Expression of the *trk* proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development

Dionisio Martin-Zanca, Mariano Barbacid,[1,2] and Luis F. Parada[3]

Molecular Embryology Group and [1]Developmental Oncology Section, BRI–Basic Research Program, National Cancer Institute–Frederick Cancer Research Facility, Frederick, Maryland 21701 USA

We have cloned and characterized the mouse homolog of the human *trk* proto-oncogene, a member of the protein tyrosine kinase (TK) receptor gene family. Here, we present the first report of a *trk*-encoded mRNA species in vivo. In situ hybridization analysis in the mouse embryo reveals a striking temporal and spatial regulation of *trk* transcription, with expression confined to the sensory cranial (trigeminal, superior, jugular) and dorsal root ganglia (DRG) of neural crest origin. Recent reports have shown that TK receptors can play regulatory roles in embryonic development. Thus, the developmental mutations *W* in mouse and *torso* and *sevenless* in *Drosophila* represent genes that code for defective TK receptors. Our data show that *trk*, a gene associated with malignancy in humans, is a specific marker for a set of neural crest-derived sensory neurons, and are consistent with the hypothesis that this proto-oncogene may have an important role in the development or phenotype of the neurons where it is expressed.

[*Key Words*: Developmental expression; in situ hybridization; proto-oncogene; *trk*; tyrosine kinase receptor]

Received January 31, 1990; revised version accepted March 8, 1990.

The elegant classical body of work on the developing vertebrate embryo has provided an essential framework for more recent studies on the molecular basis of development. Various approaches have been applied to the search for genes that are potential regulators of mammalian development. One method has been to clone and study the mammalian homologs of genes known to have developmental functions in invertebrates. An example is provided by the study of homeotic genes (first identified in *Drosophila melanogaster*) in mouse and man, which has resulted in the identification of many loci that present unique expression profiles in the embryo (Jackson et al. 1985; Joyner et al. 1985; Awgulewitsch et al. 1986; Holland and Hogan 1988; Dollé et al. 1989; Wilkinson et al. 1989). Another fruitful method for identifying genes with potential roles in embryogenesis stems from work with oncogenes. Investigators in this field have identified and isolated a diverse series of genes that, when structurally mutated or inappropriately expressed, have the common property of exerting profound effects on the regulation of cell proliferation (Land et al. 1983; Weinberg 1985; Bishop 1987). It has further emerged that many proto-oncogenes form part of highly conserved gene families and, at least in some cases, are either exclusively expressed or tightly regulated during embryogenesis (Jakobovits et al. 1985, 1986; Zimmerman et al. 1986; Wilkinson et al. 1987, 1988; R. Klein, D. Martin-Zanca, M. Barbacid, and L.F. Parada, in press), leading to the suggestion that proto-oncogenes may have important roles in development (Muller 1986; Nusse 1988).

The tyrosine kinase (TK) receptor proto-oncogenes comprise a large family of structurally related genes whose products mediate proliferation and differentiation control signals in a variety of cell types (for review, see Hanks et al. 1988). Recently, TK receptors have also been implicated in the control of embryonic cell fate decisions. The *W* locus in mouse codes for the c-*kit* proto-oncogene, a TK receptor that is required for the effective migration and proliferation of various stem cells in the embryo and in the adult (Silvers 1979; Yarden et al. 1987; Chabot et al. 1988; Geissler et al. 1988). In *Drosophila*, the *torso* and *sevenless* loci encode TK receptors that are required for terminal structure differentiation and photoreceptor morphogenesis, respectively (Basler and Hafen 1988; Sprenger et al. 1988). Here, we report the molecular cloning and developmental expression profile of the mouse *trk* proto-oncogene. The human *trk* oncogene was originally identified following transfection of NIH-3T3 cells with DNA obtained from a human colon carcinoma and remains the only example of a TK oncogene activated in a solid human tumor (Pulciani et al. 1982; Martin-Zanca et al. 1986a). Subsequent isolation and structural characterization of both the oncogene and proto-oncogene cDNAs identified a gene

[2]Present address: Squibb Institute for Medical Research, Department of Molecular Biology, Princeton, New Jersey 08543-4000 USA.
[3]Corresponding author.

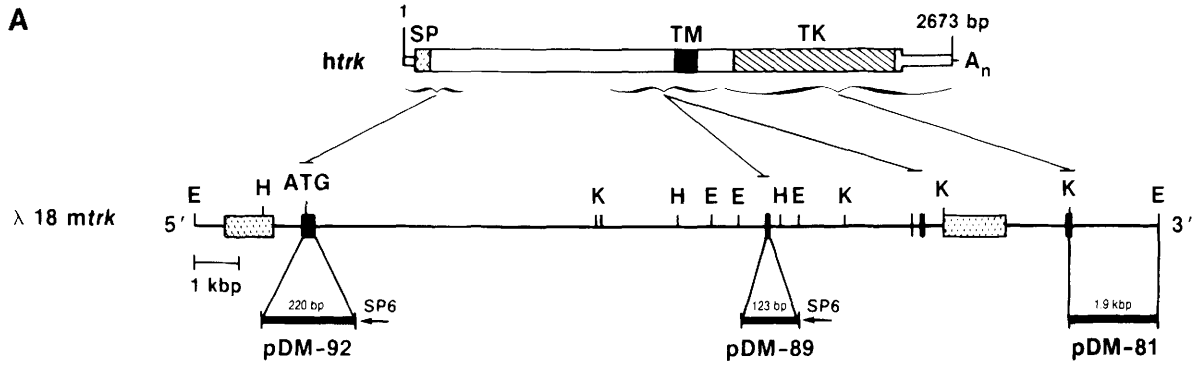
product containing all the structural motifs of a transmembrane receptor (Martin-Zanca et al. 1986a, 1989; see Fig. 1A). Oncogenic activation of *trk* occurred via a somatic genetic rearrangement that resulted in the replacement of the *trk* receptor extracellular domain by the amino-terminal region of a nonmuscle tropomyosin molecule. As has been proposed for other members of the family of transmembrane receptors, the *trk* gene product apparently becomes transforming through the disruption of its signal-receiving extracellular domain, resulting in the constitutive activation of the signal-transducing intracellular domain (Downward et al. 1984; Bargmann et al. 1986; Park et al. 1986; Roussel et al. 1987; Coulier et al. 1989). Northern blot analysis with a human *trk* cDNA probe revealed an extremely restricted pattern of expression for this gene in mRNAs from human cell lines and tissues. Abundant levels of *trk* transcripts were found only in a limited number of human tumor cell lines of diverse tissue origins (Martin-Zanca et al. 1986b). These data did not provide any useful clues about the in vivo specificity of *trk* expression. In a survey of RNA from human tissues, extremely low levels of *trk* mRNA were seen only in testes (unpubl.), an observation whose physiological relevance remains unclear. In contrast, a complex pattern of mRNA species was detected when the human *trk* cDNA probe was hybridized to adult mouse brain RNA. Studies based on this latter observation have led to the identification of a closely related gene, *trk*B (Klein et al. 1989, and in press).

The failure to detect *trk*-encoded transcripts in adult tissues prompted us to explore whether this gene was transcribed in the embryo. For this purpose, we have cloned and characterized the mouse *trk* locus. After generating exonic probes, we have performed developmental Northern blot and in situ hybridization analyses of the mouse *trk* proto-oncogene from early postimplantation through late fetal development. We detect the appearance of a *trk*-encoded mRNA in the embryo and show a striking temporal and spatial regulation of *trk* transcription. These data identify *trk* as the earliest known marker for dorsal root ganglia (DRG) and cranial sensory ganglia of neural crest origin.

