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Human trks: Molecular Cloning, Tissue Distribution, and Expression of Extracellular Domain Immunoadhesins

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Using molecular cloning techniques, human homologs of the known members of the trk family of neurotrophin receptors have been cloned and sequenced. Overall, there is a high degree of similarity between the human sequences and those from other mammals; however, there are differences in splicing patterns. There are two spliced forms of the extracellular domain of trkC in the human, a finding that has not been described in other species. In contrast, fewer spliced forms were detected of the intracellular domains of human trkB and trkC than has been described in other mammals. Northern analysis and *in situ* hybridization experiments indicate that the human trks are expressed in a similar pattern to that described in other mammals. Expression of the trk extracellular domains as fusion proteins with IgG heavy chain yields soluble molecules that mimic intact trks in their binding specificity and affinity. These soluble chimeras block the biological activity of their cognate neurotrophin(s) *in vitro*.

[Key words: tyrosine kinase, northern blot,* in situ *hybridization, immunoglobulin, neurotrophin, binding, inhibition]

Neurotrophins are a family of small, basic proteins that play a crucial role in the development and maintenance of the nervous system (Thoenen, 1991). Different members of the neurotrophin family [nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) (Leibrock et al., 1989) neurotrophin-3 (NT-3) (Hohn et al., 1990; Kaisho et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990), and neurotrophin 4/5 (NT-4/5) (Berkemeier et al., 1991; Hallböök et al., 1991; Ip et al., 1992)] exhibit actions on distinct, but overlapping, sets of peripheral and central neurons. These effects range from playing a crucial role in ensuring the survival of developing neurons (NGF in sensory and sympathetic neurons) (Levi-Montalcini and Booker, 1960) to relatively subtle effects on the morphology of neurons (NT-3 on Purkinje cells) (Lindholm et al., 1993). The activities of neurotrophins have led to increasing interest in using them as therapeutics for certain neurodegenerative diseases (Hefti and Weinder, 1986; Hefti and Schneider, 1989). However, the entire spectrum of the possible actions of the neurotrophins is incompletely understood, as they have only recently been available in sufficient quantity to permit investigation of their biological activities. The present lack of functional blocking reagents for neurotrophins other than NGF has also impeded this work.

Recently, the understanding of neurotrophin action has been greatly improved by the identification of the trk family of cell surface tyrosine kinases as signal transducing neurotrophin receptors (Cordon-Cardo et al., 1991; Kaplan et al., 1991a,b; Klein et al., 1991a,b; Lamballe et al., 1991; Soppet et al., 1991; Squinto et al., 1991). The first member of the trk family was initially identified as the result of an oncogenic transformation caused by the chromosomal translocation of tropomyosin sequences onto the catalytic domain of trkA (Martin-Zanca et al., 1986, 1989). Later work identified trkA as a signal transducing receptor for NGF (Kaplan et al., 1991a,b; Klein et al., 1991a), and led to the identification of two other related receptors, trkB (Klein et al., 1989, 1990; Middlemas et al., 1991) and trkC (Lamballe et al., 1991). Different members of the trk family can serve as signal transducing receptors for different members of the neurotrophin family. TrkA binds NGF (Kaplan et al., 1991a,b; Klein et al., 1991a), trkB binds BDNF and NT4/5 with high affinity and NT3 to a lesser extent (Klein et al., 1991b) (Soppet et al., 1991; Squinto et al., 1991), and trkC specifically binds NT3 (Lamballe et al., 1991). Overall, the structures of the trks are quite similar to each other, but alternate splicing increases the complexity of the family by giving rise to two known forms of trkA (Barker et al., 1993), three forms of trkB (two without functional tyrosine kinase (TK) domains) (Middlemas et al., 1991), and six forms of trkC (four without a functional TK domain and two with small inserts in the TK domain) (Tsoulfas et al., 1993; Valenzuela et al., 1993) (see Fig. 4).

In order to better understand the possible role of trk and neurotrophin action in normal human physiology and in various human pathological states, it would be useful to determine the distribution of the alternatively spliced forms of the various trks in both normal and pathological human tissues. As a first step toward investigating the potential involvement of the trk-neurotrophin system in human disease, we report here the cloning and sequencing of the various isoforms of the trks from the human and their expression pattern in various tissues by both Northern and *in situ* hybridization analysis. In addition, we show that expression of the extracellular domains of the human

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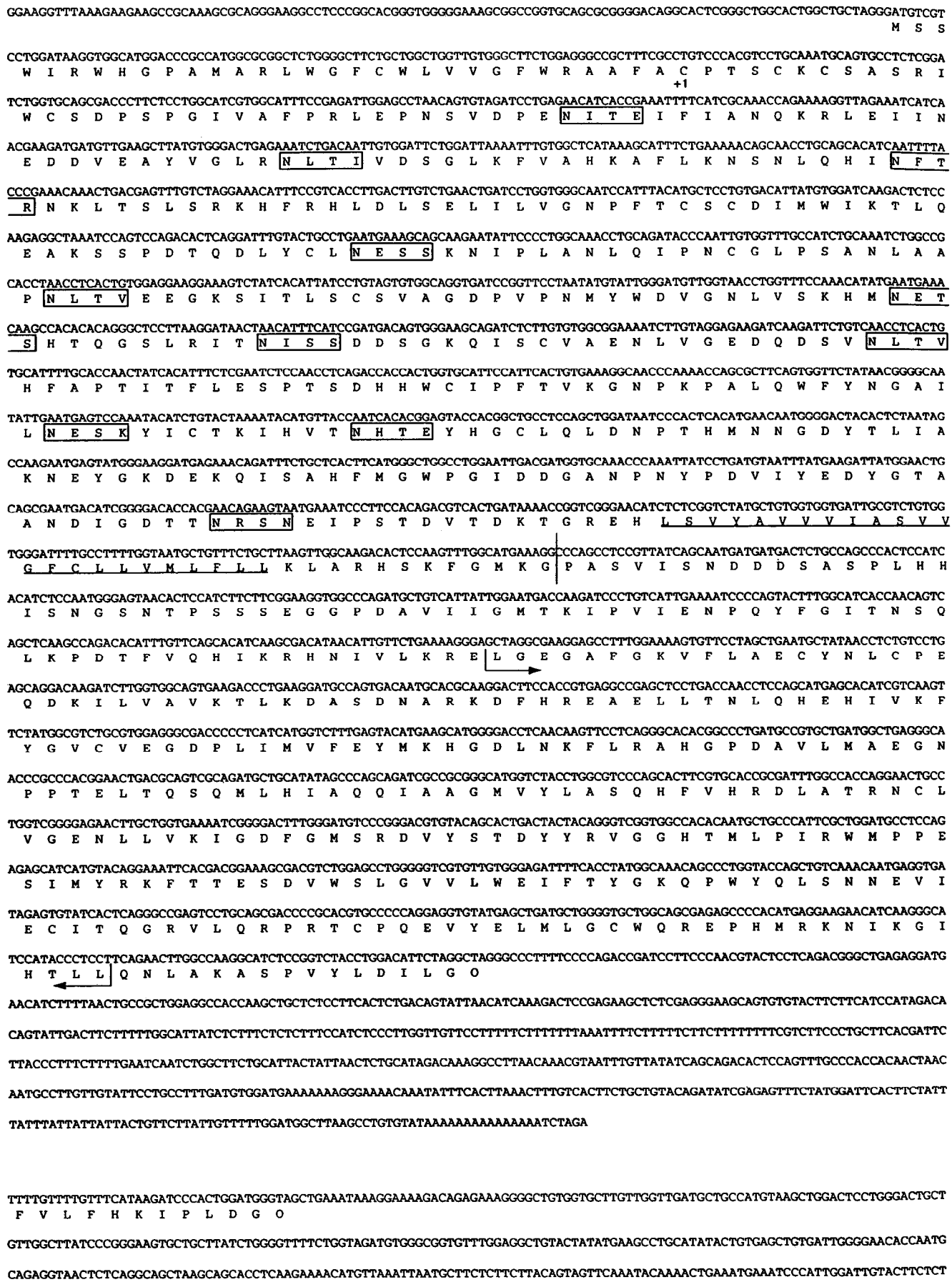

