The human tissue plasminogen activator-Cre mouse: a new tool for targeting specifically neural crest cells and their derivatives in vivo

Thomas Pietri, Olivier Eder, Martine Blanche, Jean Paul Thiery, and Sylvie Dufour*

UMR 144 Compartimentation et Dynamique Cellulaire Centre National de la Recherche Scientifique et Institut Curie, 26 rue d’Ulm, 75248 Paris Cedex 05, France

Received for publication 13 December 2002, revised 4 March 2003, accepted 5 March 2003

Abstract

The ontogeny of neural crest cells (NCC) involves a number of orchestrated variety of derivatives, including components of the peripheral nervous system and melanocytes. Thus, it represents an excellent model system to investigate mechanisms controlling epithelial–mesenchymal transitions, cell migration and differentiation, as well as cell proliferation and death. We have established a new transgenic line expressing the Cre recombinase under the control of the human tissue plasminogen activator promoter (Ht-PA). The activity of the reporter in the Ht-PA-Cre/R26R embryos is observed as early as Theiler stage 12 in the cephalic mesenchyme. Later, the targeted cells include all the known derivatives of cranial, vagal, and trunk NCC, including craniofacial structures and cranial ganglia, cardiac and endocrine derivatives, melanocytes, peripheral, and enteric nervous system. At the vagal level, the location of presumptive enteric NCC differs from their avian counterparts in their ability to invade the mesenchyme lateral to the neural tube. In contrast to the Wnt1-Cre line, the Ht-PA-Cre line does not target the central nervous system and therefore renders it more specific for NCC. Our Ht-PA-Cre mice represent a novel model to specifically target conditional mutations in migratory NCC.

© 2003 Elsevier Science (USA). All rights reserved.

Keywords: Neural crest cell; Transgenic mice; Cre recombinase; Human tissue plasminogen activator; Enteric nervous system; Peripheral nervous system

Introduction

Neural crest cells (NCC) are induced at the dorsolateral edge of the neural plate during early embryogenesis. They undergo an epithelium-to-mesenchyme transition, delaminate from the neural tube (NT), and migrate in the embryo to reach target sites where they differentiate into a variety of derivatives (Le Douarin and Kalcheim, 1999). Complementary approaches, such as grafting experiments, tracing with dye labeling, or retrovirus insertion coupled with the analysis of marker expression, have been used extensively in chick, frog, and zebrafish to define the fate of NCC (Le Douarin and Kalcheim, 1999). Complementary approaches, such as grafting experiments, tracing with dye labeling, or retrovirus insertion coupled with the analysis of marker expression, have been used extensively in chick, frog, and zebrafish to define the fate of NCC (Le Douarin and Kalcheim, 1999). Complementary approaches, such as grafting experiments, tracing with dye labeling, or retrovirus insertion coupled with the analysis of marker expression, have been used extensively in chick, frog, and zebrafish to define the fate of NCC (Le Douarin and Kalcheim, 1999). In addition, the cellular and molecular mechanisms underlying the induction, migration, and differentiation processes of NCC have been investigated by using gene transfer and perturbation experiments (reviewed in Christiansen et al., 2000; Garcia-Castro and Bronner-Fraser, 1999; Anderson, 1999). Genomic technologies in mice offer the possibility to turn on or off expression of specific genes and determine their function in embryogenesis (Lewandoski, 2002). In particular, transgenic mice expressing Cre recombinase under the control of spatially and temporally regulated promoters are powerful tools to promote a conditional deletion of floxed genes (Kilby et al., 1993). A growing number of transgenic Cre-lines are being established (Nagy, 2000), among which some are potentially useful to create conditional mutations in NCC and their derivatives (Danielian et al., 1998; Li et al., 2000; Yamauchi et al., 1999; Feltri et al., 1999; Voiculescu et al., 2000). However, until now, a specific targeting of NCC had not been achieved.

