ganglia of the stomach (s), duodenum (d) and small intestine (i).
atives of the sympathoadrenal lineage, the adrenal chromaffin cells (data not shown).

Expression in the developing central nervous system
During the period of embryogenesis that we examined, c-ret is also expressed in the developing CNS. More specifically, expression was observed in undifferentiated neuroepithelial cells of the neural tube and their postmitotic derivatives. Shown in Fig. 7A,B is a transverse section through the trunk of a 8.75 dpc embryo. Hybridization signal is localized in the neuroepithelial cells of the ventral half of the neural tube. Strong signal was also detected laterally to the epithelial somites, in the nephrotome. At 9.5 days, hybridization was further restricted to the ventrolateral compartment of the spinal cord, where the motor neurons are differentiating (not shown). An identical labelling pattern was maintained during the subsequent periods of embryogenesis that we analyzed (10.5-14.5 dpc, Fig. 7C,D). The ventrally localized domain of c-ret expression extends along the entire anteroposterior axis of the developing spinal cord, and extends anteriorly into groups of motor neurons in the hindbrain (data not shown).

We also detected c-ret mRNA in the neuroretina of the embryonic and postnatal mouse eye. Shown in Fig. 8A,B is in situ hybridization to a section through the eye of a 13.5 day mouse embryo. Hybridization is restricted to the innermost layers of the neuroretina, where the first postmitotic neurons are born. To determine whether all groups of postmitotic cells in the mammalian retina express c-ret, we analyzed sections from eyes of postnatal day 7 mice, a stage by which all classes of neurons and glial cells have been generated (Turner and Cepko, 1987). As shown in Fig. 8C,D, c-ret mRNA is localized to certain layers of the mature mammalian retina, those containing ganglion, amacrine and horizontal cells. No expression was detected in the layers that contain the bulk of the bipolar cells and the photoreceptors.

Expression in the developing excretory system
Expression of c-ret in the nephrotome of 8.5 day embryos prompted us to examine in more detail its expression in the developing excretory system of the mouse. In vertebrate embryos, the excretory system can be subdivided into three sequentially appearing organs, the pronephros, mesonephros and metanephros (Saxen, 1987). The pronephros and mesonephros are transient embryonic structures, which consist of a series of segmentally arranged tubules connected to the nephric (Wolffian) duct. In situ hybridization on sections and hybridization histochemistry on whole-mount preparations of 9.0-10.5 day embryos revealed that c-ret is expressed in the nephric duct of the pronephros (not shown) and mesonephros (Fig. 9A).

The permanent, or metanephric kidney, is formed through a series of reciprocal inductive interactions between the ureteric bud, which emerges from the caudal part of the nephric duct, and the surrounding metanephric mesenchyme (Saxen, 1987). Upon invasion of the metanephric mesenchyme (day 11.5) the ureteric bud begins to branch dichotomously. In embryonic kidneys at this stage, c-ret transcripts were detected only in the epithelial cells of the branching ureteric bud, but not in the surrounding mesenchymal cells (Fig. 9B). Subsequently, the branching tips of the ureteric epithelium induce the surrounding undifferentiated mesenchyme to condense and differentiate into the epithelial renal tubules and glomeruli, while the mesenchyme induces the ureteric bud to grow and branch, forming the collecting ducts. At more advanced stages of organogenesis, these inductive interactions take place only in a narrow region around the outer edge of the kidney, the nephrogenic zone (Saxen, 1987). In sections of embryonic
Expression of c-ret proto-oncogene in mouse

Fig. 8, c-ret expression in the mouse eye. (A,B) Bright-field and dark-field photomicrograph of a section through the eye of a 13.5 dpc embryo. Signal is observed in the innermost layer of the neuroretina (r), where the first postmitotic neurons are born (pe, pigmented epithelium). (C,D) Bright-field and dark-field photomicrograph of a section through the retina of an albino postnatal day 7 mouse. Signal is observed in the ganglion (g), the amacrine (a) and the horizontal (h) cell layers. No signal is observed over the bipolar (b) or the photoreceptor (ph) cell layers.

kidneys at these stages (13.5-17.5 dpc), c-ret mRNA was detected only in the nephrogenic zone (Fig. 9C). At high magnification, it could be seen that the hybridization signal was restricted to the growing tips of the collecting ducts, but was absent from all of the more centrally located structures, including the condensing renal vesicles and nephrons (of mesenchymal origin), and the subcortical segments of the collecting ducts (Fig. 9D).

