# *trk*B, a novel tyrosine protein kinase receptor expressed during mouse neural development

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We have isolated a novel member of the tyrosine protein kinase family of cell surface receptors. This gene. designated trkB, is highly related to the human trk protooncogene. At the amino acid level, their respective products share a 57% homology in their extracellular regions including 9 of the 11 cysteines present in the trk proto-oncogene. This homology increases to 88% within their respective tyrosine kinase catalytic domains. Both trk and trkB are equally distantly related to the other members of this gene family of receptors. A biologically active cDNA clone of trkB can direct the synthesis of gp145<sup>trkB</sup>, a glycoprotein of 145 kd of which only 93 kd correspond to its polypeptide backbone. In adult mice, trkB is preferentially expressed in brain tissue, although significant levels of trkB RNA have also been observed in lung, muscle and ovaries. In addition, trkB transcripts can be detected in mid and late gestation embryos. The trkB locus exhibits a complex pattern of transcription. At least seven RNA species ranging in size from  $\sim 9$  kb to 2 kb have been identified in brain. However, only a subset of these transcripts appears to be expressed in the other tissues. In situ hybridization analysis of 14 and 18 day old mouse embryos indicates that trkB transcripts are localized in the central (CNS) and peripheral (PNS) nervous systems, including brain, spinal cord, spinal and cranial ganglia, paravertebral trunk of the sympathetic nervous system and various innervation pathways. These results suggest that trkB may code for a novel cell surface receptor involved in neurogenesis.

Key words: cell surface receptor/neural development/tyrosine protein kinase

## Introduction

The receptors for a number of well-characterized growth factors including EGF (Downward *et al.*, 1984), insulin (Ullrich *et al.*, 1985), IGF-I (Ullrich *et al.*, 1986), CSF-1 (Coussens *et al.*, 1986) and PDGF (Yarden *et al.*, 1986) have been identified as members of the tyrosine protein kinase gene family. Other members of this gene family of receptors such as *erbB2/neu* (Schechter *et al.*, 1984), *kit* (Besmer *et al.*, 1986), *met* (Dean *et al.*, 1985), *ret* (Takahashi and Cooper, 1987), *ros* (Neckameyer *et al.*, 1986) and *trk* (Martin-Zanca *et al.*, 1986a) have become

known, not because of their physiological roles, but because of their ability to acquire oncogenic properties upon somatic mutation.

The *trk* locus was first identified in a human colon carcinoma where it became activated as an oncogene by a chromosomal rearrangement which fused its transmembrane and catalytic domains to a subset of sequences derived from a non-muscle tropomyosin gene (Martin-Zanca *et al.*, 1986a). Additional *trk* oncogenes carrying activating sequences other than tropomyosin have been generated during the course of gene transfer assays (Kozma *et al.*, 1988; Oskam *et al.*, 1988). More recently, *trk* oncogenes have been reproducibly identified in at least 25% of human papillary thyroid carcinomas (Bongarzone *et al.*, 1989).

Aside from its involvement in malignant transformation, little is known regarding the physiological role of the *trk* proto-oncogene. Expression studies using human tumor cell lines derived from different lineages did not reveal a pattern consistent with a role of the *trk* gene in a defined developmental process (Martin-Zanca *et al.*, 1986b). Since these tumor cell lines might exhibit either aberrant or ectopic *trk* gene expression, we decided to conduct similar expression studies using normal mouse tissues. These studies have not only yielded important information regarding the potential role of the *trk* proto-oncogene in mouse development (D.Martin-Zanca, L.F.Parada and M.Barbacid, submitted for publication) but have also allowed us to identify a novel *trk*-related gene that we have designated *trk*B.

In this report we describe the molecular structure and gene product of a biologically active cDNA clone of the mouse *trkB* gene. The results obtained indicate that *trkB* codes for a novel member of the tyrosine protein kinase family of cell surface receptors. In addition, analysis of the pattern of expression of the *trkB* locus in both adult and embryonic mouse tissues suggests that this novel receptor may play an important role in neural development.

# Results

## trk-related transcripts in adult mouse tissues

Previous studies have indicated that the human *trk* protooncogene is expressed in certain human cell lines derived from hematopoietic and mesenchymal tumors (Martin-Zanca *et al.*, 1986b). In order to study the pattern of *trk* protooncogene expression in normal cells, we isolated poly(A)containing RNAs from a variety of mouse tissues and submitted them to Northern blot analysis. Only RNAs isolated from brain and lung tissues appeared to contain *trk*related transcripts, albeit at very low levels. These transcripts, unlike the single 3 kb RNA species previously identified in human cells (Martin-Zanca *et al.*, 1986b), varied in size ranging from ~9 to ~2 kb (data not shown). Since these transcripts were detected with a probe of human origin under low hybridization stringency, we considered the possibility that at least some of them might have originated from other tyrosine protein kinase loci closely related to *trk*.

To test this hypothesis, we decided to characterize molecularly these trk-related transcripts. For this purpose, we screened a  $\lambda$ gt11 mouse brain cDNA library (Citri *et al.*, 1987) with a human trk tyrosine kinase-specific probe under relaxed hybridization conditions. Three recombinant bacteriophages containing identical 3.2 kb long cDNA inserts were isolated. This insert was subcloned in pBluescript and used to probe genomic DNA in order to determine whether it was derived from the mouse homolog of the human trk locus or from a related gene. Three human BamHI DNA fragments of 4.6, 3.3 and 1.5 kb were identified with this cDNA probe (not shown). None of these genomic DNA fragments corresponded to those previously shown to encompass the human trk locus (Martin-Zanca et al., 1986a). These results indicate that this 3.2 kb cDNA insert must have been derived from a mouse gene related to, but distinct from trk. This novel trk-related gene has been designated trkB.

#### Nucleotide sequence of trkB cDNA

Preliminary sequence analysis of the 5' end of this 3.2 kb trkB cDNA clone suggested that it may not contain the entire trkB coding sequence. Therefore we rescreened the above mouse brain cDNA library with a 200 bp DNA fragment derived from the 5' end of the 3.2 kb cDNA clone. Fifteen positive  $\lambda$ gt11 clones with inserts ranging from 1.3 to 2.6 kb were isolated. The longest 2.6 kb cDNA insert was subcloned in pBluescript and submitted to restriction endonuclease map analysis. Based on their respective restriction maps, we estimated that the 3.2 and 2.6 kb cDNA clones must contain an overlapping region spanning of at least 1 kb.

