

The immediate upstream sequence of the mouse *Ret* gene controls tissue-specific expression in transgenic mice

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Abstract. The *RET* gene is tightly regulated at the transcriptional level during embryo development, however *in vitro* experiments in cultured cells have failed to clarify the molecular mechanism of cell-type specificity of *RET* promoter activity. Therefore, we have generated transgenic mice in which the *LacZ* reporter gene is controlled by murine *Ret* promoter sequences to clarify in an *in vivo* model how this transcriptional regulation is achieved. We describe here the results of reporter gene expression in mice in which the transgene contained 380- and 1962-bp sequence upstream of the ATG start codon, derived from the mouse *Ret* promoter region, fused to the β -galactosidase coding sequence. Transgenic mice showed well-defined patterns of β -galactosidase staining obtained with both transgenes, suggesting that they were able *per se* to direct the reporter gene expression in specific districts, such as cranial ganglia, dorsal root ganglia, the heart and the kidney, partially recapitulating the profile of the endogenous *Ret* gene. In particular, proper expression in the developing excretory system seemed quite significant when considering that the appropriate regulation was obtained with a very short, 380 bp, fragment of *Ret* 5' flanking sequence.

Introduction

Cell proliferation and differentiation are crucial processes during embryo development and are controlled by a complex network of regulated gene expression. This can occur at various levels starting from chromatin remodelling, through the modulation of the transcriptional process, to a variety of post-transcriptional modifications (1).

The regulation of gene expression at the transcriptional level is one of the most important mechanisms of control in vertebrate embryos, and mainly depends on the specific interaction between transcription factors and their cognate recognition sequences of promoter and enhancer elements.

The *RET* proto-oncogene encodes a transmembrane receptor with tyrosine kinase (RTK) activity. So far, four specific ligands, glial cell line derived neurotrophic factor (GDNF), artemin (ARTN), neurturin (NRTN) and persephin (PSPN) with the corresponding phosphatidylinositol (GPI)-linked ligand-binding subunits have been described as components of a multimeric complex in which RET is the pivotal transducer element (2,3,4). The concerted expression of these genes during early stages of embryogenesis drives the migration and proliferation/differentiation of specific cell lineages deriving from neural crests leading to the development of enteric ganglia, thyroid C-cells, adrenal medulla, and the excretory system (5).

Accordingly, *Ret* knock-out mice (*Ret*^{-/-}) have shown the absence of enteric ganglia throughout the digestive tract and renal agenesis or dysgenesis, and the lack of superior cervical ganglion (6,7). Moreover, targeted mutagenesis of *Gdnf* and *Gdnf Receptor a1 (GFRa1)* also leads to defective ureteric bud morphogenesis, thus causing kidney agenesis or dysgenesis (8,9).

In humans, *RET* is involved in the genetic aetiology of several neurocristopathies, such as Hirschsprung disease, multiple endocrine neoplasia type 2A and 2B, and both sporadic and familial isolated medullary thyroid carcinoma (10,11).

The temporal and spatial pattern of expression of this gene during embryogenesis suggests that it is tightly regulated at the transcriptional level (5,12) and so far several studies have been carried out to identify the molecular determinants of such an expression specificity. Comparison of the human and mouse promoter sequences shows a limited degree of identity at the nucleotide level. However, there is good conservation in terms of structure: absence of canonical consensus TATA or CAAT boxes, a high GC content with several GC boxes and putative Sp1 binding sites (13), which suggests a conserved mechanism of transcriptional control.

In vitro experiments showed that a restricted region of the human *RET* promoter is sufficient to drive reporter gene

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expression and that the SP1 transcription factor is the major activator of promoter activity (14,15,16). It was also demonstrated that the *RET* promoter is responsive to the Pax 3 and Sox 10 transcription factors (17), and that its expression is influenced by epigenetic processes (18).

However, experiments in cultured cells failed to reproduce cell-type specificity of the *RET* promoter activity, consistent with the regulated spatio-temporal profile of the endogenous gene in tissues. This observation has emphasized the need to specifically generate animal models to clarify how the regulation of the *RET* gene transcription is achieved at the molecular level.

Lang *et al.* (2000) reported the generation of transient transgenic mice carrying a 5.1-kb fragment from the human *RET* promoter driving the β -galactosidase (β -gal) coding sequence as reporter gene. They found β -gal staining in the stomach and in the gut, where the endogenous gene is normally expressed. However, the observed staining pattern did not entirely encompass the endogenous profile, suggesting the lack of specific regulatory elements in the fragment used, and/or the existence of species-specific differences in the regulatory mechanisms (17).