In the present study, we have produced and characterized a new transgenic mouse line in which the transgene is...
specifically targeted to NCC. This line expresses the Cre recombinase under the control of the 3-kb fragment of the human tissue plasminogen activator (Ht-PA) promoter. This promoter was chosen because it has been reported to drive β-galactosidase expression in a spatiotemporally regulated manner in various NC-derivatives in E10.5–E14 transgenic Ht-PA-LacZ embryos (Theuring et al., 1995). In Ht-PA-Cre/R26R embryos, the earliest β-galactosidase expression is observed at Theiler Stage 12 (E8) in NCC-derived cephalic mesenchyme. At E16.5 and in the adult, the labeled cells correspond to all the known derivatives of NCC, including cardiac derivatives, the enteric nervous system, the peripheral nervous system, the endocrine derivatives, and melanocytes. We have analyzed the location of NCC during the early steps of their migration to colonize the gut. Later, the enteric ganglion networks display specific organization throughout the developing gut, which is maintained in the adult. Our Ht-PA-Cre mice and the Wnt1-Cre mice, described previously (Danielian et al., 1998; Chai et al., 2000, 2002), target the NCC derivatives similarly. However, in contrast to the Wnt1-Cre, the Ht-PA-Cre mice do not target cells in the dorsal NT. The NCC were labeled only as they begin to migrate, but not before they undergo their epithelial-to-mesenchymal transition. This new transgenic line constitutes an extremely valuable tool to target the conditional mutation of a gene of interest in the NCC at the onset of their migration and subsequently in their derivatives. Furthermore, it can also be used as a tool to mark NCC for various purposes, such as lineage tracing. Finally, this line is useful to elucidate molecular mechanisms underlying NCC ontogeny.

Materials and methods

Plasmid construction

The Ht-PA-Cre vector was constructed by fusing the proximal 3 kb of the human tissue plasminogen activator promoter (Ht-PA) upstream to the Cre recombinase coding sequence, and the mouse MT-1 polyadenylation site was chosen because it has been reported to drive β-galactosidase expression in a spatiotemporally regulated manner in various NC-derivatives in E10.5–E14 transgenic Ht-PA-LacZ embryos (Theuring et al., 1995). In Ht-PA-Cre/R26R embryos, the earliest β-galactosidase expression is observed at Theiler Stage 12 (E8) in NCC-derived cephalic mesenchyme. At E16.5 and in the adult, the labeled cells correspond to all the known derivatives of NCC, including cardiac derivatives, the enteric nervous system, the peripheral nervous system, the endocrine derivatives, and melanocytes. We have analyzed the location of NCC during the early steps of their migration to colonize the gut. Later, the enteric ganglion networks display specific organization throughout the developing gut, which is maintained in the adult. Our Ht-PA-Cre mice and the Wnt1-Cre mice, described previously (Danielian et al., 1998; Chai et al., 2000, 2002), target the NCC derivatives similarly. However, in contrast to the Wnt1-Cre, the Ht-PA-Cre mice do not target cells in the dorsal NT. The NCC were labeled only as they begin to migrate, but not before they undergo their epithelial-to-mesenchymal transition. This new transgenic line constitutes an extremely valuable tool to target the conditional mutation of a gene of interest in the NCC at the onset of their migration and subsequently in their derivatives. Furthermore, it can also be used as a tool to mark NCC for various purposes, such as lineage tracing. Finally, this line is useful to elucidate molecular mechanisms underlying NCC ontogeny.

Production of transgenic mice

The genomic DNA prepared from tail biopsies of 4-week pups was tested for the insertion of the transgene by PCR analysis using the sense (5'-TGTCTCCTCCTTCTCTTCTCTTA-3') and the antisense (5'-CGCCTGAA-GATATAGAAGATA-3') oligonucleotides, which map in the Ht-PA first intron and in the Cre recombinase coding sequence, respectively. Eleven transgenic animals were selected based on the presence of the 427-bp amplicon. The Cre recombinase expression in the transgenic animals was tested by using the ROSA26 Cre reporter (R26R) mice as follows. The transgenic males were crossed with R26R females. The founder transgenic females were crossed with wild type males, and the corresponding F2 transgenic males were then crossed with R26R females. The morning of appearance of a vaginal plug (day 0) was considered E0.5.