Results

Isolation of the mouse gene homologous to the human trk *proto-oncogene*

To isolate the mouse homolog to the human *trk* proto-oncogene, a genomic library from NIH-3T3 cells was



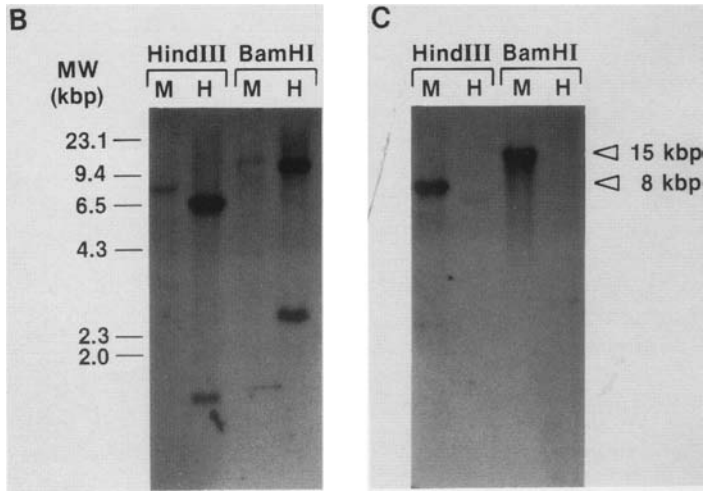


Figure 1. (*A*) Schematic representation of human and mouse *trk* proto-oncogene clones. (h*trk*) Structure of the human *trk* cDNA (Martin-Zanca et al. 1989). Thick bar represents coding sequences. (SP) Signal peptide; (TM) transmembrane domain; (TK) tyrosine kinase catalytic domain. Brackets indicate cDNA probes used to identify *trk* exons in the mouse *trk* clone shown below. (λ18m*trk*) Partial restriction map of a mouse *trk* genomic λ clone. Crossbars represent DNA fragments containing mouse repetitive sequences. Solid boxes indicate *trk* exons. Also shown are the mouse *trk* probes used for Southern blots (pDM-81) and for Northern and in situ hybridizations (pDM-89, pDM-92) (for details, see Methods). The 1-kb scale bar corresponds to the genomic clone λ18m*trk*. (*B* and *C*) Southern blot analysis of mouse tail (M) and human HeLa cell (H) DNAs. DNAs (5 μg) isolated from human HeLa cells or C57BL/6J mouse tails were digested with *Bam*HI or *Hin*dIII restriction enzymes, electrophoresed through 1% agarose gels, and transferred to filters (for details, see Methods). Mouse DNA restriction fragments hybridizing with both the human (*B*) and mouse (*C*) *trk* probes are indicated by arrowheads. Molecular weight markers (*left*) correspond to coelectrophoresed λ *Hin*dIII restriction fragments.

screened with the entire human *trk* cDNA as probe. Southern blot analysis showed that under the appropriate hybridization conditions (see Methods), the human cDNA probe hybridized strongly with a 15-kb *Bam*HI mouse genomic fragment (Fig. 1B) and weakly with another fragment of ~6 kb (not seen in Fig. 1B). Two overlapping λ clones, containing DNA inserts ~20 kb in length, were isolated and purified. A partial restriction map of the *trk* clone extending furthest 5′ is shown in Figure 1A.

To demonstrate that the genomic clones represented the mouse *trk* locus and not a related locus (Klein et al. 1989), fragments lacking repetitive sequences were identified, subcloned, and used as probes in Southern transfer analysis of mouse and human DNAs. In the Southern blots shown in Figure 1, probes that corresponded to the same coding regions from the human cDNA and from the mouse genomic clones (human TK probe and mouse probe pDM-81; Fig. 1A) were hybridized under stringent conditions to mouse and human DNAs digested with the restriction endonucleases *Hin*dIII or *Bam*HI. The resulting pattern of hybridization identifies single restriction fragments (15-kb *Bam*HI and 8-kb *Hin*dIII; Fig. 1B,C) in mouse DNA when either the exonic mouse probe or the more complex human cDNA probe was used. However, the human DNA lanes exhibit a different profile of hybridization (Fig. 1B). As expected, in this latter instance, the mouse probe recognizes single restriction fragments that comprise only a subset of those hybridized by the human probe. Similar results were obtained when probes from other regions of the human *trk* cDNA and from the corresponding mouse exonic sequences were tested (not shown).

Exon-containing genomic DNA fragments were identified by hybridization with probes corresponding to the three main domains of the human *trk* gene product: namely, the extracellular, transmembrane, and TK domains (Fig. 1A). Candidate fragments were subcloned, and subsequent partial sequence analysis of these subclones allowed us to identify and tentatively map several mouse *trk* exons (Fig. 1A). DNA sequence comparison of putative exonic mouse sequences with the corresponding regions of the human *trk* cDNA revealed a high level of identity in all cases, ranging from >90% in the TK domain to > 80% in all other domains (in prep.).

Northern analysis of trk *expression in mouse embryonic RNAs*

Two of the putative exons thus defined were selected to generate probes for use in Northern analysis and in situ hybridizations (see Methods). These probes were chosen from regions outside the TK domain of mouse *trk* to avoid recognition with other members of the TK family of genes, particularly with the closely related *trk*B gene (Klein et al. 1989). We were concerned that the small exonic components of pDM89 and pDM92 (Fig. 1A) would not provide enough probe sequence complexity to recognize the presence of a low-abundance *trk* transcript. We therefore used both plasmid inserts together, but labeled independently, as probes in a Northern analysis performed with total mouse embryo RNA. The result of this experiment is shown in Figure 2. The first appearance of *trk* transcripts was observed at E9.5 as a single, 3.2-kb species. The intensity of the *trk*-specific band increases to a maximum level at E13.5 followed by a decrease such that it is no longer detectable in adult tissues (not shown). The same filter was hybridized with a β-actin probe to assess the relative amounts of RNA loaded in each lane, and the results are shown in Figure 2. We also tested for *trk* expression in embryonic stem (ES) cells and failed to detect transcripts (Fig. 2). ES cells are derived from the inner cell mass of the mouse blastocyst and may thus be considered representative of a component of the late blastocyst (E4.5), which can participate in normal development when reintroduced into early embryos (for review, see Robertson 1987). This result is consistent with the observed expression in embryonic RNAs.

Therefore, at the resolution provided by our Northern analysis, the *trk* gene is not expressed in preneurulation embryos through 8.5 days of gestation (stage E8.5). The appearance of *trk*-specific transcripts coincides with the timing of cephalization, neural crest migration, spinal ganglia formation, and the morphogenesis of most cranial ganglia. Thus, *trk* mRNA appearance in the mouse

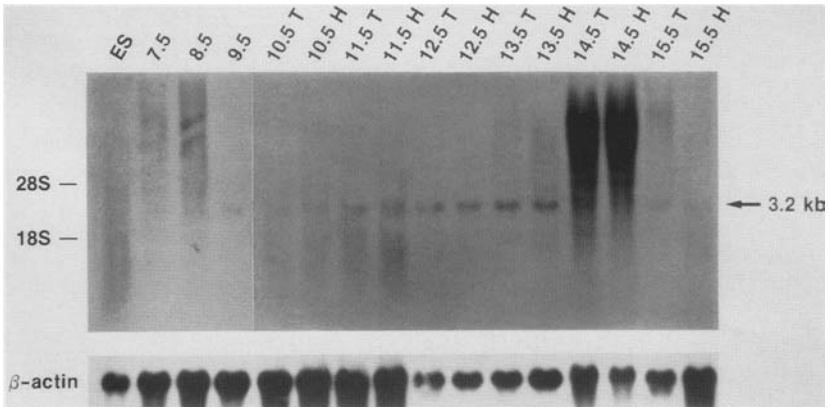


Figure 2. Northern blot hybridization of total embryo RNA with mouse genomic *trk* probes pDM89 and pDM92. Above each lane the gestation ages of the embryo samples are indicated, as well as the origin [(H) head; (T) trunk] of the RNAs. (Lane ES) Total RNA from the ES cell line CP1. Migration of the 3.2-kb *trk*-specific mRNA (arrow) and of the 28S and 18S rRNAs are indicated. Samples in lanes labeled ES, 8.5, 14.5T, and 14.5H are contaminated with embryo DNA, seen as a high-molecular-weight smear. (*Bottom*) Hybridization of the same filter with a chicken β-*actin* cDNA probe.

embryo coincides with major morphogenic events in the developing nervous system.

trk *is expressed in sensory ganglia of the peripheral nervous system*

Having ascertained that *trk* was expressed in the embryo, we performed in situ hybridization analysis on sections prepared from mouse embryos to determine the spatial distribution of transcripts in search of clues about its function. We began our analysis by using sections from the developmental stages (E12.5–E14.5) that demonstrated highest expression, as seen in Figure 2, and extended the analysis to include all stages that had been tested by Northern analysis. No *trk*-specific labeling was detected in embryo sections prior to E9.5 (data not shown), which coincides with the results seen in Figure 2.