Afterwards, Sukumaran *et al.* described the analysis of transgenic mice carrying the *LacZ* gene under the control of four genomic fragments from the mouse *Ret* promoter (19). In that study, the main construct consisted of a sequence spanning 12 kb upstream of the ATG start codon from which three other constructs were derived by progressive deletion at the 5' end, thus obtaining fragments of 6 kb, 2 kb and 0.2 kb, respectively. The authors described a modular organisation of the cis-acting elements controlling the *Ret* gene transcription: a distal region contained in the 12-kb construct, reported as specific for gene expression in the primitive streak, in the lateral mesoderm and facial ganglia; a proximal region included in the 6 kb-fragment directing *Ret* expression in the developing somatosensory system (19). Again, the pattern of the reporter gene did not recapitulate entirely the endogenous profile of *Ret*, as no staining was found in motorneurons, the enteric nervous system, autonomic ganglia and the developing urogenital system (19). Moreover, no β -gal expression was detected in transgenic embryos carrying the two smallest fragments of 2 and 0.2 kb.

Our recent work on the mouse *Ret* gene regulation has shown that a fragment as small as 380 bp upstream of the ATG start codon was able to control reporter gene expression in transfected cell lines (20). To verify whether this promoter sequence was also able to function in an *in vivo* context, we generated transgenic mice for two genomic fragments, containing 380- and 1962-bp sequence upstream of the ATG start codon, derived from the mouse *Ret* promoter region, fused to the β -gal coding sequence as reporter gene.

We describe here the results of reporter gene expression in these transgenic mice which showed well-defined patterns of β -gal staining obtained with both transgenes, suggesting that they were able *per se* to direct the reporter gene expression in specific districts, partially recapitulating the profile of the endogenous *Ret* gene. In particular, proper expression of the reporter gene was observed in the developing excretory system, consistent with what is widely reported for the endogenous *Ret*. This is significant when considering that

the appropriate regulation was obtained with a very short, 380 bp, fragment of *Ret* 5' flanking sequence.

Materials and Methods

Cloning the murine *Ret* promoter region. The 380-bp (BPr) and 1962-bp (Pr1.9) fragments from the mouse *Ret* promoter were obtained by PCR using specific sense and antisense primers: BPr-F GGTACCACACGCACCCAGCTCCGACC and BPr-R AGATCTAGGAGCAGGCGGGTCTCG; Pr1.9-F GTCCCGCCCATGCTATCTGG and Pr1.9-R AGGAGCAGGCGGGTCTCG. For the BPr fragment, forward primer carried the *KpnI* restriction site, reverse primer carried the *BglII* restriction site, suitable for direct subcloning in the same restriction sites of the p β Gal basic vector (Clontech). The PCR product corresponding to the Pr1.9 fragment was subcloned first in pCR2.1 TOPO vector (Invitrogen), then excised and subcloned into the *KpnI/XhoI* restriction sites of the p β Gal basic vector. Both constructs were verified by restriction analysis and sequencing.

Transgene construction and generation of transgenic mice. Transgenes were prepared from the two (BPr and Pr1.9) constructs by double digestion with *KpnI* and *SalI* restriction enzymes to remove vector sequences. The transgenes to be microinjected consisted of the sequences under analysis (380 and 1962 bp), *LacZ* coding sequence and 3' poly-A structures which stabilize the construct (Fig. 1). Transgenes were gel purified and prepared for microinjection.

FVB strain females were hormone-stimulated to induce superovulation and then mated with stud mice. Fertilized eggs were recovered by oviduct dissection of the plugged donor females and conserved in a microdrop culture until the microinjection. Transgene DNA was microinjected into these eggs by targeting the large male pronucleus. Manipulated embryos were transferred into the oviduct of recipient females (CD1 strain) plugged with vasectomized males.

Pregnancies were either continued until the delivery (18-20 days after the egg reimplantation in recipient females) to obtain founders for a transgenic line, or terminated at specific stages of embryonic development to allow the transient analysis of transgenic mice.

All procedures were carried out according to the principles for the use and care of animals, in order to prevent pain or unnecessary suffering. Animals were properly controlled for their well being and health, were fed *ad libitum*, and when needed sacrificed by CO₂ administration.

Identification and selection of transgenic mice was achieved by PCR and Southern blot analysis on genomic DNA extracted from mice tails, in the case of founders, and from placenta, for embryos.