These overlapping cDNA clones were assembled into a single clone, pFRK43, which was subsequently submitted to nucleotide sequence analysis. Figure 1 depicts the nucleotide sequence of the 4351 bp long insert of pFRK43. Nucleotides 1-511 are likely to represent 5' non-coding sequences. A long open reading frame (ORF) extending from nucleotides 323 to 2974 was identified. This ORF contains an ATG codon at positions 512-514 that conforms to the canonical sequences of mammalian initiator codons described by Kozak (1987). A second in-frame ATG, with potential initiating function, is present in positions 545-547, just 11 codons downstream. Although no biological data are available, we have assumed that the first of the two in-frame ATGs serves as the translational initiator (Kozak, 1989). If so, pFRK43 has sufficient information to code for a protein of 821 amino acids with a molecular mass of 92 132 daltons. A 1376 bp long 3' non-coding domain (nucleotides 2975 – 4351) completes this cDNA insert. No consensus sequences for polyadenylation signal could be identified near the 3' terminus, suggesting that additional 3' sequences might be present in the corresponding *trk*B transcript. Four ATTT(A) motifs were identified in this 3' untranslated region. These sequences, previously found in several lymphokine and proto-oncogene mRNAs, appear to play a role in mRNA instability (Shaw and Kamen, 1986). Whether they play such a role in *trkB* transcripts remains to be determined.

## Deduced amino acid sequence

The deduced amino acid sequence of the putative polypeptide coded for by pFRK43 is depicted in Figure 1. The overall sequence exhibits a structure highly reminiscent of growth factor receptors of the tyrosine protein kinase gene family (Hanks *et al.*, 1988). The putative initiating methionine leads to a relatively basic stretch of 13 amino acids followed by a highly hydrophobic domain of 17 residues likely to serve as a signal peptide. When compared with the amino-terminal sequences of other eukaryotic signal peptides, the putative *trk*B gene product is likely to be cleaved between residues 31 and 32, at the Ala-Cys junction (von Heijne, 1986). If so, the mature *trk*B product will contain 790 amino acid residues.

The ensuing extracellular domain (amino acid residues 32-429) encompasses 12 consensus N-glycosylation sites (Asn-X-Ser/Thr) and 13 cysteines, four of which are clustered immediately after the putative signal peptide. Nine of these cysteines correspond to those present in the extracellular domain of the trk proto-oncogene (Martin-Zanca et al., 1989). A highly hydrophobic region of 24 amino acids (positions 430-453) is likely to serve as the anchoring transmembrane domain. This region is separated by a stretch of 89 amino acids from the ATP-binding motif,  $GXGXXGX_{21}K$  (residues 544-571), located near the amino terminus of the putative tyrosine protein kinase domain (Hunter and Cooper, 1985). Based on amino acid sequence homology with other tyrosine protein kinases, this catalytic domain is likely to extend from residues 543 to 806 (Figure 1). Ninety-nine of these 264 amino acids are either identical (49) or conserved (50) when compared with those of the corresponding domains of other members of the tyrosine protein kinase family of growth factor receptors, including the receptors for EGF (Downward et al., 1984), insulin (Ullrich et al., 1985), IGF-I (Ullrich et al., 1986), CSF-1 (Coussens et al., 1986) and PDGF (Yarden et al., 1986); the proto-oncogenes erbB2/neu (Coussens et al., 1985), kit (Besmer et al., 1986), met (Dean et al., 1985), ret (Takahashi and Cooper, 1987), ros (Neckameyer et al., 1986), trk (Martin-Zanca et al., 1986a, 1989) and the eph gene (Hirai et al., 1987). Finally, the putative trkB receptor molecule exhibits a rather short carboxy-terminal tail of 15 amino acids (Figure 1).

#### Homology with the human trk proto-oncogene

Alignment of the deduced amino acid sequence of the mouse *trk*B gene product with that of the human *trk* proto-oncogene revealed an overall homology of 69%, a highly significant