DISCUSSION

We have cloned the murine homologue of the c-ret proto-oncogene and studied its spatial and temporal pattern of expression during mouse embryogenesis, using northern blot analysis, in situ hybridization to sections and hybridization histochemistry on whole-mount preparations. Our studies demonstrate that, in the postimplantation embryo, c-ret is expressed in a complex and dynamic pattern, predominantly in subsets of cells of the PNS, the CNS and the excretory system. These findings suggest that c-ret, in addition to its role in cellular transformation and tumour formation, plays an important role in normal mammalian embryogenesis.

In the developing nervous system, c-ret is expressed in undifferentiated neuroectodermal precursors (subsets of migrating neural crest cells and neuroepithelial cells of the ventral neural tube) as well as in their differentiated postmitotic derivatives. The expression of c-ret in cranial neural crest and its derivatives is particularly intriguing. During the migratory phase of the neural crest and the early stages of cranial gangliogenesis (8.5-9.5 days), c-ret mRNA is restricted to the r4 crest and the anlage of the facioacoustic ganglion complex. Subsequently, over a period of three days (day 10.5-13.5), the apparent segment-specific expression of c-ret dissipates and its mRNA is detected in groups of cells in all cranial ganglia irrespective of the origin of the contributing neural crest. In the posterior head, neural crest emerges from the hindbrain as distinct streams of cells (Serbedzija et al., 1992; Lumsden et al., 1991). It has been suggested that the patterning information for the mesenchymal and neuronal components of each branchial arch is contained within the neural crest that populates that arch and that this information has been imprinted on the neural crest by its origin along the anteroposterior axis of the brain neuroepithelium (Noden, 1983, 1988). It has been postulated that the combinatorial expression of members of the Hox gene clusters provides at least part of the patterning information for the specification of individual rhombomeres of the hindbrain and the neural crest cells derived from them (Hunt et al., 1991). How might such a ‘Hox code’ in the cranial neural crest translate into different neuronal and mesenchymal structures in the head of the adult animal? The differential activation of downstream target genes could provide the means of translating such positional information into diverse developmental programs. The restricted expression of the c-ret gene in the r4 neural crest of the 8.5-9.5 day embryo provides additional evidence that the various groups of cranial neural crest cells are molecularly distinct. Moreover, it raises the possibility that the ret receptor is part of the molecular cascade that leads to the establishment of the unique properties of the r4 crest derivatives.

In the PNS, c-ret is expressed in the developing autonomic nervous system, the ENS and the sensory ganglia of the head and the trunk, suggesting that the ret signal transduction pathway is involved in the organogenesis of all major branches of the PNS. Phenotypic analysis of the sympathoadrenal progenitors and the progenitors of the ENS in rodents has established that these two lineages share a number of independent molecular markers (Baetge et al., 1990; Baetge and Gershon, 1989; Carnahan et al., 1991; Johnson et al., 1990; Lo et al., 1991). This led to the suggestion that sympathoadrenal and enteric lineages are derived from progenitors that are either identical or closely related (Carnahan et al., 1991), and that the particular phenotypes eventually acquired by these progenitors are determined by the specific microenvironment at their final destination. The concomitant expression of c-ret in the anlage of the sympathetic ganglia and the precursors of the ENS.
in the mouse embryo further supports their close relationship. Moreover, it raises the possibility that c-ret plays a role in the proliferation, migration, differentiation or survival of the cells of these lineages. The expression of c-ret mRNA in the migratory ENS progenitors is particularly interesting, since, despite the established importance of signals encountered along the migratory pathway of the vagal crest and in the microenvironment of the embryonic bowel for the differentiation of the enteric neurons (Gershon et al., 1993), very little is known about the molecular nature of these signals. Our data suggest that the ret receptor might play a critical role in the cellular and molecular processes that lead to the organogenesis of the ENS, i.e. migration of the vagal crest, commitment to the neuroblast or glioblast fate, cell proliferation and survival of postmitotic cells in the enteric ganglia. This hypothesis is strongly supported by our recent finding that mouse embryos homozygous for a loss-of-function mutation of the c-ret gene, generated by homologous recombination in embryonic stem cells, fail to develop enteric ganglia (Schuchardt et al., unpublished data).