Table 1. Sequence of primers used in cloning and analysis of human neurotrophins

Use	trkA	trkB	trkC
Degenerate sense		TGYAYATHATGTGGYTNAARAC	TGGATGCARYTNTGGCARCARCA
Degenerate anti		YTCRTCYTTNCCRTAYTCRTT	CCYTCYTGRTARTAYTCNACGTG
ECD insert sense	CACGTCAACAACGGCAACTACA	GGAAGGATGAGAAACAGATTTCTGC	CATCAATGGCCACTTCCTCAAGG
ECD insert anti	AGGTGTTTCGTCCTTCTTCTTCC	GAGATGTGCCCGACCGGTTGTATC	CACAGTGATAGGAGGTGTGGGA
TK insert sense	GGATGTGGCTCCAGGCCCC	GGGCAACCCGCCCACGGAA	ACGCCAGGCCAAGGGTGAG
TK insert anti	TAACCACTCCCAGCCCCTGG	TTGGTGGCCTCCAGCGGCAG	AATTCATGACCACCAGCCACCA
Probes			
ECD sense	GCTCCTCGGGACTGCGATGC	ATGTCGCCCTGGCCGAGGTGGCAT	AAGCTCAACAGCCAGAACCTC
ECD anti	CAGCTCTGTGAGGATCCAGCC	CCGACCGGTTTTATCAGTGAC	ATGATCTTGGACTCCCGCAGAGG
TK specific sense		CTTGGCCAAGGCATCTCCGGT	ATGTGCAGCACATTAAGAGGA
TK specific anti		TTATACACAGGCTTAAGCCATCCA	AGGAGGCATCCAGCGAATG

Shown are the sequences of oligonucleotides used in the PCR reactions. Degenerate sense and antisense were used in amplifying original human clones, ECD sense and antisense were used to analyze the possible splice variants seen in the extracellular domains, and TK sense and antisense were used to analyze the possible splice variants seen in the TK domain. Oligonucleotides listed under the probe section were those used to generate extracellular domain or TK specific probes used for Northern analysis.

trks as chimeras with the Fc domains of human IgG leads to soluble molecules that retain the binding specificity of the native receptor and are capable of blocking the biological activity of their cognate neurotrophins. These should prove to be useful tools in the investigation of neurotrophin function.

Materials and Methods

The polymerase chain reaction (PCR) was used to generate human trkB and trkC fragments with the degenerate primers shown in Table 1 using human brain cDNA as a template. PCR reaction buffer consisted of 10 mM Tris pH 8.4 at room temperature, 2.0 mM $MgCl_2$, and 50 mM KCl. A "hot start" procedure was used for all reactions; samples without enzyme were incubated for 10 min at 98°C, equilibrated to 65°C, and Taq polymerase added. They were then cycled 35 times through 94°C for 45 sec, 60°C for 45 sec, and 72°C for 60 sec and a final extension at 72°C for 10 min. Fragments amplified by this procedure were subcloned into pGEM and sequenced (Sambrook et al., 1989). Inserts from clones with sequences similar to known trkB and trkC sequences were then excised, gel purified, and labeled by random priming with ^{32}P-dCTP. These were used to probe 10^6 cDNA clones from human brain or subregions thereof by standard techniques (Sambrook et al., 1989). Inserts from positive, plaque-purified phage were subcloned either by helper mediated excision (lambda DR2 libraries) or by standard subcloning into pGEM. Genomic clones hybridizing to the 5′ probe for trkB were digested with Sau3aI, and resulting fragments were subcloned into BamHI-cut M13 mp18 and rescreened to select positive clones.

Six clones were obtained using the probe for human trkB. These were mapped using the PCR and primers from vector and insert sequences, and the clones with the greatest 3′ and 5′ extent were sequenced. Sequence analysis revealed that these clones encoded a protein highly homologous to rodent trkB that contained an entire tyrosine kinase domain and were intact to the 3′ polyA$^+$ tail, but were apparently incomplete at the 5′ end. An oligonucleotide probe designed from the 5′ end of the rat trkB sequence was used to rescreen the initial library and subsequently four other oligo-dT–primed human brain libraries with no positive clones found. Four positive clones were obtained when a random primed human brain library was screened with this probe. Sequence analysis of these clones indicated that they overlapped with the previous human clones, but, by comparison with the rat, were still missing 17 bases of coding region at the 5′ end. A human genomic library was then probed with the 5′ oligonucleotide probe and genomic clones isolated. Sau3aI fragments of these clones were then subcloned into M13, rescreened, and positive subclones were sequenced to obtain the last of the coding sequence. The final nucleotide and deduced amino acid sequence of human trkB obtained from the overlapping regions of the cDNA clones is shown in Figure 1.