By E9.5, when a *trk*-hybridizing band is first seen by Northern analysis (Fig. 2), morphogenesis of the peripheral nervous system (PNS) has commenced and the DRG are condensing from the migrated neural crest lateral to the neural tube. Appearance of *trk* labeling begins here, in embryo sections, as a faint but reproducible signal that is associated with the site of condensing DRG (Fig. 3A–C). This *trk*-specific labeling of the spinal ganglia is even more apparent a day later in gestation (E10.5) when the fusiform, but distinct, spinal ganglia are clearly labeled (Fig. 3D–G). As development proceeds, and organogenesis unfolds, the cell-type complexity in the embryo increases, but *trk* expression remains restricted to the same components of the developing PNS.

trk *mRNA levels remain constant during late development*

By 13.5 days of gestation, the embryo is at a stage when the major organs are formed and easily discernible. The *trk* mRNA levels reach a peak at this stage, as shown by Northern analysis (Fig. 2). We therefore hybridized diverse sections of the embryo to search for additional sites of *trk* transcript that may account for the observed increase in levels of mRNA. Figure 4, A and B, shows a parasagittal section through an entire E13.5 embryo, demonstrating strong labeling of the ganglion of the fifth cranial nerve (trigeminal). The acoustic ganglion (VIII), a sensory ganglion derived from the otic placode (Le Douarin 1982), is visualized in the same section and does not express the *trk* gene at this stage (see arrowhead in Fig. 4B). Higher magnification (Fig. 4C,D) shows the trigeminal nerve arriving into the ganglion from the base of the myelencephalon (*) and the emanating nerve that will branch toward diverse facial structures; neither nerve bundle contains detectable *trk* transcripts, indicating the exclusive *trk* transcription within the cells of the trigeminal ganglion and the exclusion of these mRNAs from the nerve processes.

The specificity of *trk* transcription for DRG and its scope of expression along the craniocaudal axis is illustrated in Figures 5 and 6 for lumbar and cervical DRG. In Figure 5, A and B, light- and dark-field views of a frontal section through an E14 embryo are shown. The only DRG visible, at the level of the metanephros, are labeled by the *trk* probe, whereas the circumscribed spinal cord and the brain, in addition to various nonneuronal organs, are not labeled. Figure 6, A and B, affords a closer view of the hybridizing region. A sagittal section through an E14.0 embryo (Fig. 5C,D) also provides a view of various neuronal and nonneuronal structures that do not express *trk*; notably, neither the inferior ninth and tenth (petrosal and nodose) cranial ganglia nor the various brain folds (telencephalon, mesencephalon, and myelencephalon) display any reactivity with the *trk* probe. Dorsally, below the myelencephalon, the cranial-most DRG is seen, and is the only structure in this plate expressing *trk*. The hybridizing region is magnified in Figure 6, C and D. We have hybridized representative frontal sections that permitted analysis through various ventral–dorsal levels. This analysis confirmed the presence of *trk* messenger in apparently all DRG, but the absence of expression in other neuronal structures, such as brain, optic nerve, retina, and visceral and somatic motor cranial ganglia. Summary of the data indicates that *trk* expression is confined to visceral sensory ganglia, including the superior ninth and tenth cranial (superior and jugular; see Fig. 7) ganglia, which are also of neural crest origin.

We then extended this analysis for *trk* expression through the remaining stages of fetal development and observed no deviation from the patterns already described. Examples of expression in late-stage embryos are shown in Figure 7. Figure 7, A and B, shows a midsagittal section through the head region of an E17.5 embryo, which brings to view the cranial-most DRG (that form the cervical and brachial plexuses) and various sensory and motor cranial ganglia, including the fifth, seventh, eighth, and ninth/tenth inferior and superior complexes. Only sensory, crest-derived structures (DRG, fifth, and superior ninth/tenth ganglia) maintain *trk* expression. Finally, a frontal view of an E17.5 embryo (Fig. 5C,D) illustrates continued *trk* expression in DRG along the entire length of the spinal chord at this late stage. Thus, cranial ganglia that are not strictly sensory, such as the inferior ganglia of the ninth and tenth nerves (petrosal and nodose) and the facial (seventh) ganglion, or ganglia that are sensory but of placodal origin, like the acoustic (eighth) ganglion, do not express the *trk* gene. This designates the *trk* gene as a marker for a unique subset of ganglia and suggests the existence of neurons specific among these structures.

Discussion

In this study we report the molecular cloning, characterization, and developmental expression profile of the mouse *trk* proto-oncogene. Partial sequence and detailed Southern blot hybridization analysis allow us to conclude that the mouse *trk* gene is highly conserved along its entire coding region with the human counterpart, which contains an amino-terminal extracellular do-