AGGCTGCGGCGGCGGCGGCGCGCGCGCGCGCGCGCGCGCTGCT	100
GGAAGCCGCGCGGGACAGTCCTCGGTGGCCCGGCACTGTCCTGCTACCGCAGTTGCTCCCCAGCCCTGAGGTGCGCACCGATATCGTGCCGGTTTAGCGGTTCTGCGAC	220
CCAAAGAGTCCAGGGAGATCCACCGAGTGGTGCCTGGTGTATAGGACTATGCAGCCGCCTTGTGGCTCGGAGCAGCGGCCCGCGATGTCCCAGCCACTGT <u>GA</u> ACCATTTGGTCAGCGCCA	340
ACCTGCCTAGCCCCAGCACCGACAGGCTCAGCCTCTGGTACGCTCCACTCCGCGGGAGGCCACCAGCACCAAGCAGCAAGAGGGCGCAGGAAGGCCTCCCCCCCC	460
IGGE TEAGEGIAGGGACACEGCACTECEACTGACTGGCACTGGGAGTCTGGGATGTCGECCTGGGTGAAGTGGCATGGACCCGCCATGGCGCGCTTAGGGCCTGGTGTCTTG	580
MetSerProirpLeuLysirphisGiyProAlaMetAlaArgLeuTrpGiyLeutevaXLeuLeuValLeu	
GGCTTCTGGAGGAGCTCCTCTCGCCCCCACGCTCCCCCACGCTCCCCACGCTCCCCCACGCCTCCCCCCCC	700
G1yPheTrpArgA1aSerLeuA1axxProThrSerQLysQWSerSerA1aArg11eTrpQSThrG1uProSerProG1y11eVa1A1aPheProArgLeuG1uProAshSerVa1	
63	
GACCEGGAGAACATCACEGGAAATTETCATTGCAAAACCAGAAAAGGCTAGAAATCATCAATGAAGATGACGTTGAAGCTTACGTGGGGCTGAGAAACCTTACAATTGTGGATTCCCGCTTA	820
AspProGluAsnIleThrGluIleLeuIleAlaAsnGlnLysArgLeuGluIleIleAsnGluAspAspValGluAlaTyrValGlyLeuArgAsnLeuThrIleValAspSerGlyLeu	
	940
LysPheValAlaTyrLysAlaPheLeuLysAsnSerAsnLeuArgHisIleAsnPheThrArgAsnLysLeuThrSerLeuSerArgArgHisPheArgHisLeuAspLeuSerAspLeu	
143	
ATCCTGACGGGTAATCCGTTCACG70CTCC70CGACATCATGTGGCTCAAGACTCTCCAGGAGACTAAATCCAGCCCCGACACTCAGGATTTGTACG66CCCAATGAGAGCAGCAAGAAA	1060
IleLeuThrG1yAsnProPheThrQxSerQxAspI1eMetTrpLeuLysThrLeuG1nG1uThrLysSerSerProAspThrG1nAspLeuTyrQx&LeuAsnG1uSerSerLysAsn	
	1180
MetProLeuAlaAshLeuGInIleProAshCyGIyLeuProSerAlaArgLeuAlaAlaProAshLeuIhrValGIuGIuGIyLysSerValThrLeuSerCvSserValGIyGIyAsp	
223	
CCACTCCCCACCTTGTACTGGGACGTTGGGAATTTGGTTTCCAAGCACATGAAACAAGCCACAAGCACACACGGGCTCCTTAAGGATAACGAAACATTTCATCTGATGACAGTGGAAAGCAAAGCACAAGCACACACGGGCTCCTTAAGGATAACGAAACATTTCATCTGATGACAGTGGAAAGCAAAGCACAAGCACACACGGGCTCCTTAAGGATAACGAACATTTCATCTGATGACAGTGGAAAGCAAAGCACAAGCACACACGGGCTCCTTAAGGATAACGAACATTTCATCTGATGACAGTGGAAAGCAAAGCACAAGCACACACGGGCTCCTTAAGGATAACGAACATTTCATCTGATGACAGTGGAAAGCAAAGCACACACGGGCTCCTTAAGGATAACGAACATTTCATCTGATGACAGTGGAAAGCAAAGCACACACGGGCTCCTTAAGGATAACGAACATTTCATCTGATGACAGTGGAAAGCAAAGCACACACA	1300
ProLeuProThrLeuTyrTrpAspValGlyAsnLeuValSerLysHisMetAsnGluThrSerHisThrGlnGlySerLeuArgIleThrAsnleSerSerAspAspSerGlyLysGl	
	1420
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303	
CCATTCACTGTGAGAGGCAACCCCAAGCCTGCGCTTCAGTGGTCTACAATGGGGCCATACTGAATGAGTCCAAGTACATC\\ACTAAGATCCACGTCACCAATCACACGGGGTACCAT	1540
ProPheThrValArgGlyAsnProLysProAlaLeuGInTrpPheTyrAsnGlyAlaIleLeuAsnGluSerLysTyrIleAthrLysIleHisValThrAsnHisThrGluTyrHis	
	1000
BUC TEAL TEAL TO BAT ARCECTATE TATA TARA TARA GAGAR LACACET GAT GOL ARGAREDAGTA TAGGARAGAA TAGGARAGAA TAATA TEAL ATA TAGGARAGAA T	1000
GTCGACTACGAGACAAACCCAAATTACCCTGAAGTCCTCTATGAAGACTGGACCACGCCAACTGACATTGGGGATACTACGAACAAAAGTAATGAAATCCCCTCCACGGATGTTGCTGAC	1780
ValAspTyrGluThrAsnProAsnTyrProGluValLeuTyrGluAspTrpThrThrProThrAspIleGlyAspThrThrAsnLysSerAsnGluIleProSerThrAspValAlaAspTyrGluThrAsnLysSerAsnGluIleProSerThrAspValAlaAspTyrGluThrAsnLysSerAsnGluIleProSerThrAspValAlaAspTyrGluThrAsnLysSerAsnGluIleProSerThrAspValAlaAspTyrGluThrAsnLysSerAsnGluIleProSerThrAspValAlaAspTyrGluThrAspTyrGluThrAsnLysSerAsnGluIleProSerThrAspValAlaAspTyrGluThrAsnLysSerAsnGluIleProSerThrAspValAlaAspTyrGluThrAspValAlaAspTyrGluThrAsnLysSerAsnGluIleProSerThrAspValAlaAspTyrGluThrAsnLysSerAsnGluIleProSerThrAspValAlaAspTyrGluThrAspValAlaAspTyrGluThrAsnLysSerAsnGluIleProSerThrAspValAlaAspTyrGluThrAspValAlaAspTyrGluThrAspValAlaAspTyrGluThrAspValAlaAspTyrGluThrAspValAlaAspTyrGluThrAspValAlaAsp	
423	
CARAGCAR LGGGAGCALTELLEGG ET A GEGEG GG TGG TGA TGGAT EG TGG GGAT EFGET GET GGEG GAT EFGET GGEG GAT EFGET GET GGEG GAT EFGET GGEG GAT EFGET GET GGEG GAT EFGET GET GGEG GAT EFGET GGEG GAT EFG	1900
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AAAGGCCCAGGTTACTACGAGAGAGAGAGAGACCCCCCCC	2020
LysG1yProAlaSerVallleSerAsnAspAspAspSerAlaSerProLeuHisHisIleSerAsnG1ySerAsnThrProSerSerG1uG1yG1yProAspAlaVallleIleG1y	
503	
ATGACCAAGATTCCTGTTATTGAAAACCCCCAGTACTTTGGCATCACCAACAGTCAGCCAGACACATTTGTTCAGCACATCAAGAGCACAACATCGTTCTGAAGAGGAAC	2140
MetinrtysiieProvaliieGluAsnProGiniyrPheGlylieInrAsnSerGinLeuLysProAspinrPhevalGinnisiieLysArgnisAshiievalLeuLysArguidLeu	TK
GGGGAAGGCCTTCGGGAAAGTTTTCCTTGCCGAGTGCTACAACCTCTGCCCAGAGCAGGATAAGATCCTGGTGGCTGTGAAGACGCTGAAGGACGCCAGCGACAATGCACGCAAGGACGC	2260
GIyGluGIyAlaPheGIyLysValPheLeuAlaGluCysTyrAsnLeuCysProGluGInAspLysIleLeuValAlaValLysThrLeuLysAspAlaSerAspAsnAlaArgLysAsp	
583	
TTTCATCGGGAAGCTGACCTGACCAACCTCCACCACCACCACCACTTGTCAAGTTCTACGGTGTGTGT	2380
PrentsargGtualaGtuLeuLeutinrasnLeuGtinristTevalLysznetyrGtyvalLysvalGtusvalGtustyrsproLeutiemetvalrieotorytmettysinisotyrsp	
CTCAACAAGTTCCTTAGGGCACACGGGCCCGACGCAGTGCTGATGGCAGAGGGTAACCCGCCCACAGAGCTGACGCAGTGCGCAGATGCTGCACATCGCTCAGCAAATCGCAGCAGTATG	2500
LeuAsnLySPheLeuArgAlaHisGlyProAspAlaValLeuMetAlaGluGlyAsnProProThrGluLeuThrGlnSerGlnMetLeuHisIleAlaGlnGlnIleAlaAlaGlyMet	
663	
GTCTACCTGGCGTCCCAACACTTTGTGCACCGTGACCTGGCGGCACTGCCCGGAACTGCCTGGTGGGAAATTGGGACTTTGGGACTTTGGGACTTGGGACGGGGGGGAGGAACTGCCTGGTGGGGGGGG	2620
VallyrLeuAlaSerGinHisPhevalHisArgASpLeuAlainrArgAShLysLeuValGiyGluAShLeuLeuValLysLieGiyAspreeliymetserHigASpvallyrSerHi 703	
GACTACTATCGGGTCGGTGGCCACAAATGTTGCCCATCCGATGGATG	2740
$\label{eq:linear} AspTyrTyrArgValGlyGlyHisThrMetLeuProIleArgTrpMetProProGluSerIleMetTyrArgLysPheThrThrGluSerAspValTrpSerLeuGlyValValLeuTrpOrtionActi$	
743	
GAGATCITCACCTACGGCAAGCAGCCCTGGTATCAGCTATCGAACAATGAGGTGATAGAGTGCATCACCCAGGGAAGAGTCCTTCACCGACGACCTGCCACGAGGGTGATGAG	2860
Glui lePhelhr Lyr GlyLys GinProl rplyr GinLeuSerAsnAsnGluVallieGluCysileinr GinGlyArgValLeuGinArgProArginr Cysroo incluVallyr Glu	
CTCATGCTCGGATGCTGGCAGCGGGAACCACCACCACCGCGAAGAACATCAAGAGCATCCACACCCTCCTTCAGAACTTGGCCAAGGCATCTCCCGTCACCCTGGATATCCTAGGCTAGGCTAGCGT	2980
LeuMetLeuG]yCysTrpG]nArgG]uProHisThrArgLysAsnI]eLysSerI]eHisThrLeuLeuG]nAsnLeuAlaLysA]aSerProVa]TyrLeuAspI]eLeuG]y***	
ТК 🛁 821	
CETECTICTEGECAGACEGICETTECCAAGGECETECTEAGAC IGGTECTECAGACIGGECTACAGGACGAACETETTGACTGECGETGAGGACETGETGETGETGETG	3100
and induced and a standard and a standard and a standard and a standard and and a standard and a standard and and a standard an	3340
AAAGGCCTTAACAAACCTAATTTGTTATATCAGCAGACACTCCAGTTTGCCCACCACACAATGCCTTGTTGTTCCTGCCTTGTGAGGGAGAAAAAAGAGAAAAAGGGCC	3460
AGACTCTCCTGCAGGAATCGGATGAGGCCTCTGAGCTCAAGCCCGTGGAACTGGACACTTTTGAAGGAAACATCACAAAGCAACTGGTGAAGAGCTCACCTCGGCTGAGGGGCCCGTCA	3580
CTACTGACAAGCTTTTCTTTGAAGGCTCTGTTGGTAGCGAGTCTGAAGGCTGGCCGGTCCTTTCTGGATGGCAGCCTGGAAGATGCCTTCAATGGACTCTTCCTTGCATTAGACCCACACA	3700
AGAAGCAGTACAAAAGAGTTCCAAGAATCTGAACCAAGAAGTCACCTACTTGGATGATGTTCTCAAAGATGCTAAACATCTTGAGGATCAGAGACTCAATGATGCTGCTGCCGCTCCCGGATGGAGA	3820
ICACAGAGGGTGAATGAGACAACCGGAG <u>ATTTAAAAGACTGAAGGACATTTTCCCATGTGCTTCTGTGTCATCCCAAGTGTCTGGGACAGATCCCCGGAAGGCCCTTCCTACCT</u>	3940
ADADILIGLARDODODATULICLIAGULAGADADADALAUGLAGOTICIGULULIIGLAADAIIIGIULGILIALULAULAULIAULIAULILULAATAAIGIGATAAGU CAGGCIAAAGCACIGGCATAGCAGCCACATAGCAGGCIICIGGTIGGCIGCAGGCGGCGGGCGGGGGGGGGG	4180