Using a similar strategy, probes specific for the extracellular domain of human trkC were generated, and two initial clones were obtained. Both of these were found to contain sequences corresponding to the truncated form of trkC isolated from the pig and rat (Lamballe et al., 1991; Tsoulfas et al., 1993; Valenzuela et al., 1993). Both of these cDNAs encoded the complete extracellular domain of trkC, a transmembrane domain, and a short cytoplasmic domain that contained no TK sequences. In order to isolate clones encoding the tyrosine kinase domain of trkC, libraries were reprobed in duplicate with oligonucleotides corresponding to the C-terminal tail of pig trkC and the juxtamembrane region of the intracellular domain of human trkC. Double-positive clones were analyzed and found to contain sequence overlapping with the truncated trkC clones and also containing a tyrosine kinase coding sequence. The nucleotide and deduced amino acid sequence obtained from the overlapping regions of these clones is shown in Figure 2.

Trk A was cloned from human brain with the PCR by using exact-match primers and human brain cDNA as a template. Amplified DNA was subcloned and sequenced. Five discrepancies with the previously published sequence were detected. Each of these was examined by direct sequencing of several different amplification reactions, and true errors in the clone were corrected by site-specific mutagenesis. There remained one difference with the previously determined sequence, a GC for CG transposition leading to a switch from serine to cysteine at residue 300 in the deduced amino acid sequence.

DNA encoding the full coding region of trkB and trkC were reconstructed using standard techniques. DNA constructs encoding the chimeras of trk extracellular domain and IgG Fc domains were made with the Fc region clones of human IgG1 (Ashkenazi et al., 1993) with the splice between aspartate 216 of the IgG and valine 402 of trkA, threonine 422 of trkB, or threonine 413 of trkC. DNAs encoding whole receptor or IgG chimeras were subcloned into pRK (Suva et al., 1987) for transient expression in 293 cells using calcium phosphate-mediated transfection. For purification of trk-IgG chimeras, cells were changed to serum-free media the day after transfection and media collected after a further 2–3 d. Media was filtered, bound to a protein A column (Hi-Trap A, Pharmacia), the column washed with PBS, bound protein eluted with 0.1 M glycine, pH 3.0, and immediately neutralized with Tris buffer. Concentration was estimated by absorbance at 280 nm using an extinction coefficient of 1.5. SDS-PAGE analysis showed the resulting protein to be a single detectable band.

Probes used for Northern analysis were labeled using the PCR and

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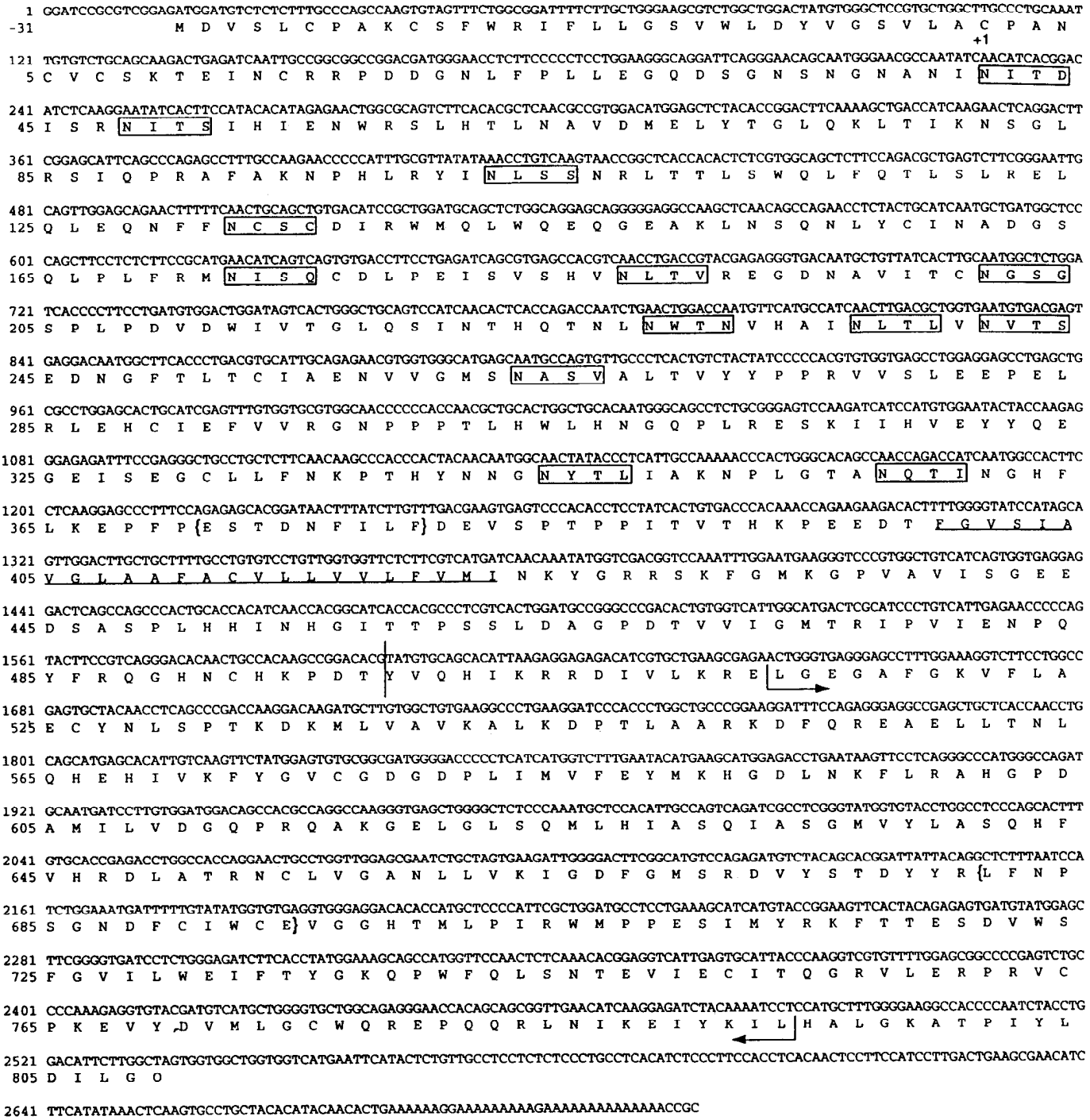
Figure 1. Nucleotide and deduced amino acid sequence of human trkB. *A,* The sequence of tyrosine kinase containing trkB is shown with potential N-linked glycosylation sites *boxed,* predicted transmembrane domain *underlined,* and tyrosine kinase domain flanked by *arrows.* The site of the splice giving rise to the truncated form is indicated by a *single vertical line. B,* The sequence of the alternately spliced truncated intracellular domain is shown.