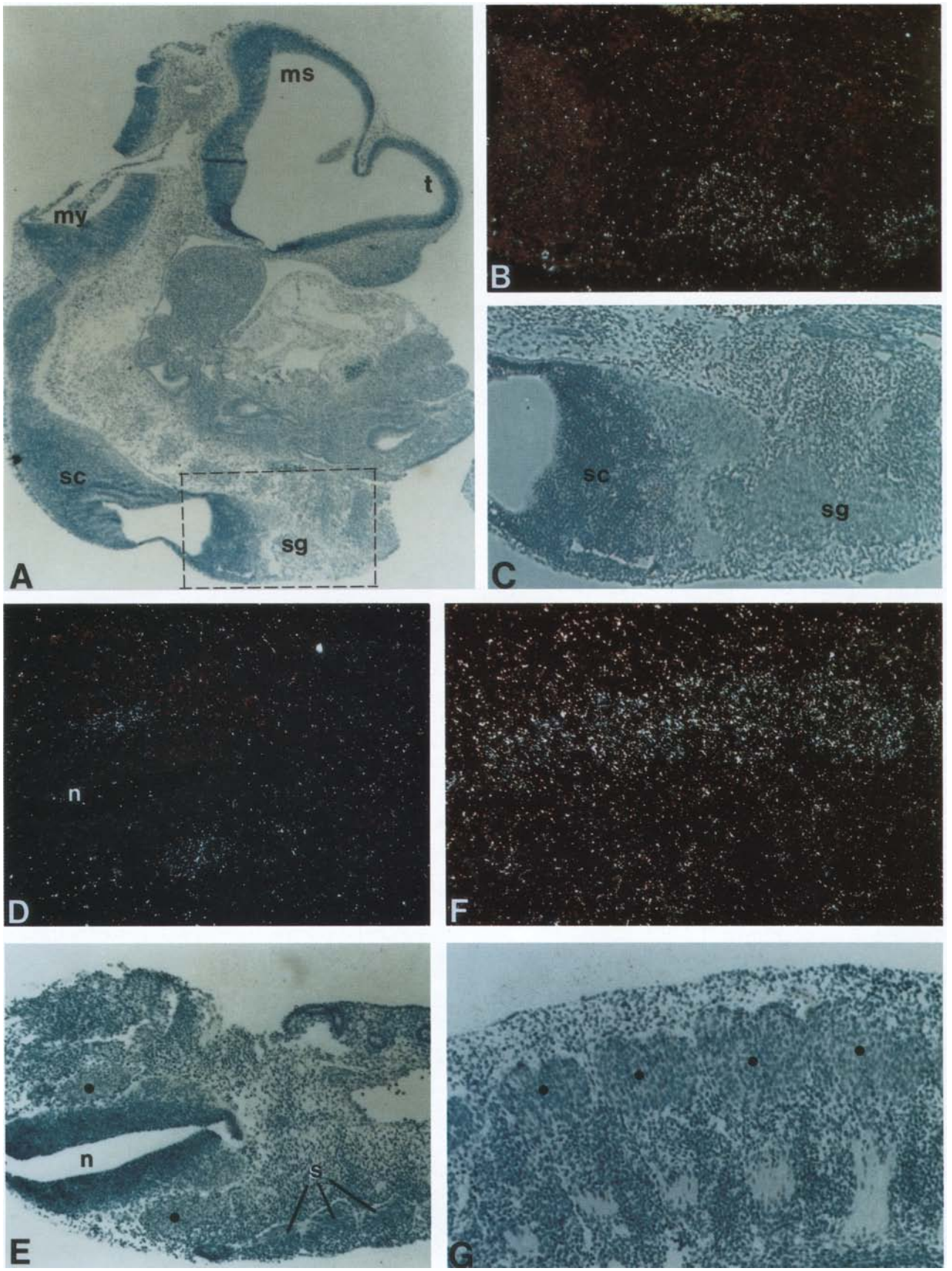


Figure 3. *(See following page for legend.)*

main, a hydrophobic transmembrane domain, and a carboxy-terminal TK domain. Throughout development, the transcript for this gene is found exclusively in the sensory cranial and spinal ganglia of neural crest origin.

The developmental profiles of *trk* expression provided by the in situ data and by Northern analysis are in agreement. We are unable to detect *trk* mRNA in preneurulation stages, but once the neural crest component commences differentiation into spinal ganglia, a faint 3.2-kb band becomes apparent by Northern analysis (Fig. 2), which is reflected, by in situ hybridization, in a weak but distinct signal seen in condensing DRG of the corresponding embryo sections (Figs. 3 and 4). By E13.5, when the spinal and cranial ganglia have enlarged and represent a significant proportion of the embryo mass, the highest levels of *trk* are also visualized by Northern analysis (cf. Figs. 2 and 5), and, by in situ analysis, the labeling of the pre-eminent sensory cranial and spinal ganglia is high.

trk *transcription is maintained at high levels*

Extensive growth and development of the CNS and internal organs occurs in the late fetal stages, resulting in a differential growth that reduces the cranial and spinal ganglia to minor components of the total embryonic mass. This resulted in a decrease of signal for *trk*-specific transcripts by Northern analysis and gave the erroneous impression that *trk* expression diminished in late fetal stages. The use of in situ hybridization has allowed the localization of these transcripts and the demonstration that *trk* expression is sustained at similar levels in the same structures of the PNS throughout embryonic development.

During embryogenesis, the cells of the PNS undergo tremendous proliferation, migration, and differentiation. Once the ganglia are formed, nerve cell bodies and neural crest (pre-Schwann) cells extend and migrate, respectively, to the sites of innervation. It is possible that *trk* plays a role in one or several of these activities. We favor the notion that *trk* is expressed in neurons, and not in satellite, glial, or Schwann cells, for the following reasons. First, Schwann cells migrate along the axonal pathways in an undifferentiated, premitotic state. The presence of *trk* transcripts in these cells would be detected in our experiments as a highlighting of the axonal tract pathways—a feature that we have observed with the highly related *trk*B gene (Klein et al. 1989 and in press) but not with *trk*. Second, various cranial ganglia that do not express *trk* are populated by satellite cells of neural crest origin (i.e., the seventh, eighth, and inferior ninth/tenth complex; see Le Douarin 1982); therefore the distinguishing feature of the *trk*-positive ganglia is the origin and functionality of their neurons. Third, histological and tritiated thymidine studies in embryonic rat and mouse DRG indicate that proliferation of satellite cells within the spinal ganglia occurs after the proliferative stage of the neurons (i.e., after E15.0; Lawson et al. 1974; for references, see Altman and Bayer 1984). Thus, the considerable *trk* expression observed prior to E15.0 is arguably present in neurons; furthermore, no relative increase in expression is observed with the onset of satellite cell proliferation. Finally, preliminary in situ analysis of adult mouse cranial and spinal ganglia, where histological discrimination of cell types is unambiguous, indicates that expression is localized exclusively to neurons (D. Martin-Zanca and L.F. Parada, unpubl.). Thus, *trk* expression is maintained throughout the life of the organism, suggesting that whatever function *trk* has, it is not solely required in early embryogenesis.

TK receptors have developmental functions

Until recently, TK receptors were primarily implicated in cell proliferation control, due mostly to the identification of many as growth factor receptors and also to the involvement of several of these genes in oncogenesis. Reports have now begun to emerge that implicate members of the TK receptor family in embryonic development. The *W* locus in mouse encodes the c-*kit* proto-oncogene, a TK receptor (Yarden et al. 1987). Mutations at this locus have pleiotropic developmental effects, including sterility, anemia, and white coat color, which result from the malfunction of this receptor in the regulation of the proliferative and/or migratory capacity of melanoblasts, primordial germ cells, and hematopoietic stem cells (Silvers 1979; Chabot et al. 1988; Geissler et al. 1988). The *Drosophila* genes *torso* (Sprenger et al. 1989) and *sevenless* (Banerjee et al. 1987; Hafen et al. 1987; Basler and Hafen 1988) also encode TK receptors. The *torso* product is required for the terminal formation of the embryonic anterior and posterior structures, whereas *sevenless* is required for the morphogenesis of photoreceptor R7 in the retina. The structural similarities of the *trk* protein with *torso* and *sevenless* and with the protein products of the proto-oncogenes c-*fms* (CSF-1 receptor) and *Erb*B (EGF receptor) and the insulin receptor (Martin-Zanca et al. 1989), among others, support the notion that the *trk* protein may function as the receptor for a morphogenic, growth, or differentiation factor in the mouse PNS.

The neural crest of the early embryo gives rise to a diverse series of cell and tissue types in the adult, including the head skeleton and mesenchyme, melanin-containing pigment cells, sympathetic and parasympathetic ganglia of the autonomous nervous system, and, of particular interest here, dorsal root, trigeminal, jugular, and superior ganglia (Le Douarin 1982, 1986;

Figure 3. In situ hybridization of early postimplantation embryos with a *trk* probe. (*A*) Bright-field sagittal section of an E9.5 embryo. Dark-field (*B*) and bright-field (*C*) view of the boxed area in *A*. Dark-field (*D*) and bright-field (*E*) longitudinal view of an E10.5 tail section. Dark-field (*F*) and bright-field (*G*) sagittal view of the spinal ganglia in an E10.5 embryo. (t) Telencephalon; (ms) mesencephalon; (my) myelencephalon; (sc) spinal cord; (sg) spinal ganglia; (n) neural canal; (s) somites; (·) spinal ganglia.