Fig. 1. Nucleotide sequence analysis of the 4351 bp long insert of pFRK43, a cDNA clone of the mouse *trkB* gene. (A) Schematic representation. The thin bar represents 5' and 3' untranslated sequences. The thick bar represents coding sequences which are flanked by the putative initiating (ATG) and terminating (TAG) codons. The putative signal peptide (SP, (A), transmembrane (TM,  $\blacksquare$ ) and tyrosine kinase (TK,  $\boxtimes$ ) domains are indicated. Other symbols represent cysteine residues ( $\bullet$ ) and consensus N-glycosylation sites ( $\nabla$ ). (B) Nucleotide and deduced amino acid sequence of the pFRK43 cDNA insert. The putative signal peptide (amino acid residues 1-31) is underlined by a cross-hatched bar. Cysteine residues in the extracellular domain are shaded. The consensus N-glycosylation sites are underlined by open bars. The putative transmembrane region (amino acids 430-453) is underlined by a solid bar. The catalytic tyrosine kinase domain (amino acid residues 543-806) is flanked by horizontal arrows. Inframe terminator codons in the 5' untranslated region and ATTT(A) motifs in the 3' noncoding region are underlined.

TCCGTGCCAAAGCAGCCTAGGGCAGGTGTATTTGAGCAGTTTCCTTTTCTG

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mouse	trkB	1	MSPWLKWHGPAMARLWGLCLLVLGFWRASLACPTSCKCSSARIWCTEPSPGIVAFPRLEPNSVDPE <u>NIT</u> EILIANOKRLEIINEDDVEAYVGLR <u>NLT</u> IVDSGLK 1	04
human	trk	1	I III I I IIIII I IIII I IIII II IIIII II IIII	04
mouse	trkB	105	FVAYKAFLKNSNLRHI <u>NFT</u> RNKLTSLSRRHFRHLDLSDLTLTGNPFT <mark>DSDDIMWLKTL.QETKSSPDTQDLYDLNES</mark> SKNMPLANLQIPMDGLPSARLAAP <u>NLT</u> VEEGK 2	12
human	trk	105	FVAPDAFHFTPRLSRL <u>NLS</u> FNALESLSWKTVQGLSLQELVLSGNPLHCSCALRWLQRWEEEGLGGVPEQKLQCHGQGPLAHMP <u>NASC</u> GVPTLKVQVP <u>NAS</u> VDVGD 2	09
mouse	trkB	213	SVTLSCSVGGDPLPTLYWDVGNLVSKHM <u>NET</u> SHTQGSLRIT <u>NIS</u> SDDSGKQISCVAENLVGEDQDSV <u>NLT</u> VHFAPTITFLESPTSDHHWCIPFTVRGNPKPALQWFY 3	19
human	trk	210	DVLLRDDVEGRGLEQAGWILTELEQSATVMKSGGLPSLGLTLA <u>NVT</u> SDLNRK <u>NLTD</u> HAENDVGRAEVSVQV <u>NVS</u> FPASVQ.LHTAVEMHHWSIPFSVDGQPAPSLRWLF	17
mouse	trkB	320	NGAIL <u>NES</u> KYICTKIHVT <u>NHT</u> EYHGCLQLD <u>NPT</u> HMNNGDYTLMAKNEYGKDERQISAHFMGRPGVDYETNPNYPEVLYEDWTTPTDIGDTT <u>NKS</u> NEIPSTDVADQSN 4	26
human	trk	318	<u>NGS</u> VL <u>NET</u> SFIFTEFLEPAA <u>NET</u> VRHGCLRLNQPTHVNNG <u>NYT</u> LLAANPFGQASASIMAAFMONPFEFNPEDPIPDT <u>NST</u> SGDPVEKKD 4	16
mouse	trkB	427	REHLSVYAVVVIASVVGFCLLVMLLLL.KLARHSKFGMKGPASVISNDDOSASPLHHISNGSNTPSSSEGGPDAVIIGMTKIPVIENPQYFGITNSQLKPDTFVQHIKR 5	34
human	trk	417	ETPFGVSVAVGLAVFACLFLSTLLLVLNKCGRRNKFGINRPA.VLAPEDGLAMSLHFMTLGGSSLSPTEGKUSGLQGHIIENPQYFSDACVHHIKR 5	,01
mouse	trkB	535	► TK HNIVLKRELGEGAFGKVFLAECYNLCPEQDKILVAVKTLKDASDNARKDFHREAELLTNLQHEHIVKFYGVCVEGDPLIMVFEYMKHGDLNKFLRAHGPDAVLMAEGN. 6	43
human	trk	502	RDIVLKWELGEGAFGKVFLAECHNLLPEQDKMLVAVKALKEASESARQDFQREAELLTMLQHQHIVRFFGVCTEGRPLLMVFEYMRHGDLNRFLRSHGPDAKLLAGGED 6	10
mouse	trk8	644	.PPTELTQSQMLHIAQQIAAGNVYLASQHFVHRDLATRNCLVGENLLVKIGDFGMSRDVYSTDYYRVGGHTMLPIRWMPPESINYRKFTTESDVWSLGVVLWEIFTYGK 7	50
human	trk	611	VAPGPLGLGQLLAVASQVAAGNVYLAGLHFVHRDLATRNCLVGQGLVVKIGDFGMSRDIYSTDYYRVGGRTMLPIRWMPPESILYRKFTTESDVWSFGVVLWEIFTYGK 7	19
mouse	trkB	751	TK - QPWYQLSNNEVIECITQGRVLQRPRTCPQEVYELMLGCWQREPHTRKNIKSIHTLLQNLAKASPVYLDILG 821	
human	trk	720	QPWYQLSNTEAIDCITQGRELERPRACPPEVYAIMRGCWQREPQQRHSIKUVHARLQALAQAPPVYLDVLG 790 TK ←J	