PCR amplification was performed with a sense oligonucleotide specific for the mouse *Ret* promoter (TR1F: 5'-CTTCATCAGTACCGCAACC-3') and an antisense primer mapping on the *LacZ* coding sequence (TR1R: 5'-GCTCAGGTCAAATTCAGACGG-3').

β -galactosidase staining. Embryos harvested at the stage of interest were fixed for 30 min in 2% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4, and 2 mM MgCl₂, and washed

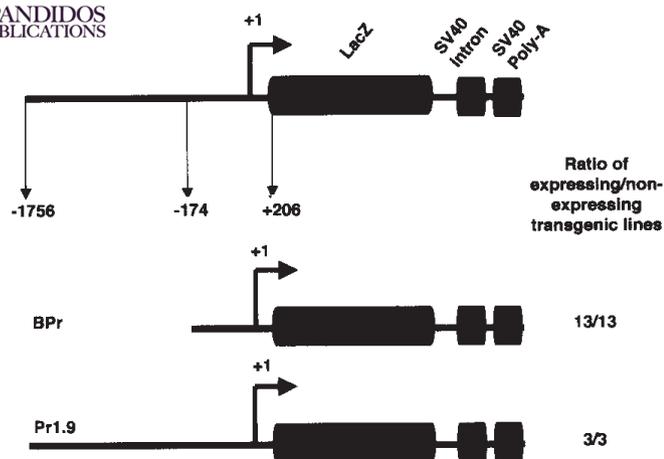


Figure 1 Schematic representation of the constructs used to generate transgenic mice (BPr and Pr1.9). *LacZ*, coding sequence for the β -galactosidase reporter enzyme with the SV40 intron, UTR and poly-A sequences.

three times for 30 min in a washing solution containing 0.1 M phosphate buffer pH 7.4, 2 mM $MgCl_2$, 0.1% sodium deoxycholate and 0.2% Nonidet P-40. β -gal staining of the whole embryos was developed by an overnight incubation at 37°C in the washing solution with 1.0 mg/ml X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside, Roche), 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide. The embryos were then washed extensively, dehydrated in an increasing ethanol scale and then made diaphanous in cedar wood oil (Aldrich), before being photographed with a Nikon digital camera under a Nikon SMZ800 stereomicroscope.

Stage matched wild-type embryos were also collected and processed as described to be used as controls. Positive embryos for β -galactosidase staining were then cleared in xylene and embedded in paraffin to obtain sagittal and transverse sections (30- μ m), subsequently dewaxed and mounted with a coverslip to be analysed and photographed.

Histology and immunohistochemistry. For sections, wild-type embryos were fixed and dehydrated as described above, cleared in xylene and then embedded in paraffin. Sagittal (5-10- μ m) cuts of embryos, obtained with a microtome, were dewaxed, rehydrated and boiled in citrate buffer (10 mM, pH 6.0) to be prepared for immunohistochemistry experiments with an anti-Ret specific antibody to evaluate the expression of the endogenous gene. Endogenous peroxidase was blocked by 3% H_2O_2 in phosphate-buffered saline (PBS) for 5 min at room temperature. After washing in PBS non-specific binding was blocked by 10% fetal calf serum (FCS; GibcoBRL) in PBS for 1 h at room temperature.

Sections were incubated overnight at 4°C with the anti-Ret antibody (Ret C-19: sc-167; rabbit polyclonal IgG, 200 mg/ml; Santa Cruz) diluted 1:200 in 10% FCS/PBS.

After washing, sections were incubated with the secondary antibody (goat anti-rabbit immunoglobulins, peroxidase conjugated; EnVision⁺, Dako) for 1 h at room temperature. AEC (amino-ethyl-carbazole, Sigma) was used as chromogenic substrate for the enzymatic reaction (AEC, 30% H_2O_2 ,

sodium acetate 0.1 M, pH 5.2). Slides were finally mounted with coverslips.

Results and discussion

Generation of transgenic mice. To study the mechanisms regulating the cell-type specific expression of the *Ret* gene, we followed an *in vivo* approach based on the generation of transgenic mice carrying the *LacZ* reporter gene driven by two different genomic fragments of 380 bp (Basal Promoter, BPr), and 1962 bp (Pr1.9), immediately upstream of the ATG start codon of the mouse *Ret* gene (Fig. 1), which we had already utilized in *in vitro* experiments. Our results had shown that the 380 bp sequence was able to control *Ret* gene expression in cultured cell lines (20). In P19 cells induced to neural differentiation, we found that *Ret* gene expression was also induced, and this correlated with a differential binding of SP1/SP3 proteins to their recognition sites in this sequence. Since these data suggested that this small fragment might play a role in regulating tissue specificity of *Ret* expression, we decided to verify whether the very short regulatory sequence contained in the BPr transgene and the longer one contained in Pr1.9 were able to function in an *in vivo* context.