Fig. 2. Early Ht-PA-Cre-dependent expression of the β-galactosidase reporter during mouse embryogenesis. In whole-mount stained Theiler Stage 13 (A), early Stage 14 (B and C), Stage 14 (D), and Stage 15 (E) embryos, and in sagittal section (C), labeled cells are found around the optic pit (red arrowhead) and in the first and second branchial arches (black arrowheads). In E9.5 embryos, an intense staining is observed in these sites and around the otic placode. More caudally, cells in the third forming branchial arch could be seen. (F and G) Transverse sections of a Stage 15 embryo, respectively. In (F), mesenchymal cells known to be of NCC origin are heavily stained in the first and second branchial arches, whereas the pharyngeal endoderm and ectoderm (black arrow) as well as few mesodermal cells (red arrow) that are not derived from NCC are not labeled. In (G), stained cells are seen lateral to the NT and in ventral position, in known NCC pathways. The neural tube itself is not labeled (arrow in C and G), (B) and (F) are at the same magnification as (A) and (G), respectively. Scale bar in (A), (D), and (E) corresponds to 500 μm. Scale bar in (C) and (G) corresponds to 200 μm.
Fig. 3. Ht-PA-Cre-dependent β-galactosidase expression in E12.5 embryos in cephalic and sensory derivatives. (A) Whole-mount staining of an embryo shows intense staining of craniofacial structures and peripheral nervous system. Sections of the head showing the mandibular process (B), the forming eye (C), the cephalic mesenchyme (D), and the trigeminal and vestibulocochlear ganglia (E). (F) Double staining for β-galactosidase (blue) and with an antibody recognizing placodal neurons and directed against Brn-3a antibody (brown) show that glial cells of the vestibulocochlear ganglia are targeted by the transgene but not the neurons from placodal origin. In (D), black arrowhead, red arrowheads, and black arrow, respectively, indicate the labeling of the ectoderm of the face, the ectomenix, and the cells associated with the vasculature of CNS that corresponds to pericytes. Lateral view (G) of the whole-mount stained E12.5 embryo and a 200-μm-thick transverse section (H) showing the subcutaneous and muscular sensory innervation network. Sagittal section of a E12.5 embryo where sympathetic and dorsal root ganglia appear heavily stained (I). Arrowheads and arrows in (H) and (I) show the dorsal and ventral roots, respectively. nc, nasal cavity; tg, trigeminal ganglia; vch, vestibulocochlear ganglia; il, internal layer; l, lens; ce, corneal epithelium; hc, hyaloid cavity; tv, telencephalic ventricle; cm, cephalic mesenchyme; drg, dorsal root ganglia; sg, sympathetic ganglia; vr, ventral ramus; dr, dorsal ramus; mi, muscular innervation; ci, cutaneous innervation; ca, cutaneous anterior ramus; cl, cutaneous lateral ramus. Scale bar in (A), (C), and (G) corresponds to 1 mm. Scale bar in (D), (B), (H), and (I) corresponds to 200 μm. Scale bar in (E) and (F) represents 100 μm.
and the pregnant females were sacrificed by cervical dislocation at E8–E16.5.

Among the 11 founders, 9 animals produced embryos expressing β-galactosidase in NCC-derived tissues. Among them, the male founder #116 was selected for further analysis on the basis of the specific expression of the reporter gene observed in E11.5–E12 embryos in NCC-derived tissues, such as craniofacial structures and peripheral nervous system.

The Ht-PA-Cre mouse line was generated by mating a founder (#116) to wild type females and then by intercrosses and backcrosses of the offspring to obtain homozygous animals. Wnt1-Cre mice were provided by A. McMahon (Danieilian et al., 1998). Transgenic Ht-PA-Cre or Wnt1-Cre males were mated with R26R females to produce embryos and pups. Ht-PA-Cre/R26R and Wnt1-Cre/R26R embryos were collected at defined stages of development. E8–E10 embryos were fine staged according to the Theiler stage table (Theiler, 1989). Newborn mice from the same type of crosses were staged according to the date of birth (P0).

Embryo processing and detection of the β-galactosidase in tissue

Embryos or organs were dissected in cold phosphate-buffered saline (PBS), pH 7.6, with 5% fetal calf serum and fixed in toto in freshly made ice-cold 1% formaldehyde/0.2% glutaraldehyde/0.02% Nonidet-P40 for 2–4 h. The detection of β-galactosidase activity was revealed at 30°C by a 16- to 20-h whole-mount X-gal staining as described before (Dufour et al., 1994). Whole-mount stained samples were photographed under a Leica MZ8 stereomicroscope (Leica Microsystems SA, France) equipped with a JVC 3CCD color camera. Some whole-mount stained embryos were serially sectioned at a thickness of 200 μm on a vibratome. For histological sections, embryos or tissue were dehydrated and embedded in paraffin plus and cut into serial sections of 7–10 μm. Sections were processed and slightly counterstained with eosin, nuclear fast Red, or the van Gieson solution (for the adult skin and gut sections; see van Gieson, 1889).