Fig. 2. Amino acid sequence comparison between the putative products of the mouse *trkB* gene and the human *trk* proto-oncogene. Homologous residues are indicated by a vertical bar. Conserved cysteines in the extracellular domain are boxed. Consensus *N*-glycosylation sites are underlined. The respective transmembrane regions are indicated with solid boxes. The catalytic tyrosine kinase domains are indicated by horizontal arrows.

value considering that these genes are derived from different species (Figure 2). As expected, the highest homology (88%) was found in the catalytic domain (residues 543-806). Indeed, the trkB protein contains 92 of the 99 amino acid residues conserved between the trk proto-oncogene product and other growth factor receptors of the tyrosine protein kinase gene family (Martin-Zanca *et al.*, 1986a, 1989). These conserved residues include  $Tyr^{705}$  the tyrosine presumably involved in autophosphorylation reactions. In trkB, Tyr<sup>705</sup> is followed by another tyrosine residue, a feature previously observed in the trk proto-oncogene and in the insulin receptor subfamily (insulin, IGF-I receptors and c-ros). In addition, the trkB protein contains each of four unique features present in the human trk receptor. They include (i) a single amino acid gap between residues 575 and 576 (residues 542 and 543 in human trk), (ii) a threonine in position 678 (Thr<sup>647</sup> in human trk), (iii) a tryptophan in position 753 ( $Trp^{722}$  in human *trk*) and (iv) the absence of the helix-breaking proline in position 797 (position 766 in human trk) (Martin-Zanca et al., 1986a, 1989). The trkB protein also resembles the human trk receptor in its carboxyterminal domain. Both molecules exhibit a very short, highly conserved tail of 15 amino acids which include a conserved tyrosine located six residues from their respective carboxy terminus (Kozma et al., 1988; Martin-Zanca et al., 1989).

The overall homology between the putative extracellular domains of the mouse trkB and the human trk proteins is 57% (38% are identical amino acids). This homology is not randomly distributed. We have identified two regions located near the amino terminus (residues 64-107) and the transmembrane (residues 343-363) domains which exhibit homologies of 77 and 90% respectively. Nine of the 12 *N*-glycosylation sites present in the trkB gene product correspond to those previously identified in the human trk



Fig. 3. Immunoprecipitation analysis of the trkB gene product. S]methionine-labeled cell extracts of (A) NIH3T3 cells transfected with the mouse trkB cDNA clone pFRK44 or (C) control NIH3T3 cells were incubated with a rabbit antiserum raised against a peptide corresponding to carboxy-terminal residues (794-808) of the putative trkB protein, either (a) in the presence or (b) in the absence of 10  $\mu$ g of competing peptide. [35S]methionine-labeled cell extracts from (B) NIH3T3 cells expressing the human trk proto-oncogene products gp140/gp110<sup>trk</sup> were incubated with a rabbit antiserum raised against a peptide corresponding to the 14 carboxy-terminal amino acid residues of the predicted trk proto-oncogene product, either (c) in the presence or (d) in the absence of 10  $\mu g$  of competing peptide. Parallel cultures were metabolically labeled either in the absence (-) or presence (+)of 10 µg/ml tunicamycin as indicated. The resulting immunoprecipitates were analyzed by SDS-PAGE as described in Materials and methods. The migration of the trkB gene product gp145'rkB and the products of the trk proto-oncogene gp140/gp110<sup>trk</sup> are indicated by arrows. The migration of the corresponding polypeptide backbones of these molecules, p93<sup>trkB</sup> and p80<sup>trk</sup> are also indicated. Co-electrophoresed mol. wt markers included myosin (200 000), phosphorylase B (92 500) and albumin (69 000).

protein. More importantly, nine of the 11 extracellular cysteines present in the *trk* receptor are conserved in the *trk*B gene product. The other two cysteines are part of a conserved



Fig. 4. Northern blot analysis of *trkB* transcripts in adult mouse tissues. Poly(A)-selected RNAs from adult mouse tissues were electrophoresed in a 1% agarose – formaldehyde gel, transferred to nitrocellulose filters and hybridized under stringent conditions (50% formamide, 42°C) to (A) a <sup>32</sup>P-labeled probe derived form a 1.4 kb DNA fragment of the *trkB* cDNA clone pFRK43 (nucleotides 1664–3105, see Figure 1). (B) The same filter hybridized to a <sup>32</sup>P-labeled  $\beta$ -actin probe to control for the amounts of RNA loaded in each lane. Filters were exposed to Kodak X-OMAT film at  $-70^{\circ}$ C with the help of intensifier screens for the indicated times. The migration of *trkB* and  $\beta$ -actin-specific transcripts is indicated by arrows. Co-electrophoresed RNA size markers included Saccharomyces cerevisiae 28S (3.4 kb) and 18S (1.8 kb) and *E. coli* 23S (2.9 kb) and 16S (1.5 kb) ribosomal RNAs.

cluster of four cysteines located immediately after the putative signal peptide region. These observations suggest that the products of the *trk* and *trk*B genes might recognize related ligands.