We obtained thirteen transgenic mice (two stable lines, A and B, and eleven transient transgenic mice) carrying the BPr transgene, and three stable lines (α , β , and δ) for the Pr1.9, as assessed by PCR with oligonucleotides specifically designed to detect the presence of these transgenes. Embryos, either from stable lines or from transient experiments, were harvested at defined developmental stages and the pattern of β -gal staining was evaluated in whole embryos or in histological samples derived from such embryos.

All the transgenic mice obtained (13/13 and 3/3 for BPr and Pr1.9 transgene, respectively) developed clearly detectable and defined β -gal staining as exemplified in Fig. 2. The staining pattern displayed in the whole embryos demonstrated that both types of regulatory sequences were active in promoting gene expression and that reporter gene expression was found in districts known to be expressing the endogenous *Ret* gene in mice of corresponding embryonic stage.

This is in contrast with a previous study in which reporter gene expression was observed in transgenic mice carrying a *Ret* 5' flanking region of 12 and 6 kb respectively, fused to *LacZ*, but not in deletion construct of 2 and 0.2 kb (19).

Cranial ganglia. Previous reports have shown that most cranial ganglia, including trigeminal and superior ganglia, are positive for *Ret* mRNA expression, in wt mouse embryos at the stage of 13.5-14.5 dpc (5).

Our 13.5-14.5 dpc transgenic embryos displayed expression of *LacZ* within glossopharyngeal (IX) ganglia in both BPr (6/13) and Pr1.9 (3/3) (Fig. 3A). Well detectable reporter gene expression was also found in the sensory epithelium of the cochlea (Fig. 3B) of all transgenic mice for both promoter fragments. This is in accordance with previous studies with rats and pigs, showing that the endogenous *Ret* is expressed in the epithelium of semicircular canals and is absent in the vestibul-ocochlear ganglion (21,22,23).

Defined β -gal staining was detected in a subset of cells in the innervations of the tongue (3/13), the mandible branches of

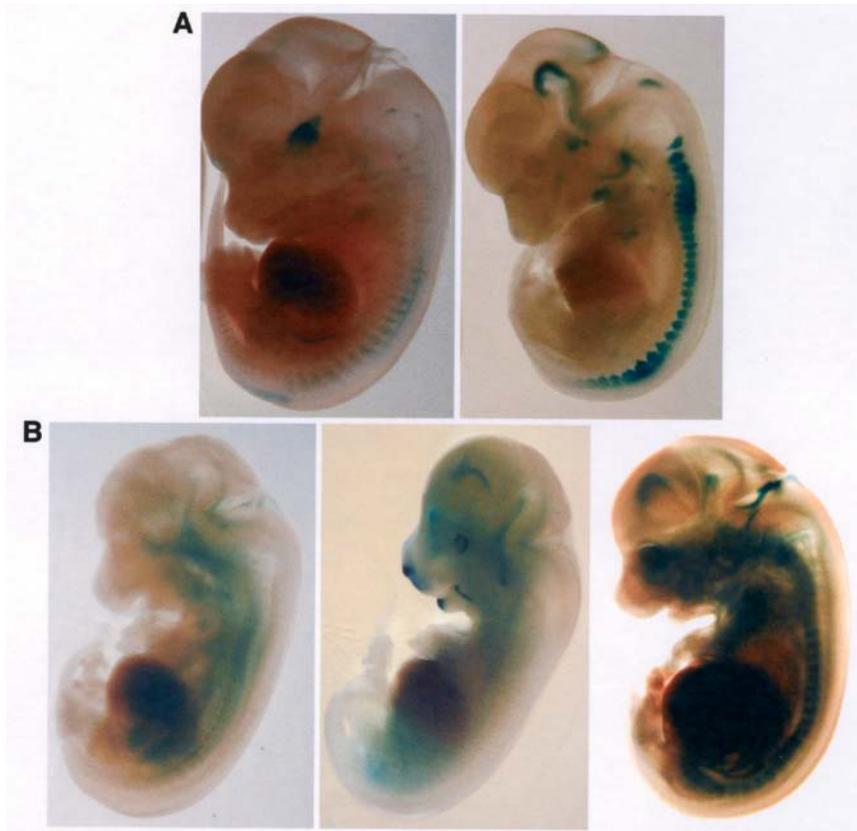


Figure 2. Tissue-specific expression of the *LacZ* reporter gene driven by the two genomic fragments from the mouse *Ret* promoter flanking region. β -galactosidase expression pattern in whole embryos harvested at E13.5. Stable lines A and B (A), were generated with the BPr construct, whereas stable lines α , β and δ (B) were obtained with the Pr1.9 transgene.