Antibodies and immunohistochemistry

Polyclonal antibodies directed against chromogranin A + B were purchased from Progen Biotechnik (Germany). The anti-Hu antibody was a gift of J. Weston (Marsisch and Weston, 1992). The antibody directed against Brn-3a was a gift of E. Turner (Artinger et al., 1998). Sections were dewaxed and processed for antigen retrieval by boiling in citrate buffer for 20 min and then kept at room temperature for 30 min. Sections were treated with 3% hydrogen peroxide in PBS containing 0.1% Tween 20 (PBST) for 1 h, rinsed several times in PBST, and blocked for 2 h in blocking solution containing 0.5% Blocking Reagent (Roche Applied Science, France) and 10% fetal calf serum in PBST. The sections were then incubated overnight at 4°C with primary antibody (anti-chromogranin A + B, 1:50; anti-Brn-3a, 1:500) in the blocking solution, rinsed several times in PBST, and incubated with anti-rabbit-HRP (1:100; Amersham Biosciences Europe, France) for 2 h at room temperature prior to revealing the HRP activity using the peroxidase substrate kit VECTOR according to manufacturer instructions (Vector Laboratories, Inc.).

Results

We have characterized the spatiotemporal pattern of the Ht-PA-Cre activity by examining the β-galactosidase staining in the Ht-PA-Cre/R26R offspring at various stages of their embryonic development and postnatally. Six generations were analyzed for β-galactosidase expression. Over all these generations, the expression pattern remained as described below, indicating that the expression of the transgene is stable.

Early activation of the Ht-PA promoter during development and expression of β-galactosidase in various NCC derivatives

The earliest expression of β-galactosidase was seen at Theiler Stage 12. By Stage 13, labeled cells were found in the mesenchyme of the first pharyngeal arch, in the frontonasal region and around the optic pit (Fig. 2A). The number of labeled cells increased with time of development. Expressing cells were found in the second and third pharyngeal arches and into the other arches from Stage 13 to 15 (Fig. 2B–E). In transverse sections, most of the mesenchymal cells in pharyngeal arches were clearly labeled, but not the pharyngeal ectoderm and endoderm (Fig. 2F). Some unlabeled cells in the center of the pharyngeal arch correspond to mesodermally derived cells (Fig. 2F; and Noden, 1978). At that stage, at both the rostral and caudal levels, cells that emerge from the neural tube and are distributed in the ventral pathway were labeled (Fig. 2G). The NT does not express the β-galactosidase.

At E12.5 and later, β-galactosidase was heavily expressed in craniofacial structures and the sensory nervous system (Fig. 3A). In the head, the maxillary (Fig. 3B) and mandibular process, the periostial mesenchyme, the cells that will form the cornea and iris (Fig. 3C), and the cells located in the hyaloid cavity were labeled. The cephalic mesenchyme surrounding telencephalic vesicle and the diencephalon were intensively stained for β-galactosidase (Fig. 3D). These cells produce pericytes of CNS and the outer table of ectomenix (arrow and red arrowhead, respectively) as well as the dermis of the face (black arrowhead). In addition, labeled cells were found in various sensory cranial ganglia, such as the vestibulocochlea and trigeminal ganglia (Fig. 3E) as well as the ciliary and node ganglia and the corresponding nerves (not shown). The labeled cells
in the vestibulocochlear ganglia are glial cells (presumably of neural crest origin) which surround Brn-3a-positive neurons (presumably of placodal origin), which do not express β-galactosidase (Fig. 3F). In the trigeminal ganglia, we observed that most of the cells are labeled for β-galactosidase (Fig. 3E). A large part of the labeled cells expressed the Hu protein, a marker of early neuronal differentiation (Marusich and Weston, 1992), whereas others are negatives (not shown). This result indicates that the labeled cells contribute to the neuronal and glial compartments of the trigeminal ganglia, which probably derived mainly from NCC. However, some Brn-3a-positive cells did not express β-galactosidase, suggesting that few neurons from placodal origin may also contribute to the trigeminal ganglia. This supports the possibility of dual origin of neurons in the trigeminal ganglia.

In the trunk, in whole-mount preparations (Fig. 3G) and on 200-μm-thick transverse sections (Fig. 3H), the developing muscular and cutaneous innervation corresponding to the primitive spinal nerve trunk were visible. They first separate into primary dorsal and ventral ramus and then into lateral and anterior branches. In all these structures, the glial cells located along the sensory and motor nerves were strongly labeled. The spinal ganglia and sympathetic ganglia (Fig. 3I) were homogenously stained, revealing that both glial and neuronal precursors were labeled in these structures. Cells in the central and peripheral roots of spinal ganglia as well as the ventral root of the spinal cord were also positive for β-galactosidase expression (Fig. 3H and I, arrows). At higher magnification, the staining observed on ventral roots corresponds to the glial cells (NCC-derived) associated with the axons, whereas the motor neurons and their axons were not labeled (not shown).