the primers indicated in Table 1 on appropriate cloned template DNA. PCR reactions were run as described for initial cloning except that unlabeled dCTP was replaced in the reaction with α-^{32}P-dCTP at a concentration of 8 mCi/ml (3,000 Ci/mmol) and the reaction was run for 20 cycles. Probes were separated from unincorporated nucleotides and boiled for 5 min before being added to Nytran blots containing 2 μg of polyA$^+$ RNA per lane (Clontech, Palo Alto, CA) that had been prehybridized in 5× SSPE, 10× Denhardt's, 100 μg/ml salmon sperm DNA, 50% formamide, and 2% SDS. Hybridization was carried out at 50°C in the same solution overnight and then blots were washed as for library filters but with the final wash at 50°C. Autoradiograms were obtained using a Fuji BAS2000 image analyzer after exposing the imaging plate for 10–20 h.

In situ hybridization was carried out by a modification of a previously published procedure (Phillips et al., 1990). Tissue was prepared for hybridization by a variety of techniques. Autolysis times on all samples were less than 24 h. Whole, unfixed embryos were embedded in a water-soluble embedding matrix (OCT, Tissue-Tek, Miles, Inc, Elkhart, IN), frozen by floating the blocks in petri dishes on liquid nitrogen, and 40 μ sections cut using a cryostat. Sections were thaw mounted onto slides (Superfrost Plus, Baxter Diagnostics, Deerfield, IL), air dried, baked at 55°C for 10 min, and stored in sealed boxes with desiccant at −70°C until use. Adult dorsal root ganglia were fixed by immersion in 4% formaldehyde and either processed for paraffin sectioning or for cryosectioning. Brain specimens were fixed by immersion for 24 hr in 4% formaldehyde, cryoprotected for 24 hr in phosphate-buffered sucrose, frozen on dry ice, and cut on a freezing sliding microtome. Sections were stored (less than 48 hr) in phosphate-buffered saline at 4°C, mounted onto gelatin-subbed slides, air dried, and stored at 4°C. Care was taken to avoid any condensation of moisture on all tissue sections during storage of the tissue.

On the day of hybridization, tissue sections were differentially pretreated, depending on the fixation and sectioning protocol employed. Unfixed tissue sections were fixed by immersion in 4% formaldehyde, 1% glutaraldehyde in 0.1 M sodium phosphate for 30 min at 4°C, rinsed in 0.5× SSC, and placed directly into prehybridization solution. Cryosections of immersion-fixed tissue were fixed in 4% formaldehyde in 0.1 M sodium phosphate for 5 min, rinsed in 0.5× SSC, digested for 30 min at room temperature with proteinase-K (Boehringer–Mannheim; 25 μ/ml in 0.5 M NaCl and 10 mM Tris, pH 8.0), rinsed, refixed for 10 min in 4% formaldehyde, dehydrated in a series of alcohols (50% ethanol containing 0.3% ammonium acetate; 70% ethanol containing ammonium acetate; 100% ethanol; 2 min per incubation), rehydrated through the same series of ethanols, and rinsed again in 0.5× SSC prior to prehybridization. For paraffin-embedded tissue, deparaffinization was performed by two rinses in xylene (2 min each), after which tissue was rehydrated through a series of alcohol solutions (100% ethanol twice, 95% ethanol, 70% ethanol; 2 min each). Tissue sections were then fixed in 4% formaldehyde for 10 min, digested for 30 min with proteinase K (25 or 50 μg/ml; room temperature or 37°C), rinsed, refixed for 10 min, and rinsed again in 0.5× SSC prior to prehybridization.

Prehybridization, hybridization, and posthybridization RNAase treatment and stringency washes were identical for all tissues carried out as previously described (Phillips et al., 1990).

Binding assays were performed by coating 96-well plates (Nunc, Naperville, IL) with 5 μg/ml Fab fragments of goat anti-human Fc (Organon Teknika Cappel, Malvern, PA), washing with PBS, and binding the trk-IgG chimera at 8 ng/ml. Plates were then washed with PBS, blocked with 1% fetal bovine serum, and incubated with a mixture of iodinated tracer neurotrophin (30–40 pmol) and varying concentrations of non-radioactive neurotrophin for 1 hr at room temperature. Unbound radioactivity was removed by washing the plate six times in PBS with 0.05% Tween 20, leaving the plate dry after the last wash. Individual wells were then counted in a gamma counter and competitive displacement curves were fit using a four parameter nonlinear least squares equation. Neurotrophin incubations were done in L15 media supplemented with 20 mM HEPES, pH 7.0, 1 mg/ml BSA, and 0.5 mg/ml cytochrome *c*. Neurotrophins were obtained from Dr. Louis Burton (Genentech, Inc.) and iodinated with Enzymobeads (Bio-Rad, Richmond, CA) as described (Escandón et al., 1993).

Neurotrophin bioactivity assays were done on peripheral neurons from chick. Dorsal root ganglion neurons from E8 chick were used for NGF and BDNF assays, and sympathetic ganglion neurons from E9 chick were used for assays of NT3. Ganglia were dissociated with 0.02% trypsin, washed in media with 10% FBS, dissociated by gentle trituration in serum-free media, and neurons enriched by preplating on tissue culture plastic previously incubated with 10% fetal bovine serum and washed with serum-free media. Neurons were cultured in serum-free media in six-well dishes precoated with poly-ornithine and laminin at 1–2000 neurons per dish for 2–3 d, and then a defined grid comprising 1 cm^2 was counted for live neurons. Serum-free media was based on L15 with additives as described (Davies, 1989).

Human brain cDNA, polyA$^+$ RNA (from pooled donors taken at autopsy); genomic and cDNA libraries were from Clontech (Palo Alto, CA.); pGEM was obtained from Promega (Madison, WI); restriction enzymes from New England Biolabs (Beverly, MA); Taq polymerase was from Perkin-Elmer (Norwalk, CT); all other enzymes, frozen competent *E. coli*, and tissue culture media were from GIBCO-BRL (Gaithersburg, MD).