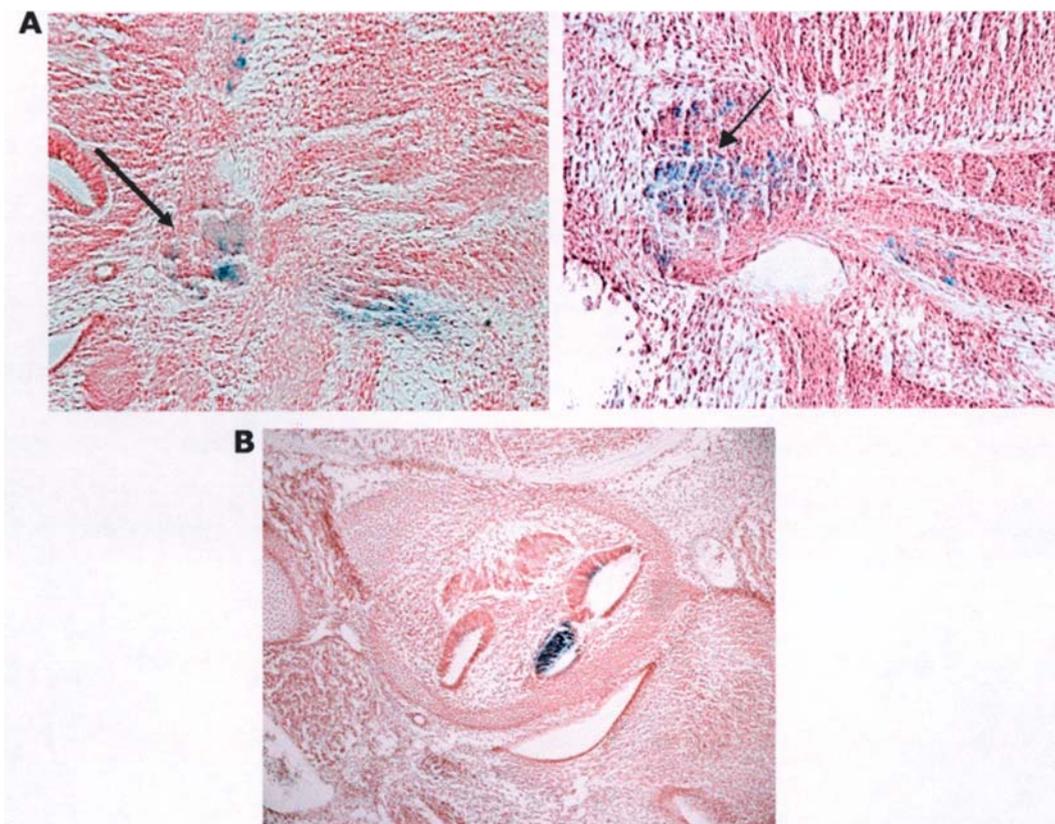


Figure 3. Histological sections of E13.5 transgenic mice showing the representative staining pattern of the *LacZ* reporter gene expression induced in the glossopharyngeal ganglion (A, arrows) and in the sensory epithelium of the cochlea (B) of transgenic mice for both promoter fragments.