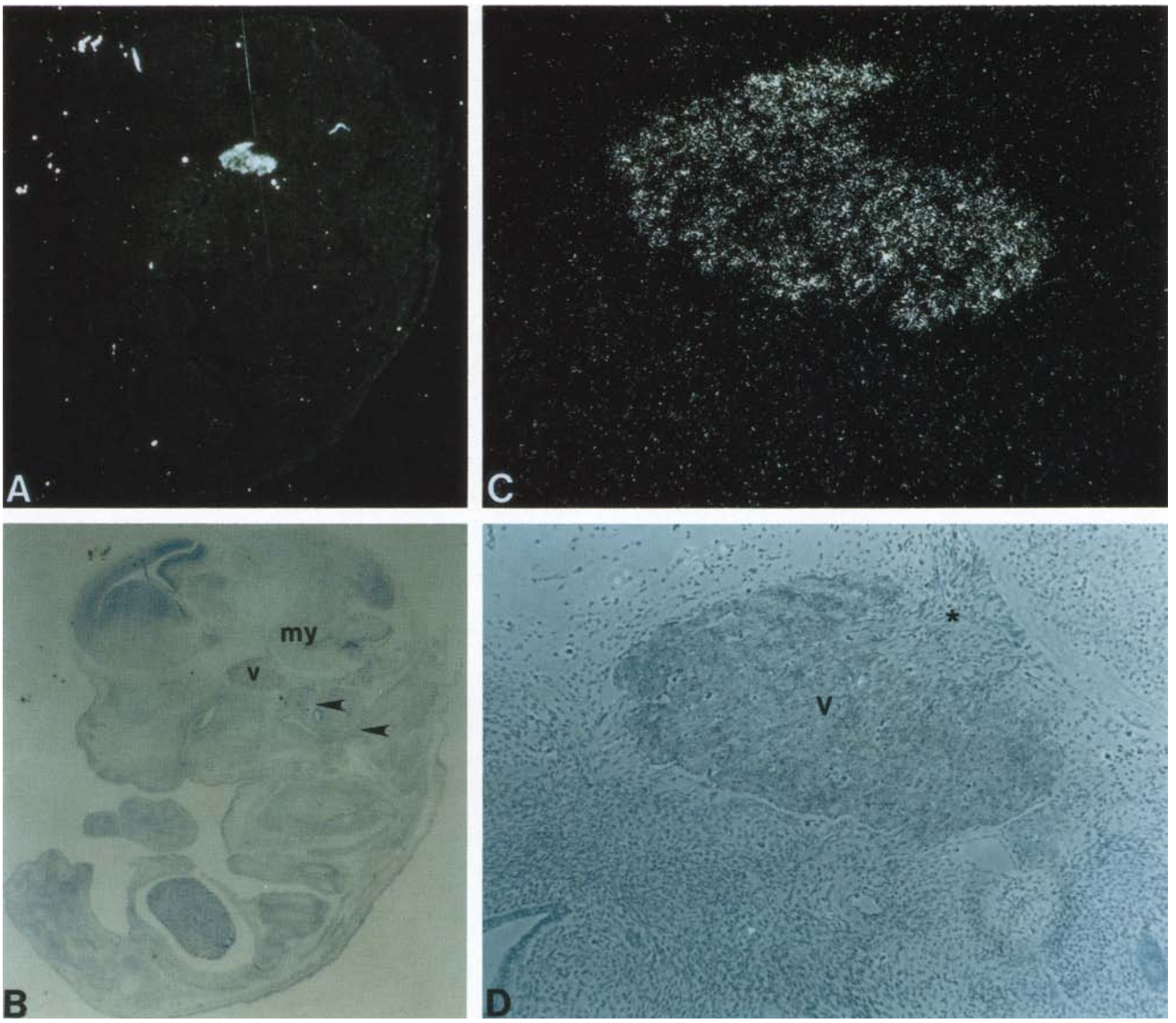


Figure 4. Expression of *trk* in trigeminal ganglion. Dark-field (*A*) and bright-field (*B*) view of a sagittal section from an E13.5 embryo. (V) Fifth ganglion; (my) myelencephalon. Arrowheads indicate acoustic and tenth ganglion. Dark-field (*C*) and bright-field (*D*) enlarged view of the trigeminal ganglion. (*) Boundary cap abutting the myelencephalon.

Sieber-Blum 1989). We show a specific and exclusive expression of a TK transmembrane receptor in these sensory ganglia early in development, whereas sensory ganglia of placodal origin (i.e., olfactory, optic, acoustic) do not express the *trk* gene. This work identifies the *trk* proto-oncogene as an early molecular marker for the sensory ganglia of cranial nerves V, IX, and X and for DRG, suggesting the existence of cell types unique to these sensory neural-crest-derived structures. By analogy to other TK receptors with roles in embryogenesis, and in light of the tight regulation observed for this gene, it is possible that the *trk* protein performs an important function in the development and/or specific phenotype of neurons within these sensory ganglia.

Methods

Mice and embryos

Mouse embryos were derived from C57BL/6 NCR × C3H/HeN MTV$^-$ F_2 litters. For RNA preparation and paraffin imbedding, embryos were staged under the dissecting microscope by counting somites through midgestation, and when somites were no longer visible in older embryos, external markers such as state-of-limb development were used (Theiler 1972). The morning of vaginal plug was considered day 0.5 (E0.5).

In situ hybridization

In situ hybridization protocols were as described (Hogan et al. 1986). Briefly, dissected embryos were fixed overnight in 4%

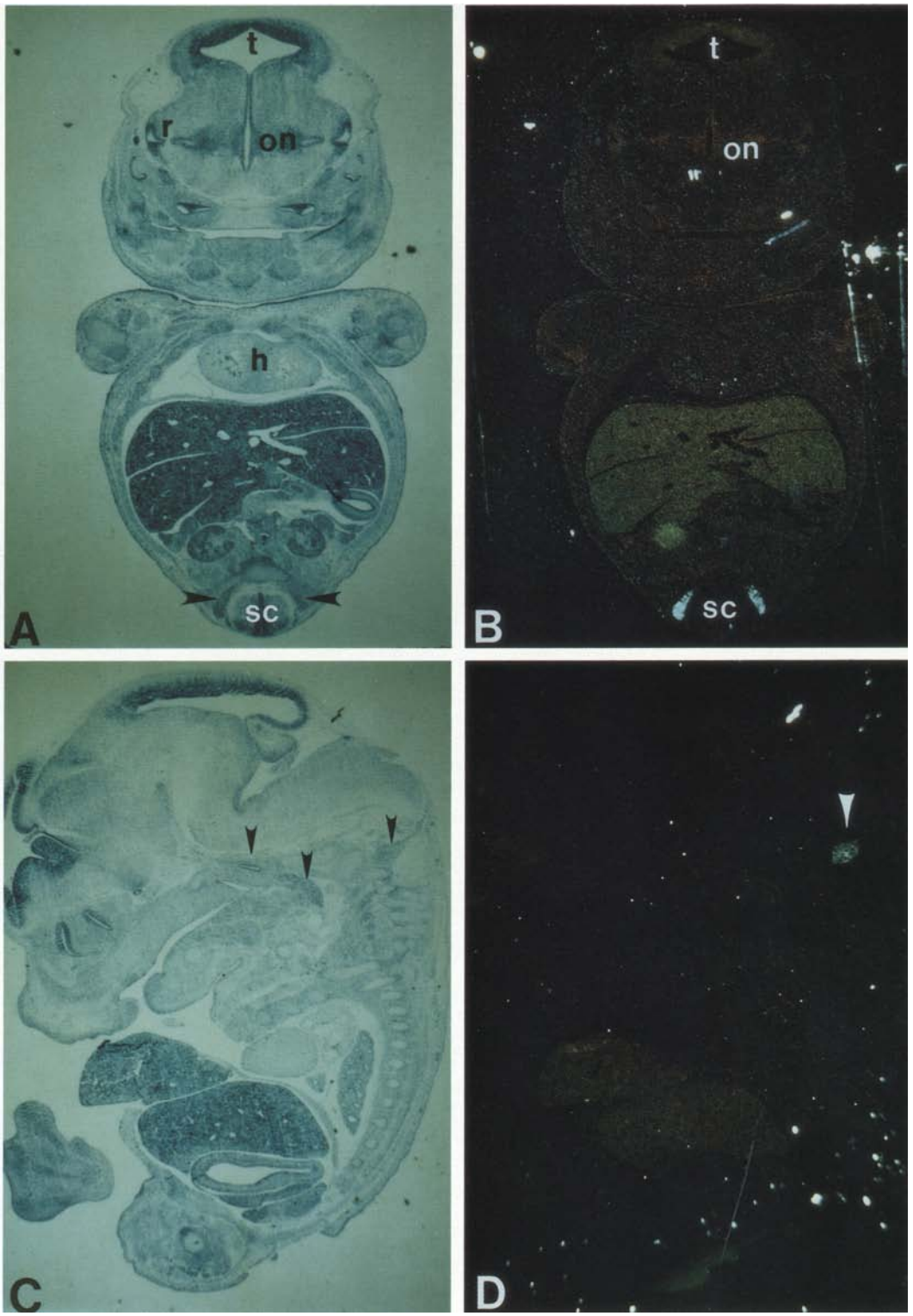


Figure 5. *trk* expression in caudal and rostral DRG. Bright-field (*A*) and dark-field (*B*) frontal section of an E13.5 embryo illustrating *trk* expression in the DRG at the level of embryonic kidney. (t) Telencephalon; (r) retina; (on) optic nerve; (h) heart; (sc) spinal cord. Arrowheads in *A* indicate DRG (see also Fig. 6A,B). Bright-field (*C*) and dark-field (*D*) parasagittal views of an E13.5 embryo. Arrowheads in *C* indicate cranial ganglia VIII, IX, and X (inferior complex) and the rostral-most DRG, which are also noted in *D* (see also Fig. 6C,D).