#### Identification of the trkB product

The 4.4 kb DNA insert of pFRK43 was subcloned in pMEX-NEO, a pUC18-derived mammalian expression vector in which the polylinker sequences are flanked by the LTR of the Moloney strain of murine sarcoma virus and the SV40 polyadenylation signal. This vector also carries the bacterial neo gene under the regulatory control of the SV40 early promoter. The resulting plasmid, pFRK44, was transfected into NIH3T3 cells and several G418-resistant colonies selected for immunoprecipitation analysis. As shown in Figure 3, antibodies raised against the carboxy-terminal sequences of the trkB gene product (amino acids 794-808), specifically recognized a glycoprotein of an apparent mol. wt of 145 kd, which is likely to represent the product of the mouse trkB gene. The glycoproteinic nature of this molecule was demonstrated by the significant reduction in its apparent mol. wt when cells were treated with tunicamycin, an inhibitor of N-glycosylation (Figure 3). The mol. wt of the trkB protein expressed in these tunicamycin-treated cells, 93 kd,



Fig. 5. Northern blot analysis of trkB transcripts in mouse adult ovaries and in embryonic tissues. (A) 14 and 18 day old mouse embryos were separated into head and trunk tissues and their RNAs isolated as described in Materials and methods. Poly(A)-selected RNAs from adult brain and ovaries and from embryonic tissues were electrophoresed in 1.2% agarose-formaldehyde gels, transferred to nitrocellulose filters and hybridized under stringent conditions (50% formamide, 42°C) to a <sup>32</sup>P-labeled probe derived from pFRK1, a plasmid containing a partial trkB cDNA clone (nucleotides 1181-4351, see Figure 1). (B) The same filter, this time hybridized to a <sup>32</sup>P-labeled  $\beta$ -actin probe in order to control for the amount of RNA loaded in each lane. Filters were exposed to Kodak X-OMAT film at  $-70^{\circ}$ C for either (A) 5 days or (B) 8 h. The migration of the trkB and  $\beta$ -actin transcripts is indicated by arrows. Brain trkB transcripts are shown for comparison. Co-electrophoresed RNA size markers were those indicated in the legend to Figure 4.

is in good agreement with the predicted molecular mass for the polypeptide backbone of gp145<sup>trkB</sup>.

#### trkB expression

The pattern of *trkB* gene expression in adult mouse tissues was next investigated. As shown in Figure 4, *trkB* transcripts are most abundant in brain. These transcripts included poly(A)containing RNA species of 9.0, 8.2, 8.0, 5.5, 3.5, 2.5 and 2.0 kb. Their expression appears to be developmentally regulated. Whereas the 9.0 and 5.5 kb species are only observed in the brain, all other transcripts can also be found in lung and muscle, albeit at lower levels (Figure 4). Traces of the two smallest transcripts (2.5 and 2.0 kb) can also be seen in RNA preparations derived from heart and kidney tissue. Reproductive organs exhibit their own peculiar pattern of *trkB* expression. Whereas testes express only traces of two distinct RNA species of 2.8 and 2.5 kb, ovaries contain significant levels of 8.2 and 2.5 kb transcripts (Figure 5).

The mouse trkB locus is also expressed during embryonic development. A 2.5 kb RNA species, indistinguishable in size to that found in ovaries, can be readily identified in 14 and 18 day old embryos (Figure 5). Consistent with the overall expression pattern of the trkB gene in adult mice, the bulk of this embryonic trkB transcript is present in head tissue. However, detectable levels of expression are also found in the trunks (Figure 5).



Fig. 6. trkB expression in (A,B) midsagittal and (C,D) parasagittal sections of a 14 day old mouse embryo. (E,F) magnified view of the dorsal root ganglia region of the midsagittal section. Panels, A, C and E are bright-field views of the sections. Panels B, D and F show the corresponding dark-field views after *in situ* hybridization to a <sup>35</sup>S-labeled *trkB* antisense cRNA probe (pFRK43 nucleotides 1181–1663, see Figure 1). Arrowheads in **panels A** and **B** indicate the sympathetic plexus. Other symbols include SC, spinal cord; TG, trigeminal ganglion; O, ophthalmic nerve; Mx, maxillary nerve; Md, mandibular nerve; e, eye; asterisk (\*), tooth bud; and DRG, dorsal root ganglion.

# In situ hybridization

To define precisely the tissue distribution of embryonic *trkB* transcripts in the embryo, we conducted a series of *in situ* hybridizations on 14 and 18 day old mouse embryo sections. <sup>35</sup>S-labeled 483 bp long antisense and sense cRNA probes (corresponding to nucleotides 1181 - 1663 in the *trkB* cDNA clone pFRK43; see Figure 1) were prepared as described in Materials and methods. These probes were selected

because this region of the *trkB* gene does not cross-react, even at low stringency conditions, with *trk* proto-oncogene sequences. In addition, this probe detects all of the *trkB* transcripts described above. The results of the *trkB* antisense probe hybridization to the 14 day old embryo sections are shown in Figure 6. A midsagittal section reveals *trkB* expression in brain, spinal cord, spinal ganglia, paravertebral trunk of the sympathetic nervous system and various facial



Fig. 7. trkB expression in 18 day old mouse embryos. (A) Bright-field and (B) dark-field views of a midsagittal section through the head region of an 18 day old mouse embryo hybridized to the <sup>35</sup>S-labeled trkB antisense cRNA probe described in the legend to Figure 6. (C) Depicts a dark-field view of an adjacent section of the same embryo after *in situ* hybridization with the corresponding sense trkB cRNA probe. Symbols include Br, brain; SC, spinal cord; SG, submaxillary gland.

structures which represent either neural crest-derived mesenchyme or innervation pathways from cranial ganglia (Figure 6A and B). The parasagittal view depicted in Figure 6 (C and D) highlights the presence of trkB transcripts in brain but not in other organs in which central or peripheral nervous system structures are absent.

The parasagittal section exhibits the three nerve branches stemming from the trigeminal ganglion: the ophthalmic, maxillary and mandibular nerves. The pathways of these nerves can be traced by trkB hybridization to their respective general sites of innervation; that is the eye, the upper dental papillae and snout, and the lower dental papillae respectively (Figure 6D). Whereas the sensory innervation of the eve by the ophthalmic nerve exhibits intense trkB hybridization, the optic nerve which is visible in transverse section at the center of the eye is negative. Intense trkB hybridization can also be observed in a tooth bud (marked with an asterisk in Figure 6C). In addition, the parasagittal view of the 14 day old embryo shows a distinctive punctate labeling of the Vth cranial (trigeminal) ganglion (Figure 6C and D). This punctate labeling is similar to that observed in the dorsal root ganglia shown in the enlarged midsagittal section which is highlighted in Figure 6, panels E (light field) and F (dark field). The presence of silver grains over a subset of the 'large light' cell bodies suggests that trkB might be only expressed in a subset of neurons within the dorsal root and trigeminal ganglia.

In situ hybridization of a midsagittal section of an 18 day old embryo also exhibits strong trkB expression in brain and spinal cord (Figure 7A and B). Figure 7C demonstrates the lack of reactivity when the corresponding sense probe was hybridized to an adjacent section. In brain, the levels of trkBexpression exhibit a good correlation with the cell density present in the various morphological structures. These observations are consistent with the presence of trkBtranscripts in diverse cell types of the developing nervous system. However, the possibility that *trk*B could be expressed in an as yet undefined cell type that may exist throughout the entire CNS cannot be ruled out at the present time. As observed in the 14 day old embryo, additional labeling is seen in facial structures such as the upper dental papillae, the submaxillary gland and the snout. This hybridization may reflect either sites of innervation or neural crest-derived mesenchymal structures. These results suggest that the pattern of *trk*B expression in mid-gestation embryos remains constant throughout the late stages of fetal development.