**Ht-PA-Cre-dependent expression of β-galactosidase in the enteric nervous system**

At Theiler Stage 15 (E9.75), from the cranial level to the first and second somites formed, labeled cells were found close to the NT and located laterally between the ectoderm and the condensing cervical myotome (Fig. 4B, arrows). They were also found in the space between the endoderm of the pharynx and the splanchnic mesoderm (Fig. 4A, arrows), between the myotome and the NT and in the pharyngeal pouch (Fig. 4C); but the labeled cells were never found in the perichordal mesenchyme (arrowhead in Fig. 4A). β-Galactosidase-expressing cells were closely apposed to the lateral and ventral parts of the left and right dorsal aorta (Fig. 4C–F, arrowheads). Others were found entering the primitive gut (Fig. 4E and F, arrows). Sections from several Theiler Stage 15 embryos were analyzed for the presence of labeled cells, and a schematic representation of their localization is shown in Fig. 4G. At E12.5, labeled cells were found in the developing gut (Fig. 5A). At that stage, they were present in the descending colon and the front of their migration is easily seen (arrow). Labeled cells were also seen in the pelvic mesenchyme at the level of the cecum (arrowhead). By E16.5, the whole gut contains labeled cells which were organized into interconnected ganglia, characteristic of the enteric nervous system (Fig. 5B and D, and for review, see Gershon et al., 1993). On transverse sections of an E16.5 gut, the β-galactosidase staining corresponds to ganglia located in the outer layer of the wall (Fig. 5C and E). Few positive ganglia were also seen in a submucosal position at this stage. In the adult gut, whole-mount staining revealed the organization and density of enteric ganglia which differ in the proximal part and the distal part of the gut (not shown), as previously observed (Furness et al., 1987). At the level of the jejunum, an intermediate density of labeled aggregates with thin interconnections are shown (Fig. 5F) which correspond to the outer myenteric (Fig. 5G, arrows) and inner submucosal ganglia (Fig. 5G, arrowheads) as shown on transverse section.

**Ht-PA-Cre-dependent expression of β-galactosidase in the heart and other derivatives**

β-Galactosidase-labeled cells were present along the endoderm in the aortic sac at Theiler Stage 15 (Fig. 6A, arrow). At E12.5, labeled cells were found surrounding the thymus primordium, forming the wall of innominate artery, aorta, and ductus arteriosus (Fig. 6B and C). At E16.5 (Fig. 6D) and in the adult (Fig. 6E and F), the β-galactosidase staining was found in the outflow tract of the heart, a site where the presence of NCC has recently been confirmed (Jiang et al., 2000). Parasympathetic innervation and sensory ganglia of the heart were labeled as shown on whole-mount E16.5 and adult heart (Fig. 6D and F, black arrow). On transverse sections of a E16.5 heart, the subendothelial layer of the pulmonary trunk septum and the aortic valve were heavily stained as well as scattered cells in the interventricular septum. The endothelium was not labeled, nor was the wall of the aorta, as previously described (arrow in Fig. 7E; and Jiang et al., 2000). In the adult, extensive staining of the ascending aorta was observed as well in the innominate artery, in the left and right carotid arteries, in the right but not in the left subclavian arteries nor in the dorsal aorta (Fig. 6F, arrowhead). The ligamentum arteriosum, which derives from the ductus arteriosus, was strongly stained in the adult heart (Fig. 6F, red arrow).

Other NCC derivatives, such as the stromal components of the thyroid and parathyroid glands (not shown), the thymus (see Fig. 6B), and the adrenal gland (Fig. 7A–C), were labeled as well as the melanocytes (Fig. 7D and E). The adrenal anlagen was stained with β-galactosidase at E12.5 (not shown). The positive cells found in the anlagen were most likely chromaffin cell precursors, as deduced from their typical location in the medullar compartment of the adrenal glands at E16.5 (Fig. 7A). Indeed, these cells expressed both β-galactosidase and chromogranins (Fig. 7B and C). The sensory innervation of the gland is also stained (Fig. 7A, arrow). In adult skin, the melanocytes, revealed by
their intense pigmentation in the hair matrix of the follicles (Fig. 7E), were heavily stained with β-galactosidase as well as those in interaction with the basement membrane of the epidermis (Fig. 7D, arrow) or in the dermis.