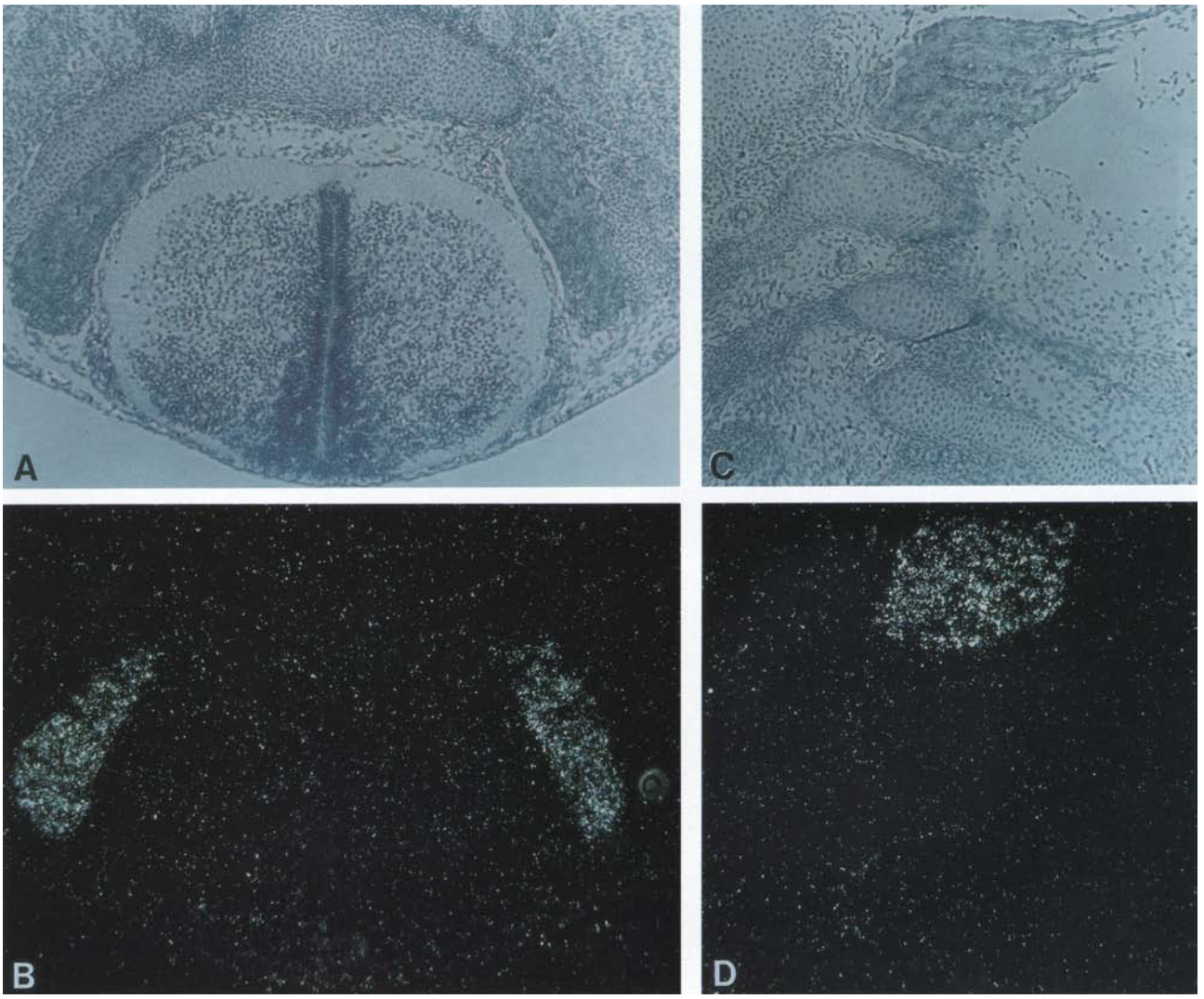


Figure 6. *trk* expression in DRG. Bright-field (*A*) and dark-field (*B*) are magnified views of the caudal DRG shown in Fig. 5, A and B. (*C* and *D*) Magnified views of the cervical DRG shown in Fig. 5, C and D.

paraformaldehyde, dehydrated with alcohols and xylenes, and embedded in paraffin. Embryos were sectioned at 5-μm thickness and mounted on gelatin-coated slides. Slides were deparaffinized, digested with proteinase K (Boehringer–Mannheim), acetylated with triethanolamine, dehydrated, and hybridized overnight with 5×10^5 cpm of ^{35}S-labeled riboprobe at 50°C. The slides were washed in hybridization buffer at 37°C for several hours, treated with ribonucleases at 65°C for 30 min, rinsed in 2× SSC, dehydrated, and dipped in Kodak emulsion NTB-2. After drying, the slides were stored for 8 days at 4°C, developed in Kodak D-19, and fixed as recommended by the manufacturer. All dark- and bright-field photomicroscopy was done on a Zeiss Axiophot microscope. RNA probes labeled with ^{35}S were prepared by standard procedures (Krieg and Melton 1987) by using either UTP or CTP (Amersham) as the labeled nucleotide.

The specificity of our *trk* probe is supported by the use of probes from different regions of the cDNA, which we have recently isolated from embryonic RNA. Furthermore, heterologous probes applied to sections adjacent to those presented here, including the related *trk*B gene, display distinct patterns of expression, recognizing different neuronal components (Klein et. al. 1989, and in prep.; D. Sassoon and L.F. Parada, in press).

Southern transfer analysis

DNAs (5 μg) isolated from human HeLa cells or C57BL/6J mouse tails were digested with *Bam*HI or *Hin*dIII restriction enzymes and electrophoresed through 1% agarose gels. Gels were irradiated with UV light (λ = 254 μm) for 4 min, soaked in alkali (0.5 M NaOH, 1.5 M NaCl) for 45 min, and transferred to ZETABIND membranes (Cuno, Inc.), using the same alkali solution.

Probes were ^{32}P-labeled either by nick-translation (Amersham kit) or by random priming procedures (Boehringer–Mannheim kit). Hybridizations were carried out at 65°C in 4× SSCP, 1× Denhardt's, and 1% SDS, when high stringency was required, or at 55°C in 5× SSCP, 1× Denhardt's, and 1% SDS for low stringency. Filters were washed twice in 0.1× SSCP,