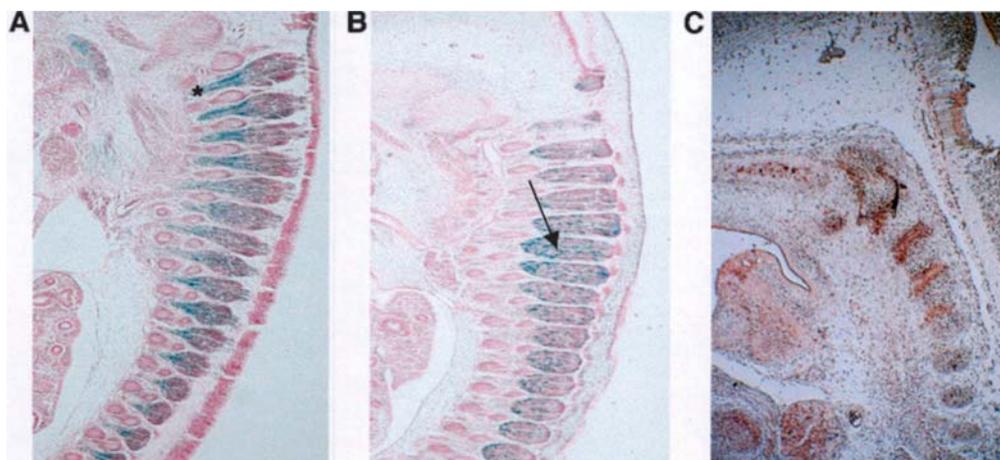


Figure 4. *LacZ* reporter gene expression in developing dorsal root ganglia. Parasagittal section of E13.5 transgenic embryos for the BPr construct. In the BPr transgenic line β -galactosidase staining is detectable in a subset of cells within the ganglia and in emerging nerve roots (A); in the BPr line B, the expression of the reporter gene is found only confined to a subset of neurons (B, arrow) and it is completely overlapping with the expression of the endogenous *ret* gene (C), as assessed by immunohistochemistry with specific anti-*Ret* antibodies.

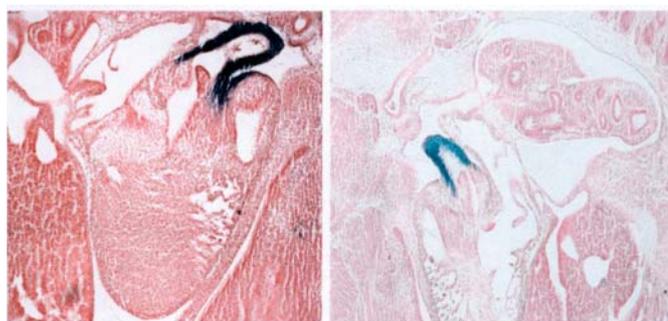


Figure 5. Histological sections of E13.5 embryos showing that the BPr transgene can induce β -galactosidase expression in the outflow tract of the developing heart.

the vagus (X) ganglia (4/13), and in the SCG (superior cranial ganglia) in 5/13 mice carrying the smallest BPr construct.

Moreover, the BPr transgenic mice also showed the reporter gene expression in the hair follicles of the vibrissae innervated by the maxillary branch of the trigeminal nerve (3/13). β -gal expression in the nucleus of the trigeminal nerve (V) was observed only in 1/13 BPr transgenic mice.

Dorsal root ganglia. In the two BPr stable lines, A and B, we found β -gal staining in a subset of cells within all the dorsal root ganglia (DRG). In BPr line A, the reporter gene was detected both in a subset of cells within the ganglia and in the emerging nerve roots (Fig. 4A). In BPr line B, *LacZ* staining was observed first within a few cells of DRG at the E10.5 dpc stage, and later, from 11.5 to 14.5, this became an intense punctate signal in DRG of the entire embryo axis and was still absent from the emerging peripheral spinal nerves.

The expression of endogenous *Ret* in DRG is well documented in the literature and, in our case, the β -gal staining observed in the transgenic line B corresponded to the immunohistochemical staining observed in a control mouse at the same embryonic stage (Fig. 4B and C). However, this expression pattern was not observed in the other BPr transgenic

mice nor in DRG from the Pr1.9 transgenic mice. This variable expression can be explained by strong dependence of both transgenes on position effect due to the random integration of constructs in the mouse genome.

As previously reported, *Ret* expression is found in cells derived from trunk neural crest. These cells also migrate ventrally in the sclerotome where they condense to form DRG. Later on, the expression shows a defined punctate pattern suggesting that it is present in a restricted subtype of neurons and is absent in emerging roots (5). In late embryonic development and the early postnatal period *Ret* expression is confined to the large diameter, isolectin B4 (IB4⁺) binding DRG neurons, representing a subset of nociceptive sensory neurons. Later on in the postnatal life also the small and intermediate diameter IB4⁺ neurons become *Ret* positive (19,24,25).