Comparison between Ht-PA-Cre and Wnt1-Cre line to target NCC derivatives

The Wnt1-Cre line described by McMahon and coworkers (Danielian et al., 1998) has been used to determine the fate of the mammalian cranial and cardiac NCC (Chai et al., 2000; Jiang et al., 2000). We compared the Wnt1-Cre with the Ht-PA-Cre line for each of their targeted derivatives. In whole-mount stained embryos, it is obvious that the two types of markers are able to target the cephalic mesenchyme and the cells of branchial arches as shown in Fig. 8A and B (arrowheads; Wnt1-Cre/R26R at E9.75 and in Fig. 2C and D (Ht-PA-Cre/R26R E9–E9.5). In E12–E12.5 whole-mount embryos, a similar staining in craniofacial structures and the developing sensory nervous system was observed for the two types of embryos (Fig. 8D and E). A major difference between the two embryos, however, is that Wnt1-Cre can target the dorsal NT as shown on sagittal sections at E9.75 (Fig. 8B and C, arrows) and at E12.5 (Fig. 8E and G, arrows), whereas the Ht-PA-Cre line never targeted this structure (Fig. 2C and G, arrow; Fig. 8D and F). In transverse sections at the trunk level, it appeared that the ventral and dorsal roots as well as the sensory ganglia and the developing muscular and cutaneous innervation are labeled in the Ht-PA-Cre/R26R and Wnt1-Cre/R26R (Fig. 8H and I). However, it was clear that only the Wnt1-Cre/R26R embryo exhibited a strong staining in the dorsal NT (black arrow) and to a lesser extent in the mantle layer of the intermediate NT, and the floor plate (red arrow and arrowhead, respectively). An extensive comparison of the stained derivatives in the head was made for these two transgenic lines (Fig. 9), showing that they expressed β-galactosidase in the NCC-derived glial and neuronal cells of the sensory nervous system as well as in the choroid plexuses and pericytes (Etchevers et al., 2001). NCC-derived mesenchymal derivatives are similarly stained, including the cephalic mesenchyme rostral to the mesencephalon, the perioptic mesenchyme, and that of maxillary process. During the course of the heart development and in the adult, the labeled cells found in our Ht-PA-Cre/R26R animals (see Fig. 6) were similar to those described for the Wnt1-Cre/R26R animals (Jiang et al., 2000). In addition, a similar labeling of the enteric nervous system throughout its development was observed for the two types of animals (not shown).
Fig. 6. Ht-PA-Cre-dependent β-galactosidase expression in NCC-derived cardiac derivatives. (A) Transverse section of a Theiler Stage 15 embryo at the level where labeled cells (arrow) are seen along the endoderm in the aortic sac. (B and C) On transverse section of a E12.5 embryo, labeled cells are shown around the thymic primordium and are located at the conotruncus region, the pulmonary trunk, and in the wall of the aorta and innominate artery (arrow in B). On whole-mount stained E16.5 (D) and adult heart (F), extensive staining of the ascending aorta, the arch of the aorta, the left and right carotid arteries, and right subclavian artery is observed as well as the sensory innervation (black arrow in D and F). (E) Transverse section of a E16.5 heart reveals the localization of labeled cells in the subendothelial layer of the pulmonary trunk and of the aortic valve, whereas the endothelium and the wall of the aorta are unlabeled. Some cells in the muscular part of the interventricular septum are also stained. Sg, sympathetic ganglia; inn, innominate artery; th, thymus primordium; du, ductus arteriosis; Ao, aorta; dAo, dorsal aorta; pt, pulmonary trunk; rv, right ventricle; ivs, interventricular septum; Aov, aortic valve; ct, conotruncus; Ica and rca, left and right carotid arteries; lsca and rsca, right and left subclavian arteries; aAo and dAo, ascending and descending aorta. The ligamentum arteriosum (red arrow). Scale bar in (A), (B), and (E) corresponds to 200 μM. Scale bar in (D) and (F) corresponds to 1 mm.