## Discussion

Hybridization of RNAs isolated from normal mouse tissues with a probe specific for the tyrosine protein kinase domain of the human *trk* proto-oncogene revealed a series of weakly hybridizing transcripts ranging in size from ~9 to ~2 kb instead of the expected 3 kb transcript previously identified in human tumor cell lines (Martin-Zanca *et al.*, 1986b). Molecular cloning of some of these *trk*-related sequences from a mouse brain cDNA library revealed the existence of *trk*B, a novel *trk*-related locus. A 4.4 kb long *trk*B cDNA clone, generated by assembling two overlapping clones, directed the synthesis of a glycoprotein with an apparent mol. wt of 145 kd. As expected from its deduced amino acid sequence, gp145<sup>trkB</sup> displays a protein kinase activity specific for tyrosine residues and is tightly associated with cellular membranes (unpublished observations).

Sequence analysis of this biologically active *trk*B cDNA clone revealed the classical domains of cell surface receptors of the tyrosine protein kinase gene family. A putative signal peptide (residues 2-31), likely to be cleaved at the Ala-Cys junction, is followed by an extracellular domain presumably responsible for ligand recognition and binding. This region does not offer any relevant structural features except for its high homology (57% at the amino acid level) to the

corresponding domain of the *trk* proto-oncogene product. Two regions of the *trk*B molecule (residues 64-107 and 343-363) displayed homologies of 77 and 90% respectively, with the *trk* protein, suggesting that they may play an important role in ligand recognition. Interestingly, a 53 amino acid deletion capable of conferring transforming properties to the *trk* proto-oncogene (unpublished observations), encompasses the latter of the above highly conserved extracellular regions. In addition, 9 of the 11 extracellular cysteines present in the *trk* gene product are conserved in the putative *trk*B protein.

The 264 amino acid long tyrosine protein kinase catalytic domain of the trkB product is highly reminiscent of the corresponding region of other growth factor receptors. As many as 99 of its 264 residues are homologous to those present in each of the other members of the receptor tyrosine protein kinase gene family (Hanks et al., 1988). As expected, the highest degree of homology was found with the trk proto-oncogene product. These molecules have 233 homologous residues (88%) with a single 2 amino acid insert in trkB within the region corresponding to the putative intercalating domain as defined in the PDGF receptor-like kinases. Moreover, the trkB product exhibits each of the distinctive features previously identified in the human trk protein (Martin-Zanca *et al.*, 1986a, 1989) including Thr<sup>678</sup> instead of alanine,  $Trp^{753}$  instead of tyrosine, and the absence of a proline residue at position 797. Finally, the trkB gene codes for a molecule with a short carboxy-terminal tail of 15 residues, 11 of which are identical to those of the trk protein, including a tyrosine located five residues from the carboxy terminus (Kozma et al., 1988; Martin-Zanca et al., 1989). These similarities, taken together, strongly support the concept that the products of the trk and trkB genes represent a subfamily of tyrosine protein kinase cell surface receptors.

One of the most intriguing properties of the trkB locus is its complex pattern of transcription. At least eight distinct transcripts ranging in size from ~9 to 2 kb have been identified. Seven of these transcripts are expressed in brain, whereas a barely detectable 2.8 kb RNA species appears to be unique to testes. Four other tissues, including lung, muscle, kidney and heart, depict their own pattern of expression which consists of the 8.2/8.0 kb RNA species, the 3.5 kb species and the two small 2.5- and 2.0-kb transcripts. Ovaries also exhibit a similar pattern of expression except for the absence of the smallest 2.0 kb RNA species. Whether these trkB transcripts are expressed in cell types specific for each of these tissues or represent expression in cells derived from a multi-organ system such as the nervous system remains to be determined.

The *trkB* gene is also expressed during embryonic development. The major transcript detected in 14 and 18 day old embryos is a 2.5 kb RNA species. Whether other *trkB* RNA species can be expressed during different stages of embryonic development is currently under investigation. Unveiling the biological significance of the complex pattern of expression of the *trkB* locus will require the isolation and molecular characterization of cDNA clones specific for each of the observed transcripts. Preliminary information suggests that the different patterns of expression of *trkB* might reflect the presence of distinct, developmentally regulated promoters and/or alternative splicing events. Even more intriguing is the presence of the 2.0 kb transcript, whose small size makes

it unlikely to code for a molecule of 818 amino acids such as  $gp145^{trkB}$ , thus suggesting that the *trkB* locus may code for more than one protein.

The preferential expression of trkB in both embryonic and adult mouse brain tissue raises the possibility that this novel tyrosine protein kinase gene might play a role in the development and/or maintenance of the nervous system. Results obtained by in situ hybridization with 14 and 18 day old embryo sections are consistent with data generated by Northern blot analysis. Highest levels of trkB gene expression were detected in the developing CNS (brain and spinal cord) and PNS, including cranial and spinal ganglia. The observed pattern of trkB expression does not evoke an obvious physiological interpretation. trkB transcripts in the brain and spinal cord appear to be in good correlation with cell density. However, they are neither preferentially localized in areas of active mitosis nor in areas comprised by postmitotic, differentiated cells. In the ganglia, trkB is transcribed to high levels only in a subset of the large cell body neurons. The observed hybridization in the innervation pathways stemming from these ganglia suggests that trkB might be expressed either in the neural crest-derived Schwann cells which migrate along the axonal pathways or in the mesenchyme surrounding such axonal pathways. Although precise localization will require detailed biochemical and immunohistochemical analysis, we favor the former possibility since all tissues in which trkB expression could be unequivocally confirmed are part of the central or peripheral nervous systems.

The localization of *trk*B transcripts differs from that observed for the closely related *trk* proto-oncogene. This gene, which appears to encode only one mRNA species, is expressed only in dorsal root and trigeminal ganglia. Moreover, the pattern of expression suggests that most, if not all, neuroblasts in the ganglia contain *trk* transcripts (D.Martin-Zanca, L.F.Parada and M.Barbacid, submitted for publication). These observations strongly suggest that *trk* and *trk*B genes code for a family of cell surface receptors likely to be involved in distinct, perhaps complimentary functions in the development and/or maintenance of the nervous system.