Results

In order to amplify fragments of the human trkB and trkC sequences for use in probing cDNA libraries, the PCR with degenerate primers based on known sequences of rat trkB or pig trkC (see Table 1), was employed. Using human brain cDNA as the template, fragments of the predicted molecular weight were obtained, which were then subcloned. Sequence analysis of these cloned fragments verified that they represented the human homologs of the known rat trkB and pig trkC sequences. These cloned fragments were then used to probe a human brain cDNA library at high stringency to obtain full coding length cDNAs.

Examination of the sequences obtained from the human clones and comparison to the known structure of rat and mouse trkB and rat and pig trkC indicates that there is a very high degree of overall sequence similarity across these mammalian species. The overall structural motifs identified by Schneider and Schweiger (1991) are maintained, namely, a signal sequence, two cysteine-rich domains flanking a leucine-rich domain, two Ig-like domains of the C2 type, a transmembrane domain, and a tyrosine kinase domain showing high similarity to other known tyrosine kinases. The signal sequences are predicted to be clipped at residue 31 in both trkB and trkC, and this was confirmed by N-terminal sequence analysis of expressed immunoadhesins (see below) (data not shown). There are 11 and 13 potential N-linked glycosylation sites in the extracellular domains of trkB and trkC, respectively. An alignment of the human trk peptide sequences and the similarity of different regions of the known trks within and across species is shown in Figure 3.

During sequence analysis of several different clones obtained for trkB and trkC, unique forms apparently arising from alternate splicing were identified: (1) a deletion in the extracellular domain of trkC, (2) truncated, non-TK forms of trkB and trkC, and (3) a potential insert within the TK domain of trkC. Using library screening with specific oligonucleotide probes and the

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Figure 2. Nucleotide and deduced amino acid sequence of human trkC. *A,* The sequence of tyrosine kinase containing trkC is shown with potential N-linked glycosylation sites *boxed*, predicted transmembrane domain *underlined*, and tyrosine kinase domain flanked by *arrows*. The site of the splice giving rise to the truncated form is indicated by a *single vertical line*. The sequence of the potential inserts in the extracellular and tyrosine kinase domains are flanked by *brackets*. *B,* The sequence of the alternately spliced truncated intracellular domain is shown.

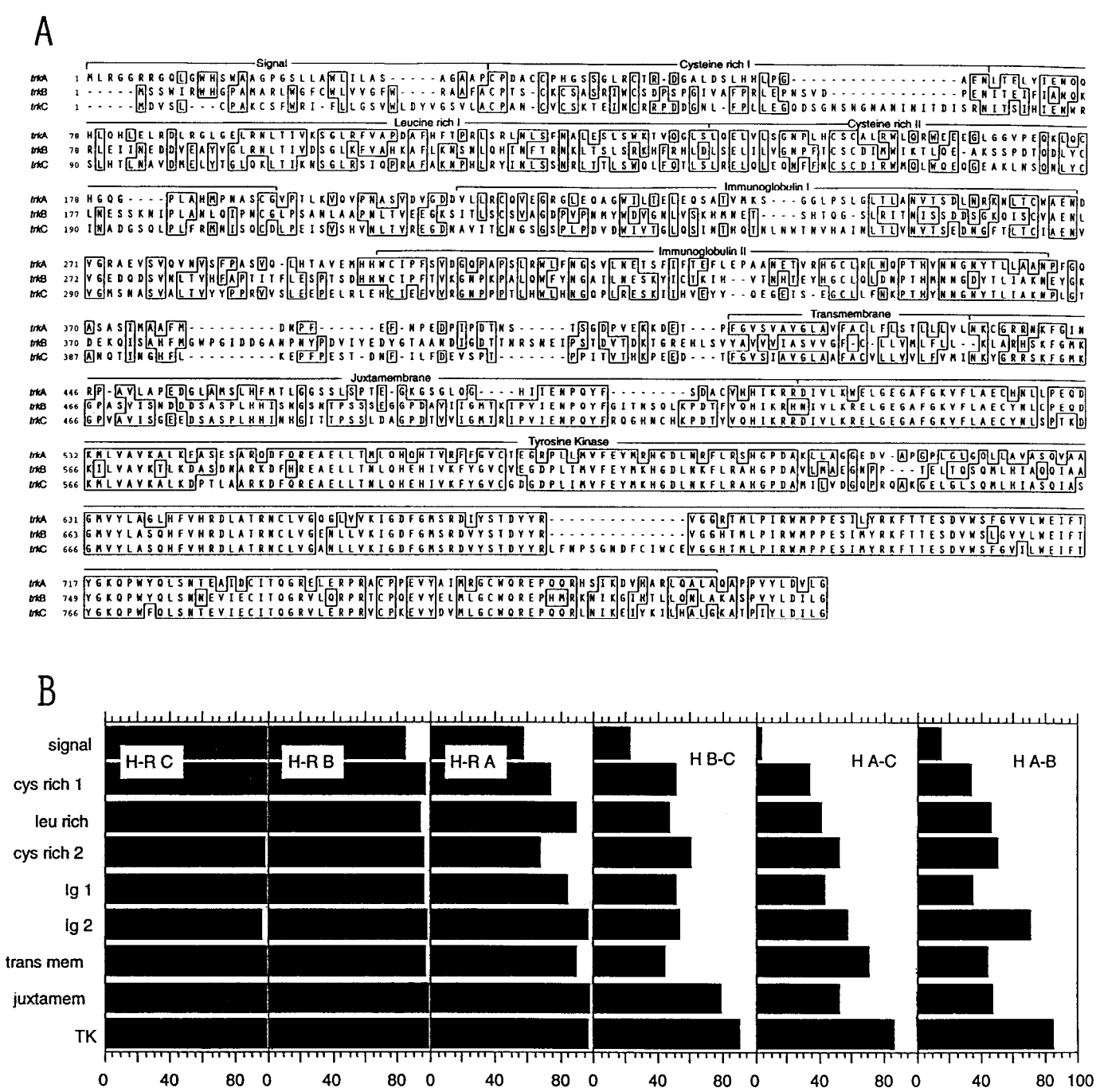

Figure 3. Comparison of human and rat trk sequences by domain. *A,* Alignment of peptide sequences of three human trks, with domains as defined by Schneider and Schweiger (1991) indicated. *Insert* in TK domain of trkC is included. *B,* Analysis of similarity of domains of human and rat trks. Percent similarity based on the PAM250 matrix (Dayhoff et al., 1983) was determined for each trk domain. Pair-wise comparisons were made between human trkC and rat trkC (*H-R C*), human trkB and rat trkB (*H-R B*), human trkA and rat trkA (*H-R A*), human trkB and human trkC (*H B-C*), human trkA and human trkC (*H A-C*), and human trkA and human trkB (*H A-B*). For this comparison, trkC TK insert was not included.