Fig. 7. Ht-PA-Cre-dependent β-galactosidase expression in adrenal gland and melanocytes. An intense labelling of the medulla is observed on E16.5 adrenal gland (A) as well as on the sensory innervation of the cortex (arrow). In the adult adrenal gland (B and C), the β-galactosidase-expressing cells (B and C) correspond to chromaffin cells as revealed by a colocalization of the anti-chromogranin A and B immunostaining (C). Transverse section of the skin dissected from the back of adult mice (D and detail of a hair follicle in E) where sensory nerves (arrowhead) and pigmented melanocytes located close to the basement membrane of the epidermis and in the hair matrix of follicles (arrows) are heavily stained for β-galactosidase. Scale bar in (A) represents 500 μm, and in (B) and (E) corresponds to 50 μm. Scale bar in (D) corresponds to 100 μm. e, epidermis; d, dermis; h, hypodermis; m, muscular layer.
Discussion

Specific expression of the transgene in the NCC derivatives

We have generated transgenic mouse lines that express the Cre recombinase under the control of the proximal 3-kb promoter sequences of human tissue plasminogen activator. By crossing the transgenic founder animals with ROSA26 Cre reporter mice, we observed that the β-galactosidase was expressed in NCC-derived tissues. In addition to marking migratory cells, this indicates that the transgene is functional to promote Cre-dependent DNA recombination and that the proximal 3-kb Ht-PA promoter sequence contains information to be activated in NCC as previously observed (Theuring et al., 1995). We observed that, depending on the founders, the transgene was also expressed in other tissues that are not from a neural crest origin. These differences...
among the founders analyzed probably reflects the effect of
the integration site into the genome on the modulation of the
3-kb Ht-PA promoter activity. In the Ht-PA-Cre/R26R an-
imals derived from the founder #116, we observed a speci-
cfic expression of the reporter gene in the NCC-derived tissues.
Only one ectopic site of β-galactosidase expression was
found in the mesenchyme of the limb at E12.5, a non-NCC-
derived tissue. In the adult, the stained cells in the limb were
detected as a minor component of the periostum and ten-
dons (not shown) that can be easily distinguished from the
NCC-derived labeled cells. The Ht-PA promoter has been
suggested to be activated at sites of tissue remodeling
(Theuring et al., 1995). An extensive tissue remodelling
occurs in the limb at E12.5 that could explain the localized
expression of β-galactosidase found in Ht-PA-Cre/R26R
embryos. Therefore, in the line #116, the Ht-PA-Cre trans-
gene is efficiently and almost specifically activated in NCC
and their derivatives. In contrast to various promoters pre-
viously used to target NCC derivatives by a Cre-dependent DNA recombination, such as Krox20 (Voiculescu et al., 2000), Pax-3 (Li et al., 2000), or P0 promoters (Yamauchi et al., 1999; Feltri et al., 1999), the Ht-PA promoter activity appears not to be restricted to specific NCC subpopulations but rather behaves as a pan-NCC marker of craniofacial structures, peripheral neuroblasts, and glial precursors, as well as neuroendocrine cells all along axial levels.

**Targeting of neural crest cells when they are migratory**

The earliest expression of the β-galactosidase in Ht-PA-Cre/R26R embryos starts at Theiler Stage 12 among mesenchymal cells in the cephalic region that appear distally from the NT. It is difficult to discern whether NCC should have been detected earlier and hence proximal to the NT. A delay between the induction of the Ht-PA promoter and the expression of β-galactosidase may arise from the Cre-dependent DNA recombination at the ROSA26 locus. On plausible explanation of this phenomenon is that β-galactosidase is observed only once these early cephalic NCC have already dispersed far from the NT. In the trunk, labeled NCC are visualized close to the neural tube, suggesting that they stay in the so-called staging area before they actively migrate (Weston, 1991). Subsequently, the Ht-PA promoter targets a large variety of crest-derived cells, such as cranial, vagal, cardiac, and trunk crest cells and their derivatives. In addition, we have observed that, in our transgenic Ht-PA-Cre line #116, the promoter is not activated in the NT. Therefore, the transgene does not mark premigratory NCC or other cells in the CNS. Rather, this promoter is specifically activated in NCC when they begin to migrate. This is consistent with previous observations showing that migrating NCC express tissue plasminogen activator (Valinsky and Le Douarin, 1985; Menoud et al., 1989; Erickson and Isseroff, 1989) and the Ht-PA promoter activity in vivo in NCC (Theuring et al., 1995).