The apparent localization of c-ret mRNA in subsets of neurons of the sensory ganglia (cranial nerve ganglia and DRG) is consistent with previous studies indicating that distinct gene expression programmes exist in the various functional classes of the sensory neurons. Using monoclonal antibodies, Dodd and colleagues were able to identify subsets of primary sensory neurons in rat DRG expressing unique carbohydrate differentiation antigens (Dodd et al., 1984; Dodd and Jessell, 1985). More recently, in situ hybridization analysis of the pattern of expression of members of the trk RTK subfamily has revealed that, similarly to the ret receptor, individual members of the trk subfamily are expressed in subclasses of DRG cells (Carroll et al., 1992). Furthermore, immune deprivation of NGF in utero resulted in selective ablation of trk-expressing cells of the DRG (Carroll et al., 1992; Ruit et al., 1992). Our data suggest that, as is the case for the trk subfamily of RTKs, the ret receptor

Fig. 9. c-ret expression in the excretory system of the mouse embryo. (A) Whole-mount hybridization histochemistry of the posterior trunk region of a 9.5 dpc embryo. c-ret mRNA is expressed throughout the nephric duct (nd), with highest levels observed in its caudal part (open triangles). (B) In situ hybridization of a section through the metanephric region of an 11.5dpc embryo. High levels of c-ret mRNA is detected in the ureteric bud and its branches (u), while no signal is detected in the surrounding mesenchyme (m). (C) A section through the kidney of a 17.5 dpc embryo. Signal is restricted in the outer zone of the kidney where induction of new nephrons is still taking place. (D) Bright-field photograph of a high magnification view of part of kidney shown in C. The c-ret-positive collecting ducts (arrows) are derivatives of the ureteric bud.
plays a role in the differentiation and survival of a specific class of primary sensory neurons. The identity of this class and the particular sensory modality that it conveys are currently unknown.

In the CNS, expression has been detected predominantly in the motor neuron lineages of the spinal cord and the hindbrain, and in subsets of cells of the neuretina. Lineage analysis of the cells of the vertebrate retina has shown that all cell types of the mature retina are derived from an early common progenitor (Turner and Cepko, 1987; Turner et al., 1990; Wetts et al., 1989), and strongly suggests that cellular interactions in the developing vertebrate retina play a critical role in the establishment of the various cellular phenotypes. Although certain growth factors have been identified as playing an important role in the differentiation of retinal cells in vitro (Lillien and Cepko, 1992; Guillemot and Cepko, 1992), the molecules that mediate such cellular interactions in vivo have yet to be identified. Our expression data, combined with the nature of the ret protein, suggest that it may play a role in the cell-cell interactions that determine the cellular phenotypes of particular classes of neurons in the mammalian retina.

The molecular mechanisms underlying the potential role of c-ret in the development of the mammalian nervous system are currently unknown. However, the structural similarity of ret to other members of the RTK family and its pattern of expression in the developing PNS and CNS, suggest that it functions as the receptor to a (potentially novel) neurotrophic factor(s) required for the migration, and the particular sensory modality that it conveys are.

Molecular characterization of c-ret cDNAs from patients with MEN2A syndrome suggests strongly that gain-of-function mutations in the c-ret proto-oncogene are responsible for this inherited cancer syndrome (Mulligan et al., 1993). Although the documented expression of c-ret in cell lines of neural crest origin is consistent with the neural crest origin of medullary thyroid carcinoma (C-cells of the thyroid) and pheochromocytoma (sympathoadrenal progenitor), the occurrence of parathyroid hyperplasia in patients with MEN2A syndrome has been puzzling, as parathyroid cells are derived embryologically from the endoderm of the fourth pharyngeal pouch (Balinsky, 1970). This led to the suggestion that the tumours of the MEN2A syndrome may result from defects in closely linked genes that independently control neural crest or endocrine tissue development (Lairmore et al., 1991). Our expression studies further support the suggestion that the entire MEN2A syndrome is caused by mutations in the c-ret proto-oncogene by providing evidence that all lineages affected in this syndrome are likely to express the c-ret proto-oncogene. The precursors of the C-cells, originating from the posterior hindbrain, are transiently localized, along with the precursors to the ENS, in the mesenchyme of the posterior branchial arches (Le Douarin, 1982), an area where c-ret is strongly expressed (Fig. 3E-H). This, along with the high levels of c-ret expression in C-cell derived medullary thyroid carcinomas (Santoro et al., 1990) and our preliminary experiments, which show low levels of expression in the C-cells of adult mouse thyroid (P. Durbec, B. M. and V. P., unpublished data), suggests that the C-cell lineage expresses c-ret mRNA from the early embryonic stages. Despite lack of expression in the adrenal medulla, it is likely that the chromaffin cell precursors are also expressing c-ret mRNA since they originate from the ret-positive cells of the sympathetic ganglia condensations (Fig. 6A; (Pankratz, 1931)). Finally, the parathyroid cell precursors are derived from the c-ret-positive endoderm of the posterior branchial arches (Fig. 3E,F). Overall, the common expression of c-ret reveals a developmental link between the cell types affected in the MEN2A syndrome and provides further support for the hypothesis that germ-line mutations in c-ret are responsible for all the manifestations of the MEN2A syndrome.