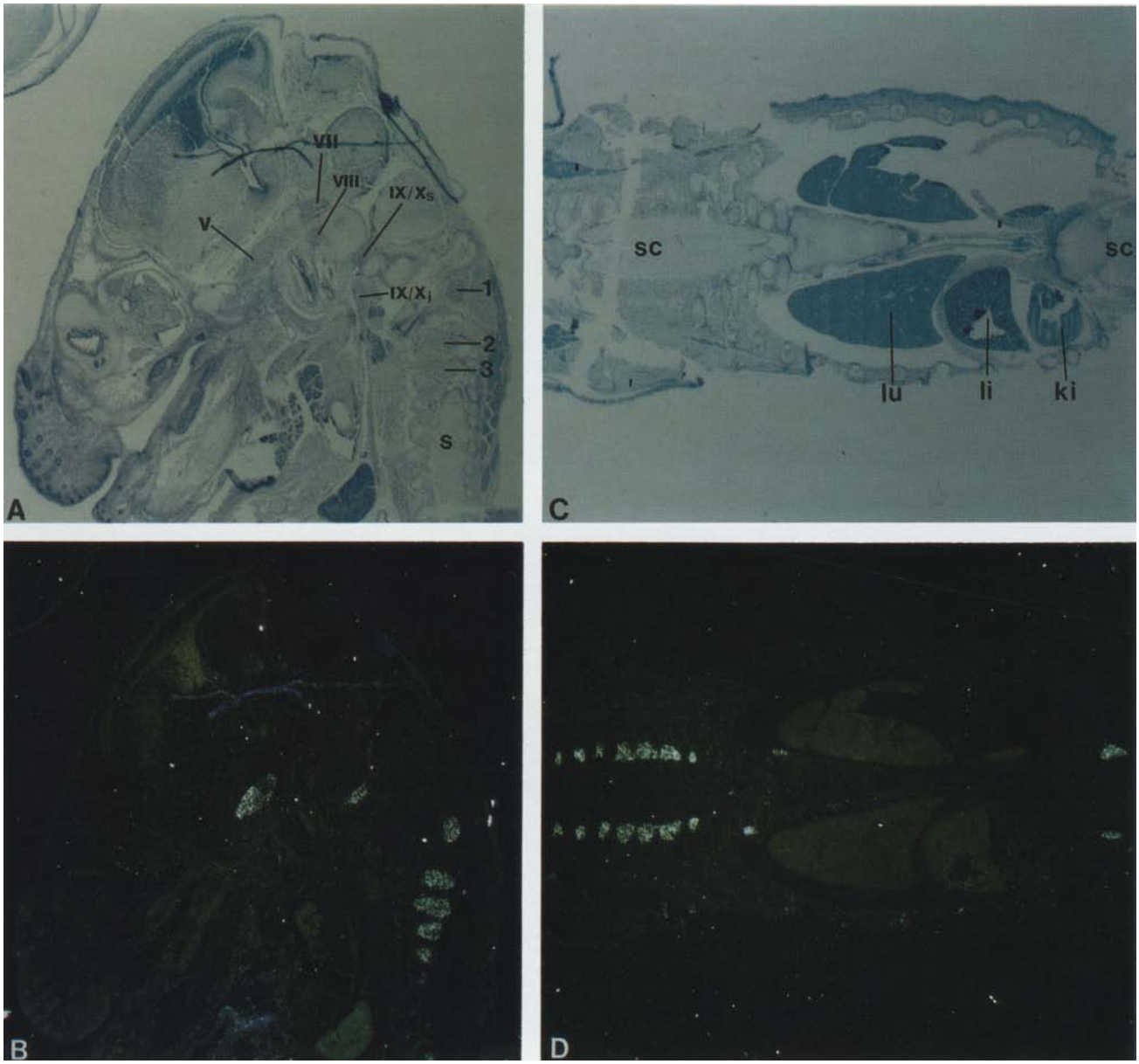


Figure 7. *trk* expression in late-stage embryos. (*A* and *C*) Bright-field views of E17.5 embryo sections, with corresponding dark-field views (*B* and *D*). (V) Fifth cranial ganglion; (VII) seventh cranial, (VIII) eighth cranial, (IX/X_s) ninth/tenth superior complex; (IX/X_i) ninth/tenth inferior complex. (1, 2, and 3) The first through third DRG (cervical plexus); (s and sc) spinal cord; (lu) lung; (li) liver; (ki) kidney.

0.1% SDS at 65°C, or in 0.2× SSCP, 0.1% SDS at 55°C, according to the stringency required.

Library screening

A genomic mouse NIH-3T3 library (from cells transformed with the human *vav* oncogene) (Katzav et al. 1989) was prepared. For this purpose NIH-3T3 DNA was partially digested with *Sau*3A1 and fractionated in sucrose gradients. Fractions containing 15- to 22-kb-long DNA fragments were ligated to phage λEMBL-4 arms (obtained by double digestion of this vector with the enzymes *Bam*HI and *Sal*I, followed by isopropanol precipitation). One and a half million recombinant phage λ clones were hybridized with a nick-translated probe corresponding to the complete 2.7-kb human *trk* proto-oncogene cDNA (Martin-Zanca et al. 1989) under conditions of moderate stringency (42°C, 40% formamide, 5× SSC). Filters were washed twice at 45°C in 0.1× SSC, 0.1% SDS.

RNA isolation and Northern analysis

Embryos were dissected under the microscope, as detailed above, frozen in liquid nitrogen, and stored at −70°C. RNA was extracted by using RNAzol (Cinna/Biotecx) following the manufacturer's recommendations. For Northern analysis, 20 μg of total RNA was electrophoresed through 1.2% agarose gels containing 0.37 M formaldehyde, as described (Maniatis et al. 1982), transferred to ZETABIND membranes, and hybridized at 65°C

according to Church and Gilbert (1984). Washes were carried out at 65°C in 0.5% BSA, 40 mM phosphate buffer (pH 7.2), 1% SDS.

Construction of probes

The *trk*-specific DNA fragments used for Northern analysis and in situ studies were inserted into SP6 or T7 promoter-containing plasmids such that antisense exonic sequences could serve as templates for RNA probes (Fig. 1A). pDM-81 contains a 1.9-kb *Kpn*I–*Eco*RI fragment that hybridizes with the human TK domain. pDM-89 was generated by subcloning a 123-bp *Bgl*II–*Hin*dIII DNA fragment, corresponding to a mouse *trk* exon 88% identical to nucleotides 814–934 of the human *trk* proto-oncogene cDNA (Fig. 1A; Martin-Zanca et al. 1989), between the *Bam*HI and *Hin*dIII sites of the vector pGEM-3Z (Promega). The *Bgl*II site located at the 5′ end of this exon occurs naturally, whereas the *Hin*dIII site located at the 3′ end was artificially introduced by DNA amplification using *Taq* polymerase (Mullis and Faloona 1987). The fidelity of the amplification was confirmed by sequencing of the resulting plasmid pDM-89 (not shown). pDM-92 was generated by subcloning of a blunt-ended 201-bp *Nar*I DNA fragment, corresponding to a portion of the mouse *trk* first exon (Fig. 2A), into the *Hin*cII site of the vector pGEM-3Zf(+) (Promega).

Acknowledgments

We thank Gretchen White and Aliki Grammatikakis for their assistance with embryo preparations. Shula Katzav provided the mouse genomic library, and Jose-Luis Barredo assisted with screening. We are grateful to the members of the Mammalian Genetics Laboratory for their encouragement and discussions. We thank Tom Jessell for helpful discussions, Neal Copeland and Peter Donovan for critically reading the manuscript, and Robin Handley for help in its preparation. This research was supported by the National Cancer Institute, Department of Health and Human Services, under contract N01-CO-74101 with BRI. The NCI–Frederick Cancer Research Facility is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

References

Altman. J. and S.A. Bayer. 1984. The development of the rat spinal cord. In *Advances in anatomy, embryology, and cell biology*, vol. 85. Springer-Verlag, Heidelberg.

Awgulewitsch, A., M.F. Utset, C.P. Hart, W. McGinnis, and F.H. Ruddle. 1986. Spatial restriction in expression of a mouse homeo box locus within the central nervous system. *Nature* **320:** 328–335.

Banerjee, U., P.J. Renfranz, J.A. Pollock, and S. Benzer. 1987. Molecular characterization and expression of *sevenless*, a gene involved in neuronal pattern formation in the Drosophila eye. *Cell* **49:** 281–291.

Bargmann, C.I., M.-C. Hung, and R.A. Weinberg. 1986. Multiple independent activations of the *neu* oncogene by a point mutation altering the transmembrane domain of p185. *Cell* **45:** 649–657.

Basler, K. and E. Hafen. 1988. *sevenless* and *Drosophila* eye development: A tyrosine kinase controls cell fate. *Trends Genet.* **3:** 74–79.

Bishop, J.M. 1987. The molecular genetics of cancer. *Science* **235:** 305–311.

Chabot, B., D.A. Stephenson, V.M. Chapman, P. Besmer, and A. Bernstein. 1988. The proto-oncogene c-*kit* encoding a transmembrane tyrosine kinase receptor maps to the mouse *W* locus. *Nature* **335:** 88–89.