**Ht-PA-Cre expression in the developing gut: a tool to investigate enteric nervous system ontogeny and function**

The Ht-PA-Cre line #116 appears to be a powerful tool to mark NCC and to analyze their derivatives. As an example, we have further investigated the localization of cephalic and vagal NCC in the early embryo and later during the development of the gut. Neural crest-derived cells are known to give rise to both neurons and glial cells of the enteric nervous system (Le Douarin, 1982; Ciment and Weston, 1983; Young and Newgreen, 2001). Several analyses have investigated the contribution of vagal and sacral NCC to the gut, and how the mammalian and avian enteric nervous system develops (Rothman and Gershon, 1982; Pomeranz et al., 1991; Serbedzija et al., 1991; Kapur et al., 1992; Rothman et al., 1993; Serbedzija and McMahon, 1997; Taraviras and Pachnis, 1999; Kapur, 2000; Burns and Le Douarin, 1998, 2001). The migratory pathways of NC cells in the developing gut have been characterized in the chicken embryo (Thiery et al., 1982; Tucker et al., 1986), but they are poorly understood in mammals. In our model, we can mark migratory NCC as soon as they escape from the NT all along the axis of the embryo. By comparing the location of labeled cells with those defined in the avian embryo, we observed similarities and differences. Murine vagal NCC, like their avian counterparts, were found located laterally along the ectoderm and at the apex of the pharynx and avoided the space around the notochord. However, in contrast to avian embryo, they were additionally seen located in the sclerotome. This difference in the pathways followed in these two species may suggest some variation in the requirement of particular guidance cues or mesenchyme extracellular matrix for the migration of the vagal NCC.

Later during the development of the gut, the enteric nervous system is heavily stained. The staining illustrates the distinct organization and density of the enteric ganglia from the proximal to distal part of the gut, which correlates with the specific control of the contractility of the intrinsic smooth muscles and of the activity of secretory glands associated with the function of the gastrointestinal tract (Furness and Costa, 1987; Gershon, et al., 1994). This topological organization and its function could be further investigated in normal and pathological conditions, taking advantage of our transgenic line.

**Ht-PA-Cre line a good tool to target conditional mutations in NCC derivatives**

Among the transgenic lines available, the closest to our line in term of its ability to target all the NCC derivatives is the Wnt1-Cre line described by McMahon and coworkers (Echelard et al., 1994; Danielian et al., 1998). The use of Wnt1-Cre line has allowed the detailed analysis of the fate of cranial NCC during tooth and skull vault development and the fate of cardiac NCC (Chai et al., 2000; Jiang et al., 2000, 2002). A comparison between Ht-PA-Cre/R26R and Wnt1-Cre/R26R embryos revealed that the two lines similarly target the NCC-derived structures, such as the craniofacial structures, the cardiac derivatives, the peripheral and enteric nervous systems, as well as the endocrine derivatives. However, a difference between the Ht-PA and the Wnt1 promoter activity in transgenic animals concerns the targeting of the NT. The Wnt1 promoter is activated in the dorsal NT during early embryogenesis (Theiler Stage 11). Thus, the use of Wnt1-Cre line allows all the tissues derived from the dorsal NT to be targeted, including the NCC derivatives, but also the dorsal part of the CNS. The Ht-PA promoter in our transgenic line does not label dorsal NT nor the premigratory NCC (Theiler Stage 12). We suggest that this characteristic of the Ht-PA-Cre line renders it more specific for NCC than the Wnt1-Cre line. Because of its specificity, the Ht-PA-Cre line allows a more precise manipulation of gene expression in NCC without targeting the
CNS itself. The difference in the ability of the Ht-PA and Wnt1 promoters to affect the NT, in addition to NCC, may be crucial for the analysis of the control of gene expression involved in the development of NT, NCC, and their derivatives. For example, the inactivation of such genes in the dorsal NT could produce strong early defects in the CNS leading to morphological abnormalities and therefore impairing the analysis of NCC-specific defects at later stages. In this respect, this new Ht-PA-Cre transgenic line constitutes a unique and useful model which is able to specifically target conditional mutation in the NCC derivatives.

Acknowledgments

We thank F. Theuring for the gift of the construct carrying the human tissue plasminogen activator promoter and M. Soriano for the gift of ROSA26 Cre reporter mouse line. We acknowledge A. McMahon for the generous gift of Wnt1-Cre mice. J.B Sibarita, D. Meur, and D. Morineau are acknowledged for their help with imaging. We thank the staff of the animal facilities at the Institut Curie and I. Cerutti and the staff of the Service d’expérimentation Animale et de Transgenese in Villejuif for taking care of the transgenic animals. We are grateful to J.A. Weston for fruitful discussions and comments and acknowledge J. Veltmaat and M. Morgan for suggestions on the manuscript. This work was supported by the Centre National de la Recherche Scientifique, the Institut Curie, and the Association pour la Recherche sur le Cancer (Grant no. 5653).

References