In addition to its postulated role in the developing nervous system, the expression pattern of the c-ret gene suggests that the ret receptor could function during the development of the excretory system. c-ret mRNA was first detected at day 8.5 in the nephrotome, a region of lateral mesoderm from which the embryonic (pronephric and mesonephric) and permanent (metanephric) kidneys are formed and continued to be detected in the nephric (Wolffian) ducts during the development of the pronephric and mesonephric kidneys (day 9.5-10.5). The expression pattern of c-ret at this stage suggests that the ret receptor might play a role in the formation of the pronephros and mesonephros.

The development of the mammalian metanephric kidney has been extensively studied as a model system for the role of inductive tissue interactions during organogenesis (Saxen, 1987). Formation of the kidney is initiated on day 11 of mouse embryogenesis, when the ureteric bud emerges near the caudal end of the nephric duct and grows dorsally, eventually contacting the metanephric blastema, a specific condensate of mesenchymal cells. At this point, the ureteric bud begins to grow and branch repeatedly, dependent upon specific induction by the metanephric mesenchymal cells (Grostein, 1953, 1955; Erickson, 1968), and eventually giving rise to the entire renal collecting system (calyces, papillae and collecting ducts). At the same time, the tips of the branching ureteric bud induce the surrounding mesenchymal cells to condense into epithelial renal vesicles, which eventually differentiate to form the various segments of the nephron, including the proximal and distal tubules and glomeruli (Grostein, 1955, 1956; Saxen, 1987). At a stage when the ureteric bud has first branched within the metanephric mesenchyme, we observed that c-ret was strongly expressed in the ureteric bud epithelium, but was not expressed at detectable levels in the surrounding undifferentiated mesenchyme. At later stages of renal organogenesis, growth and branching of the ureteric derivatives, as well as the induction of new nephrons, is limited to a narrow ‘nephrogenic’ zone at the perimeter of the kidney (Saxen, 1987). At these stages (day 13.5-17.5), c-ret mRNA was observed only in the tips of the growing collecting ducts in the outer nephrogenic zone, and not in the previously formed and more centrally located collecting ducts. Like the undifferentiated metanephric mesenchyme, both the newly condensed renal vesicles and the more mature mesenchymal derivatives continued to be negative for c-ret expression.

Based on this restricted pattern of expression, it appears likely that the ret receptor could play a role in the growth.
and development of the ureteric bud and its derivative structures in the kidney. More specifically, ret might serve as the receptor for an inductive factor, produced by the metanephric mesenchymal cells, which stimulates the growth and/or branching of the ureteric bud epithelium. Support for the hypothesis that the c-ret gene serves an important function in renal organogenesis has recently been obtained by the production of mice carrying a targeted, loss-of-function mutation of the c-ret gene (Schuchardt et al., unpublished data). The homozygous mutant mice, in addition to the ENS defects noted above, never develop normal kidneys, and display a spectrum of excretory development ranging from tiny, hypoplastic kidney rudiments, to blind-ending ureters with no renal tissue, to the complete absence of ureters and kidneys.

Analysis of cDNA clones derived from the human (Tahira et al., 1990), the murine (C. Marcos, M. Sukumaran and V. P., unpublished observations) and the chicken (A. Schuchardt, V. P. and F. C.) c-ret locus indicates that its transcripts are capable of encoding at least two distinct receptors differing at their intracellular carboxy-terminal tails. Since the probe that we used for our in situ hybridization analysis includes cRNA sequences common to all transcripts, we are currently unable to distinguish the two potential murine ret isoforms. However, it is possible that activation of the ret receptor by its cognate ligand could lead to distinct cellular responses depending on the predominant ret isoform present on the cell surface. It would therefore be of interest to examine the differential distribution of the various c-ret transcripts and their protein products during mouse embryogenesis, using transcript-specific probes and isoform-specific antibodies.

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Expression of c-ret proto-oncogene in mouse