PCR, a more systematic search was then undertaken to search for potential other variants at these sites in the different human trks. A diagram of the forms found in human trks and a comparison to those found in other known trks is shown in Figure 4.

In the extracellular domain of human trkC, there is a variant that has a deletion of nine amino acids compared to rat and pig trkC at a site near to that where an extracellular insert was described in rat and human trkA (Barker et al., 1993) (Fig. 2). PCR analysis of this region in human trkC amplified two bands corresponding in length to that expected for the insert-containing and insert-deleted forms. PCR analysis of this region in human trkB showed no detectable length polymorphisms but amplification using trkA specific primers did show two distinct bands that were cloned and sequenced. The potential nucleotide insert was TCTCCTTCTCGCCGGTGG after position 1297, coding for the identical peptide insert previously described in rat and human trkA (Barker et al., 1993).

From the human brain libraries, both trkB and trkC clones

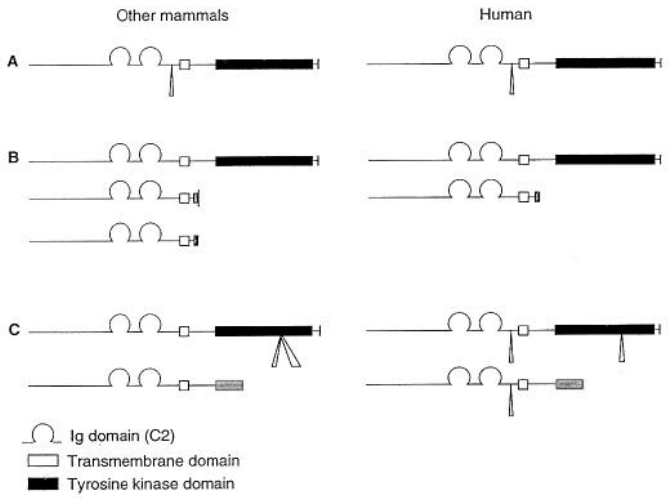

Figure 4. Summary of the splice forms seen in human and other mammalian trks. Shown are schematic representations of the forms of the various trks arising from alternate splicing. Domains are after Schneider and Schweiger (1991). Data is redrawn from the literature for rat trkA (Meakin et al., 1992; Barker et al., 1993), rat and mouse trkB (Klein et al., 1989, 1991; Middlemas et al., 1991) and rat and pig trkC (Lamballe et al., 1991; Tsoulfas et al., 1993; Valenzuela et al., 1993). Alternate forms of truncated rat trkC described by Valenzuela et al. (1993) are omitted for clarity.

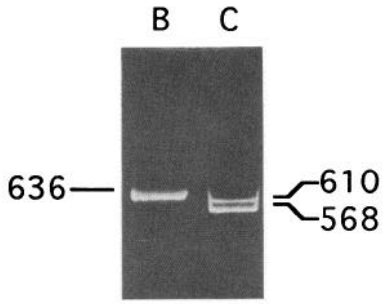

Figure 5. Amplification of region containing potential insert of tyrosine kinase domain of trkB and trkC. Brain cDNA was amplified with primers selective for the region surrounding the site of the observed insert in the TK domain of trkC. Using primers selective for trkC (*C*), two bands of sizes corresponding to the no insert (*568*) or 14 amino acid insert (*610*) form are amplified, with no evidence for any larger forms. Using primers selective for trkB (*B*), only one band corresponding to the no insert form (*636*) is detected.

were obtained that did not encode a TK domain but, instead, showed an alternate, truncated intracellular domain. In trkB, this consisted of 11 new amino acids added after position 435, which are identical to those added in the isoform of trkB previously identified in the rat as t1 (Middlemas et al., 1991) and in the mouse as the truncated form (Klein et al., 1989). All attempts using cDNA libraries probed with oligonucleotides or using PCR failed to yield sequences from the human similar to those identified in the rat as the t2 isoform of trkB (Middlemas et al., 1991). The PCR readily amplified sequences similar to the t2 isoform when either mouse or rat brain cDNA was used as a template, indicating that t2 is not unique to the rat and that the techniques used were capable of detecting t2-like sequences (data not shown).

The truncated form of trkC was longer than that of trkB and similar to that previously described in the pig (Lamballe et al., 1991) and in the rat (Tsoulfas et al., 1993) or as the ic158 form of rat trkC (Valenzuela et al., 1993). This form consisted of the replacement of the TK domain by 83 novel amino acids starting at position 498. These 83 residues were highly conserved in rat, pig, and human, with only two differences, an aspartate to glutamate and a serine to proline substitution, among all three species.

The TK domain of trkC obtained in the cDNA clones contained an apparent insert of 14 amino acids between subdomains VII and VIII (Hanks et al., 1988; Hanks and Quinn, 1991). This sequence is identical in both sequence and site of insertion to one of the previously described insertions in the rat trkC TK domain (Tsoulfas et al., 1993; Valenzuela et al., 1993). In addition to the 14 amino acid insert seen in rat trkC, longer inserts of 25 (Tsoulfas et al., 1993) and 39 (Valenzuela et al., 1993) amino acids have been identified in the rat. In an attempt to determine if these longer inserts were expressed in the human, brain cDNA was used as a template for PCR amplification across the region containing the insert (see Fig. 5). These experiments consistently amplified two DNA fragments of lengths corresponding to the two splice forms (with and without the 14 amino acid insert) and yielded no evidence of longer fragments corresponding to the 25 or 39 amino acid insert identified in rat trkC. Cloning and sequencing of these two DNA fragments verified that they correspond to the forms with and without the previously seen 14 amino acid insert. Interestingly, this splicing was tissue specific as only the band corresponding to the insert-free form was detected in amplifications using cDNA from testis, a non-neural tissue expressing high levels of trkC (data not shown). PCR amplification of human brain cDNA using oligonucleotides specific for this region of the trkB TK domain showed no evidence for length polymorphisms in the subdomain VII/VII boundary (see Fig 5).