# Materials and methods

# Isolation of trkB cDNA clones

 $2 \times 10^{6}$  phages of a  $\lambda$ gt11 cDNA library prepared from poly(A)selected RNA isolated from adult mouse brain (Citri et al., 1987) were plated on a lawn of Escherichia coli LE 392. Phages were absorbed onto nitrocellulose filters, lysed and their DNAs hybridized at 42°C for 72 h under relaxed stringency conditions (5 × SSC, 40% formamide, 1 × Denhardt's solution and 10% dextran sulfate) to a <sup>32</sup>P-labeled probe derived from the 1.2 kb Ball-EcoRI DNA insert of pDM17, a plasmid which contains a partial cDNA clone of the human trk oncogene that encompasses its transmembrane and tyrosine protein kinase domains (Martin-Zanca et al., 1989). cDNA clones containing 5' trkB sequences were isolated by rescreening the above library with a <sup>32</sup>P-labeled 200 bp *Eco*RI-*Xho*I DNA fragment derived from the 5' end of pFRK1, a partial cDNA clone of the mouse trkB gene containing a portion of the extracellular coding region and the complete transmembrane and tyrosine kinase catalytic domains. In this case, however, the cDNA library was hybridized under stringent conditions (42°C,  $5 \times SSC$ , 50% formamide,  $1 \times Denhardt's$  solution and 10% dextran sulfate). Filters were washed three times at room temperature with a solution containing  $2 \times SSC$  and 0.1% SDS and once at either  $42^{\circ}C$ (pDM17-derived probe) or 60°C (pFRK1-derived probe) with a solution containing  $0.1 \times SSC$  and 0.1% SDS. Positive phages were plaque-purified as described (Maniatis et al., 1982) and their inserts subcloned in pBluescript (Stratagene).

#### Nucleotide sequencing

Two overlapping clones encompassing the most 5' and 3' cDNA sequences of the mouse trkB gene were assembled into a single clone and inserted into the unique *Eco*RI site of pBluescript to generate pFRK43. The nucleotide sequence of pFRK43 was determined by the dideoxy chain termination method using double-stranded plasmid DNA, synthetic oligonucleotides and modified T7 DNA polymerase (Sequenase, USB).

#### Southern blot analysis

Human genomic DNA (20  $\mu$ g) was digested with *Bam*HI (Boehringer Mannheim) for 2 h at 37°C. The DNA was electrophoresed on a 0.8% agarose gel and blotted onto nitrocellulose (Southern, 1975). One half of the filter was hybridized for 72 h under high stringency (42°C in 5 × SSC, 50% formamide, 1 × Denhardt's solution and 10% dextran sulfate) with a <sup>32</sup>P-labeled 2.7 kb *Eco*RI DNA insert of pLM7, a plasmid which contains a full-length cDNA clone of the filter was hybridized for 72 h under relaxed stringency (42°C in 5 × SSC, 40% formamide, 1 × Denhardt's solution and 10% dextran sulfate) with a <sup>32</sup>P-labeled 3.2 kb *Eco*RI DNA insert of pFRK1 containing a partial cDNA clone of the mouse *trkB* gene. Filters were washed three times with 2 × SSC, 0.1% SDS and once in 0.1 × SSC, 0.1% SDS at either 60°C (pLM7-derived probe) or 42°C (pFRK1-derived probe).

#### Northern blot analysis

Total cellular RNA was prepared from adult and embryonic (14 and 18 day old embryos) tissues of BALB/c mice by the guanidine thiocyanate and CsCl<sub>2</sub> procedure. The poly(A) containing fraction was isolated by affinity chromatography on oligo(dT) – cellulose columns (Collaborative Research) (Maniatis *et al.*, 1982), electrophoresed on agarose – formaldehyde gels, transferred to nitrocellulose filters, and hybridized under high-stringency conditions (42°C in 5 × SSC, 50% formamide, 1 × Denhardt's and 10% dextran sulfate) for 48 h. Hybridized filters were washed three times in 2 × SSC, 0.1% SDS and once in 0.1 × SSC, 0.1% SDS at 60°C for 30 min.

#### trkB gene expression

The 4.4 kb mouse *trkB* cDNA insert of pFRK43 was subcloned into the mammalian expression vector pMEX-NEO (Martin-Zanca *et al.*, 1989) to generate pFRK44. Mouse NIH3T3 cells were transfected with pFRK44 DNA by the calcium phosphate precipitation technique as previously described (Graham and van der Eb, 1973). G418-resistant colonies were isolated, metabolically labeled with [ $^{35}$ S]methionine for 3 h and submitted to immunoprecipitation analysis as described (Martin-Zanca *et al.*, 1989). Antiserum against the *trkB* product was obtained by immunizing rabbits with a synthetic peptide corresponding to residues 794–808 of the putative mouse *trkB* protein. Incorporation of *N*-linked carbohydrate residues was prevented by preincubating cells with 10 µg/ml of tunicamycin (Sigma) for 30 min. The resulting immunocomplexes were analyzed by SDS–PAGE on 8% slab gels as previously described (Martin-Zanca *et al.*, 1989).

#### In situ hybridization

A trkB-specific cRNA probe was synthesized from a 483 bp HincII DNA fragment of pFRK1 which encompasses sequences encoding a portion of the putative extracellular domain of the trkB protein (see Figure 1, nucleotides 1181-1663). This HincII DNA fragment was subcloned into the pGEM-3Z vector (Promega) to generate pFRK16. In order to synthesize a <sup>35</sup>S-labeled single-stranded antisense cRNA probe, pFRK 16, was linearized by digestion with HindIII and in vitro-transcribed with T7 RNA polymerase (Promega) in the presence of <sup>35</sup>S-labeled UTP (>1000 Ci/mmol, Amersham). The resulting antisense cRNA probe was mildly degraded by alkaline hydrolysis to an average of 100-200 bases in length to facilitate its accessibility to the tissues. After treatment with DNase RQ1 (Promega) the probe was purified by using a Sephadex G-50 column (Pharmacia), precipitated, dried and resuspended in 10 mM DTT. This antisense cRNA probe was used to hybridize 10  $\mu$ m thick sections of 14 and 18 day old mouse embryos. Sections were collected on poly-L-lysine coated glass slides, deparafinized in xylene and rehydrated in graded (100-30%) ethanol solutions. After fixing in 4% paraformaldehyde, the tissues were pretreated with proteinase K (20  $\mu$ g/ml), refixed and immersed in triethanolamine buffer containing acetic anhydrate. After dehydration, tissues were hybridized with the antisense cRNA probe  $(2-4 \times 10^5 \text{ c.p.m.})$  in a buffer containing 50% formamide, 10% dextran sulfate, 1 × Denhardt's solution, 0.5 mg/ml yeast tRNA and 10 mM DTT at 51°C for 16 h. After hybridization, sections were washed in 5  $\times$  SSC and 10 mM DTT for 30 min at 51°C followed by an additional 20 min wash at 65 °C in 50% formamide,  $2 \times SSC$  and 10 mM DTT. The slides were then incubated for 1 h at 37°C with RNase

A (20  $\mu$ g/ml) and RNAse T<sub>1</sub> (2  $\mu$ g/ml) followed by a 3 h incubation at 37°C in 50% formamide, 2 × SSC and 10 mM DTT, and washed in 0.1 × SSC for 15 min at 37°C. After dehydration in graded (30–100%) ethanol solutions containing 0.3 M ammonium acetate, the slides were air dried, dipped into NTB-2 nuclear track emulsion (Kodak) and exposed for 7 days at 4°C. After developing, the slides were stained with 0.2% toluidine blue, dehydrated, mounted with cover slips and photomicrographed under both dark-field and light-field microscopes. Control sections were hybridized as described above with a <sup>35</sup>S-labeled single-stranded sense cRNA probe transcribed from the SP6 promoter of *Bam*HI-linearized pFRK16 DNA.

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