Several works have demonstrated that specific DRG neuron populations are sensitive to the trophic action of GDNF, on which they depend for their phenotypic maintenance and survival (24). However, the role of the GDNF/GFR α /*Ret* signalling in sensory neurons seems to be different in embryos compared to the postnatal life. During embryogenesis the activation of the *Ret* mediated pathway leads to the down-regulation of several cytoskeletal and cell adhesion related genes, with a suppressive effect on neurite growth and/or branching (26). Therefore, it has been hypothesized that it may be involved in terminal innervation (sensory terminal innervation) (26). During late embryogenesis and postnatal development, GDNF/GFR α /*Ret* signalling exerts a potent anti-apoptotic effect on DRG neurons (27), supporting their survival *in vitro* and in dissected intact ganglia, and their rescue after axon injury (24).

Heart. Outside the nervous system, we found specific β -gal expression in the heart in 6/13 BPr transgenic mice. The staining was confined to a specific region around the cardiac outflow tract (Fig. 5). This finding is in accordance with several lines of evidence showing that *Ret* is involved in the normal development of the heart, at the level of both

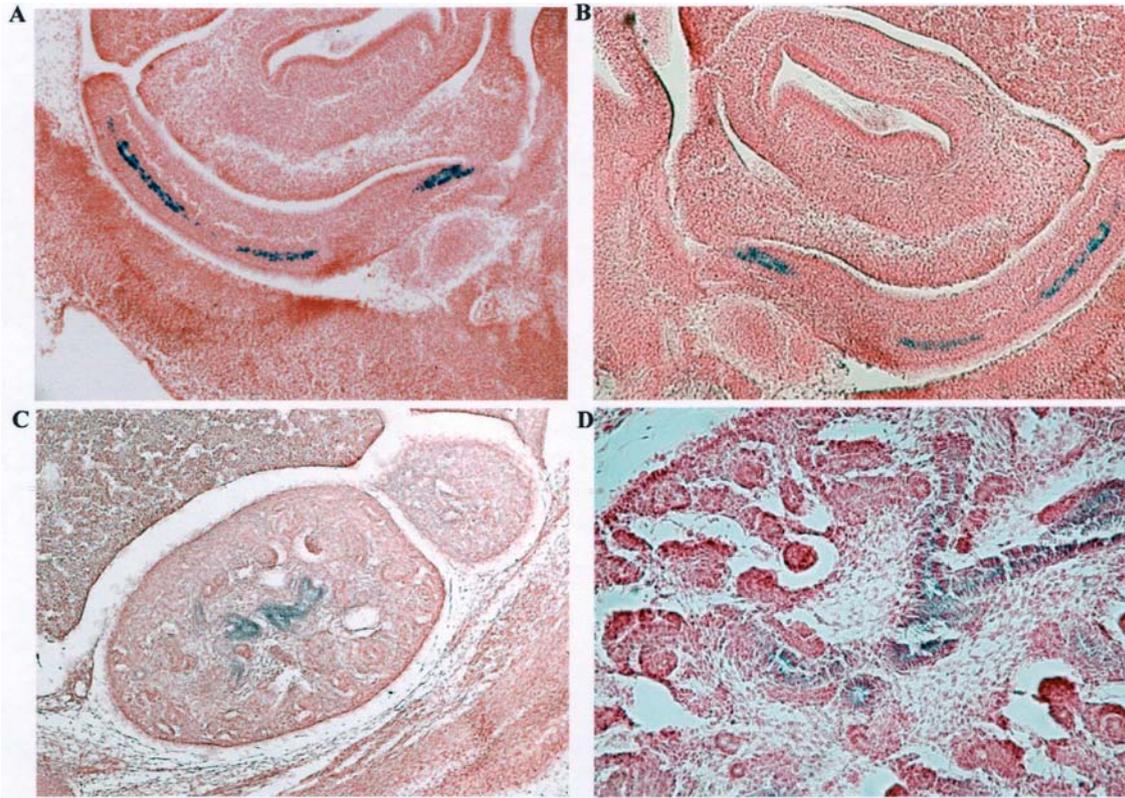


Figure 6. Both BPr and Pr1.9 transgenes can induce the *LacZ* reporter gene expression in the developing kidney. Parasagittal sections of E11.5 (A and B) and E13.5-14.5 (C and D) transgenic embryos showing the specific β -galactosidase staining pattern in the nephric duct (A and B) and in a restricted number of cells in the collecting ducts of metanephros (C and D).