Northern analysis

The expression pattern and transcript size of the trks in human tissues was examined by Northern analysis (Fig. 6). Hybridization with probes for trkB yielded a simple pattern, with a transcript of 6.9 kilobase (kb) hybridizing to both an extracellular and TK-specific probes, and a transcript of 8.1 kb hybridizing only to the TK-specific probe. This result suggests that the 8.1 kb transcript corresponds to the full length, TK-containing message while the 6.9 kb transcript corresponds to the single truncated form seen in human. As might be expected from the greater number of potential splice variants detected while cloning trkC, probing Northerns for this mRNA resulted in a more complex pattern of hybridization. Transcripts of 11.7, 7.9, and 4.9 kb were detected with a probe specific for the TK domain, while an additional transcript of 4.4 kb was detected with the extracellular domain probe (see Fig. 6).

Of the human tissues examined, both trkB and trkC are expressed in greatest abundance in the brain. However, expression is detected in a variety of locations outside the nervous system in adult and especially fetal tissues. The 8.1 kb transcript of trkB containing the TK domain was expressed in kidney, skeletal muscle, and pancreas, while the truncated form was detected in heart, spleen, and ovary. In fetal tissues, TK-containing trkB was found not only in brain, but also in kidney and lung, while truncated trkB was found in brain, kidney, lung, and heart. It is apparent that the ratio of TK-encoding to truncated trkB transcripts was much higher in fetal than adult brain.

Although the highest expression level of trkC is in brain, there

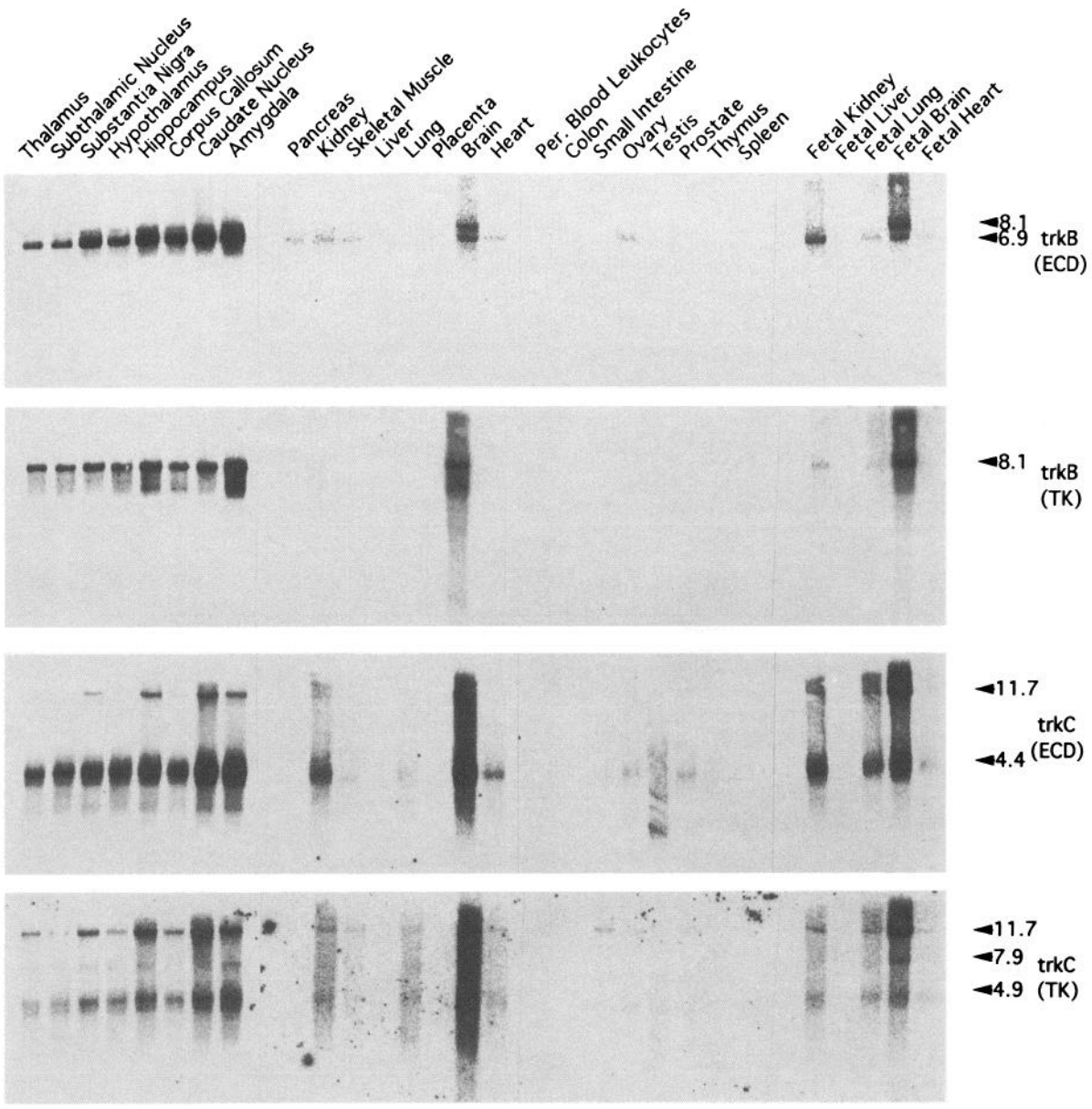

Figure 6. Northern analysis of trkB and trkC expression in human tissues. Two micrograms of polyA$^+$ RNA from the regions indicated was hybridized with probes specific for the trkB extracellular domain (*ECD*) or tyrosine kinase domain (*TK*) or the trkC extracellular (*ECD*) or tyrosine kinase (*TK*) domains. Note that the blot containing the brain regions was image processed differently than those containing the other tissues. In order to better display the range of hybridization signals present in the wide variety of tissues examined, a higher contrast setting was used for the brain regions hybridized with the trkB probes and a lower sensitivity was used for brain regions hybridized with the trkC probes.

is widespread expression of trkC outside the nervous system. In the adult, TK-containing trkC is expressed in kidney, skeletal muscle, lung, heart, small intestine, ovary, testis, and prostate, while in the fetus, the greatest expression is in brain, kidney, lung, and heart. The 4.4 kb transcript corresponding to the truncated form of trkC is detected in all tissues examined except peripheral blood leukocytes. Again, the ratio of TK-containing to truncated trkC transcripts is higher in fetal compared to adult brain.

In situ *hybridization*

In situ hybridization with probes to human trkA, and the TK-containing forms of trkB and trkC was conducted on embryonic and adult human tissue prepared by a variety of protocols. In two embryos of 6 and 8 weeks gestation (fresh frozen), trkA expression is restricted to dorsal root and cranial sensory ganglia, including the trigeminal ganglion (Fig. 7*A*). In contrast, trkB and trkC are not only expressed in sensory ganglia, but prominent expression is also seen within the developing brain and spinal cord (Fig. 7*B,C*). In addition, trkC expression is observed in the developing vasculature.