Church, G.M. and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci.* **81:** 1991–1995.

Coulier, F., D. Martin-Zanca, M. Ernst, and M. Barbacid. 1989. Mechanism of Activation of the Human *trk* oncogene. *Mol. Cell. Biol.* **9:** 15–23.

Dollé, P., J.-C. Izpisua-Belmonte, H. Falkenstein, A. Renucci, and D. Duboule. 1989. Coordinate expression of the murine *Hox*-5 complex homoeobox-containing genes during limb pattern formation. *Nature* **342:** 767–772.

Downward, J., Y. Yarden, E. Mayes, G. Scrace, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger, and M.D. Waterfield. 1984. Close similarity of epidermal growth factor receptor and v-*Erb*-B oncogene protein sequences. *Nature* **307:** 521–527.

Geissler, E.N., M.A. Ryan, and D.E. Housman. 1988. The dominant-white spotting (W) locus of the mouse encodes the c-*kit* proto-oncogene. *Cell* **55:** 185–192.

Hafen, E., K. Basler, J.E. Edstroem, and G.M. Rubin. 1987. *sevenless*, a cell-specific homeotic gene of *Drosophila*, encodes a putative transmembrane receptor with a tyrosine kinase domain. *Science* **236:** 55–63.

Hanks, S.K., A.M. Quinn, and T. Hunter. 1988. The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. *Science* **241:** 42–52.

Hogan, B., F. Costantini, and E. Lacy. 1986. *Manipulating the mouse embryo: A laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Holland, P.W.H. and B.L.M. Hogan. 1988. Expression of homeo box genes during mouse development: A review. *Genes Dev.* **2:** 773–782.

Jackson, I.J., P. Schofield, and B. Hogan. 1985. A mouse homoeo box gene is expressed during embryogenesis and in adult kidney. *Nature* **317:** 745–748.

Jakobovits, A., M. Schwab, J.M. Bishop, and G.R. Martin. 1985. Expression of N-*myc* in teratocarcinoma stem cells and mouse embryos. *Nature* **318:** 188–191.

Jakobovits, A., G.M. Shackleford, H.E. Varmus, and G.R. Martin. 1986. Two proto-oncogenes implicated in mammary carcinogenesis, *int*-1 and *int*-2, are independently regulated during mouse development. *Proc. Natl. Acad. Sci.* **83:** 7806–7810.

Joyner, A.L., T. Kornberg, K.G. Coleman, D.R. Cox, and G.R. Martin. 1985. Expression during embryogenesis of a mouse gene with sequence homology to the *Drosophila engrailed* gene. *Cell* **43:** 29–37.

Katzav, S., D. Martin-Zanca, and M. Barbacid. 1989. *vav* a novel human oncogene derived from a locus ubiquitously expressed in hematopoietic cells. *EMBO J.* **8:** 2283–2290.

Klein, R., L.F. Parada, F. Coulier, and M. Barbacid. 1989. *trk*B, a novel tyrosine protein kinase receptor expressed during mouse neural development. *EMBO J.* **8:** 3701–3709.

Klein, R., D. Martin-Zanca, M. Barbacid, and L.F. Parada. Expression of the tyrosine kinase receptor gene *trk*B is confined to the murine embryonic and adult nervous system. *Development* (in press).

Krieg, P.A. and D.A. Melton. 1987. In vitro RNA synthesis with SP6 RNA polymerase. *Methods Enzymol.* **155:** 397–415.

Land, H., L.F. Parada, and R.A. Weinberg. 1983. Cellular oncogenes and multistep carcinogenesis. *Science* **222:** 771–778.

Lawson, S.N., K.W.T. Caddy, and T.J. Biscoe. 1974. Development of rat dorsal root ganglion neurones. *Cell Tiss. Res.* **153:** 399–413.

Le Douarin, N.M. 1982. *The neural crest.* Cambridge Univer-

sity Press, Cambridge, England.
———. 1986. Cell line segregation during peripheral nervous system ontogeny. *Science* **231:** 1515–1522.
Maniatis, T., E.F. Fritsch, and J. Sambrook, 1982. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Martin-Zanca, D., S.H. Hughes, and M. Barbacid. 1986a. A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. *Nature* **319:** 743–748.
Martin-Zanca, D., G. Mitra, L.K. Long, and M. Barbacid. 1986b. Molecular characterization of the human *trk* oncogene. *Cold Spring Harbor Symp. Quant. Biol.* **51:** 983–992.
Martin-Zanca, D., R. Oskam, G. Mitra, T. Copeland, and M. Barbacid. 1989. Molecular and biochemical characterization of the human *trk* proto-oncogene. *Mol. Cell. Biol.* **9:** 24–33.
Muller, R. 1986. Proto-oncogenes and differentiation. *Trends Biochem. Sci.* **11:** 129–132.
Mullis, K.B. and F.A. Faloona. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* **155:** 335–350.
Nusse, R. 1988. The *int* genes in mammary tumorigenesis and in normal development. *Trends Genet.* **4:** 291–295.
Park, M., M. Dean, C.S. Cooper, M. Schmidt, S.J. O'Brien, D.G. Blair, and G.F. Vande Woude. 1986. Mechanism of *met* oncogene activation. *Cell* **45:** 895–904.
Pulciani, S., E. Santos, A.V. Lauver, L.K. Long, S.A. Aaronson, and M. Barbacid. 1982. Oncogenes in solid human tumors. *Nature* **300:** 539–542.
Robertson, E.J. 1987. Embryo-derived stem cell lines. *Teratocarcinomas and embryonic stem cells: A practical approach*, pp. 71–112. IRL Press, Washington, D.C.
Roussel, M.F., T.J. Dull, C.W. Rettenmier, P. Ralph, A. Ullrich, and C.J. Sherr. 1987. Transforming potential of the c-*fms* proto-oncogene (CSF-1 receptor). *Nature* **325:** 549–552.
Sieber-Blum, M. 1989. Commitment of neural crest cells to the sensory neuron lineage. *Science* **243:** 1608–1611.
Silvers, W.K. 1979. White-spotting, patch and rump-white. In: *The new coat colors of mice: A model for gene action and interaction*, pp. 206–241. Springer-Verlag, New York.
Sprenger, F., L.M. Stevens, and C. Nusslein-Volhard. 1989. The *Drosophila* gene *torso* encodes a putative receptor tyrosine kinase. *Nature* **338:** 478–483.
Theiler, K. 1972. *The house mouse: Development and normal stages from fertilization to 4 weeks of age*. Springer-Verlag, Berlin, Heidelberg.
Weinberg, R.A. 1985. The action of oncogenes in the cytoplasm and nucleus. *Science* **230:** 770–776.
Wilkinson, D.G., J.A. Bailes, and A.P. McMahon. 1987. Expression of the proto-oncogene *int*-1 is restricted to specific neural cells in the developing mouse embryo. *Cell* **50:** 79–88.
Wilkinson, D.G., G. Peters, C. Dickson, and A.P. McMahon. 1988. Expression of the FGF-related proto-oncogene *int*-2 during gastrulation and neurulation in the mouse. *EMBO J.* **7:** 691–695.
Wilkinson, D.G., S. Bhatt, M. Cook, E. Boncinelli, and R. Krumlauf. 1989. Segmental expression of *Hox*-2 homoeobox-containing genes in the developing mouse hindbrain. *Nature* **341:** 405–409.
Yarden, Y., W. Kuang, T. Yang-Feng, and A. Ullrich. 1987. Human proto-oncogene c-*kit:* A new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO J.* **6:** 3341–3351.
Zimmerman, K.A., G.D. Yancopoulos, R.G. Collum, R.K. Smith, N.E. Kohl, K.A. Denis, M.M. Nau, O.N. Witte, D. Toran-Allerand, C.E. Gee, J.D. Minna, and F.W. Alt. 1986. Differential expression of *myc* family genes during murine development. *Nature* **319:** 780–783.

Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development.

D Martin-Zanca, M Barbacid and L F Parada

Genes Dev. 1990, **4:**
Access the most recent version at doi:10.1101/gad.4.5.683