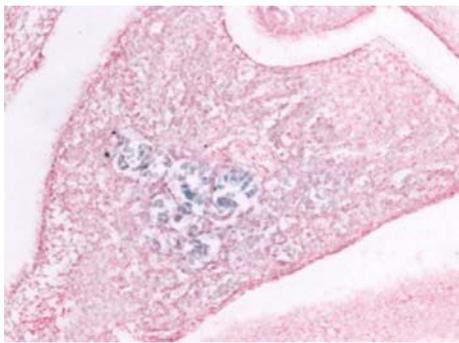


Figure 7. β -galactosidase staining within a subset of cells surrounding the pancreatic ducts in the pancreas primordium.

innervation and specification of cardiac structures. *Ret* KO mice have a reduced volume of cardiac ganglia and a defective innervation of the ventricular conduction system (28). The ablation of a premigratory cranial neural crest cell subpopulation performed in chick embryos led to malformation of the cardiac outflow tract and to septation defects. Moreover, myocardial development also was affected (29,30). These data lead to the definition of a cardiac neural crest cell subpopulation which migrates between the distal end of the outflow tract and the pharyngeal endoderm, before invading the outflow tract itself and contributing to the formation of septal tissues (31,32). The *Ret* gene, together with *Mash-1*, is one of the earliest markers appearing in the undifferentiated

neural crest cells committed to migrate through the common cardinal vein and to differentiate in cardiac ganglion cells (32).

Interestingly, our results indicate that the *Ret* regulatory sequence contained in the very short BPr transgene is sensitive to regulation in the described heart district in a significant number of transgenic mice.

Kidney. All the transgenic mice showed β -gal staining (BPr 13/13 and Pr1.9 3/3) in the metanephric region. The expression pattern of the reporter gene in this region was well defined and completely overlapping for both BPr and Pr1.9 fragments. Sections of 10.5 dpc embryos revealed the expression of the reporter gene in the nephric duct of the pro-mesonephros (Fig. 6A and B). Upon invasion of the metanephric mesenchyme (day 11-12.5) the ureteric bud began to branch to generate the collecting system. At these stages, we detected β -gal staining in epithelial cells of the ureteric bud which was absent in the surrounding mesenchyme (Fig. 6C and D).

The glial cell line-derived neurotrophic factor (GDNF) and its receptors *Ret* and *GFRa1* are essential positive regulators of primary ureteric budding and subsequent branching. GDNF is expressed by the metanephric mesenchyme and its receptors are expressed by the target tissues, the Wolffian duct and ureteric bud epithelium. A low level of GDNF expression is present in the uninduced metanephric mesenchyme and highly increased expression becomes evident in the cap condensates (33,34). GDNF binds to a receptor complex consisting of *Ret* receptor tyrosine kinase and GDNF family



$\alpha 1$ (GFR $\alpha 1$) in the Wolffian duct-derived neurons. Binding results in the activation of Ret (2) which, in turn, regulates the expression of Wnt11 (35,36), thus contributing to the regulation of ureteric branching (37). Ret is also involved in the regulation of the ureteric branching through another mechanism related to peritubular renal stroma. The stromal cells convert vitamin A to retinoic acid, which controls the expression of Ret in the ureteric bud tips by a yet unknown mechanism (38,39). Our results suggest that cis regulatory elements contained in a very short fragment of Ret 5' flanking sequence are able to control gene expression in the developing kidney, which addresses very precisely the search for transacting factors for which little is known in the pathway upstream of the GDNF receptors.

Enteric nervous system. As extensively documented, the Ret-mediated signalling pathway is important for the development of the enteric nervous system. However, we did not find LacZ expression within the neurons and glia that are organized into enteric ganglia distributed throughout the gut wall.

In transgenic mice carrying the Pr1.9 fragment we found defined β -gal staining within a subset of cells around the pancreatic ducts in the pancreatic primordium at a stage of 13.5 dpc (Pr1.9 3/3) (Fig. 7).

Only 1/13 BPr (line B) transgenic mice showed LacZ expression within a subset of cells of the thyroid follicles in E13.5 embryos, where the endogenous gene is reported to be expressed (5).

In conclusion, all transgenic mice that we generated were able to express the reporter gene, although expression patterns were variable, particularly when considering cranial ganglia, dorsal root ganglia and the heart. Such variability is probably dependent on the effect of a transgene integration locus in the mouse genome which would affect the regulating potential of cis-acting elements in the cells of neuronal tissue.

Position effect seems to be ineffective on expression in the developing kidney, which might be explained by full responsiveness of cis-acting elements in the short promoter to their cognate transcription factors. To our knowledge, little is known about these factors and Ret transcriptional regulation, however, our data can be a useful basis to refine further search inside a 380 bp sequence.

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