Within developing dorsal root ganglia, trkC is strongly expressed in ganglia from both the 6 and 8 week embryos. Curiously, in both embryos, there is a marked tendency for trkC-expressing cells to localize in the ventral end of the ganglia (Fig. 8). In contrast, trkA positive cells are largely restricted to dorsal portions of the ganglia (Fig. 8). In adult dorsal root ganglia (paraffin-embedded or cryosectioned fixed tissue), a subpopu-

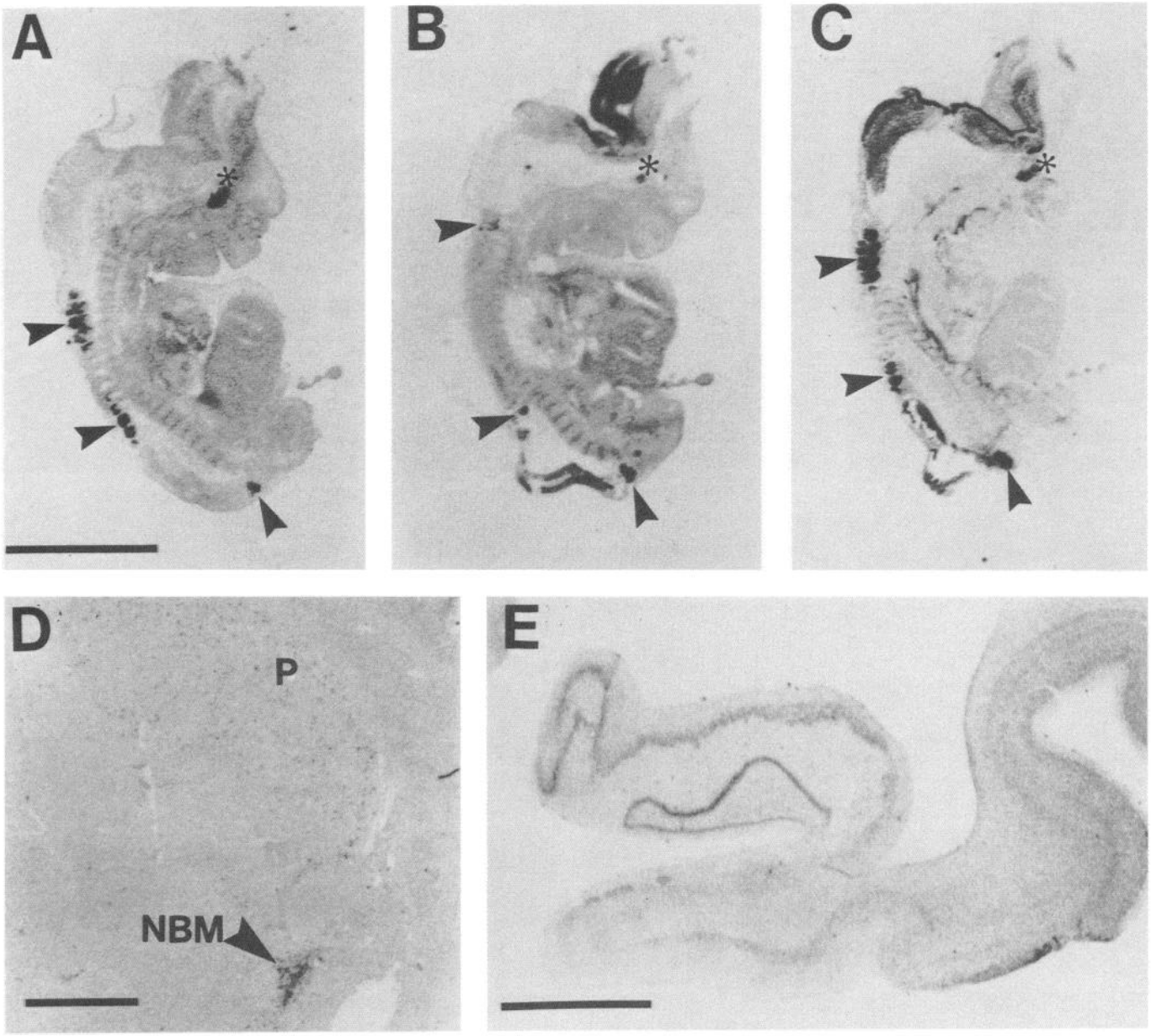

Figure 7. *In situ* hybridization analysis of embryos and adult brain. *In situ* hybridization using probes for trkA (*A* and *D*) TK-containing trkB (*B*), and TK-containing trkC (*C* and *E*). Shown are sheet film autoradiographs of sagittal sections of 8-week-old human embryos (*A, B,* and *C*) with *arrowheads* pointing to developing DRG and *asterisks* signifying trigeminal ganglion. *D* shows hybridization pattern of trkA in a coronal section through nucleus basalis of Meynert (NBM) and the putamen (P), while *E* shows the pattern of trkC expression in a coronal section through hippocampus and adjacent cortex. All scale bars are 500 μ.

lation of DRG neurons is labeled with each of the three trk probes (trkB, Fig. 9B,C; trkA and C, data not shown). Cells labeled with probes to each of the three trks appeared to be randomly distributed, with no sign of the polarization observed in the embryonic ganglia. No labeling of non-neuronal cells was observed with any of the probes.

In the adult human forebrain (fixed, cryosectioned tissue), cells strongly labeled for trkA expression were observed in the nucleus basalis of Meynert and scattered throughout the putamen (Fig. 7*D*). Labeled cells were of large diameter and conform to the expected appearance of cholinergic cells (Fig. 9*A*). trkC was widely expressed throughout the human forebrain, including prominent expression in hippocampus and neocortex (Fig. 7*E*; 9*D,E*), and labeled cells appeared to be exclusively of neuronal morphology (Fig. 9).

Expression of trk immunoadhesins

To further understanding of the roles of endogenous neurotrophins, it would be useful to have reagents that block their biological activities. Using standard techniques, sequences encoding the extracellular domains of the human trks were fused in frame with sequences encoding the Fc domain of human IgG heavy chain. The protein encoded for by these constructs would be expected to be a secreted molecule containing two trk extracellular domains linked via the association of the IgG Fc regions (Ashkenazi et al., 1993). Such chimeric molecules have been

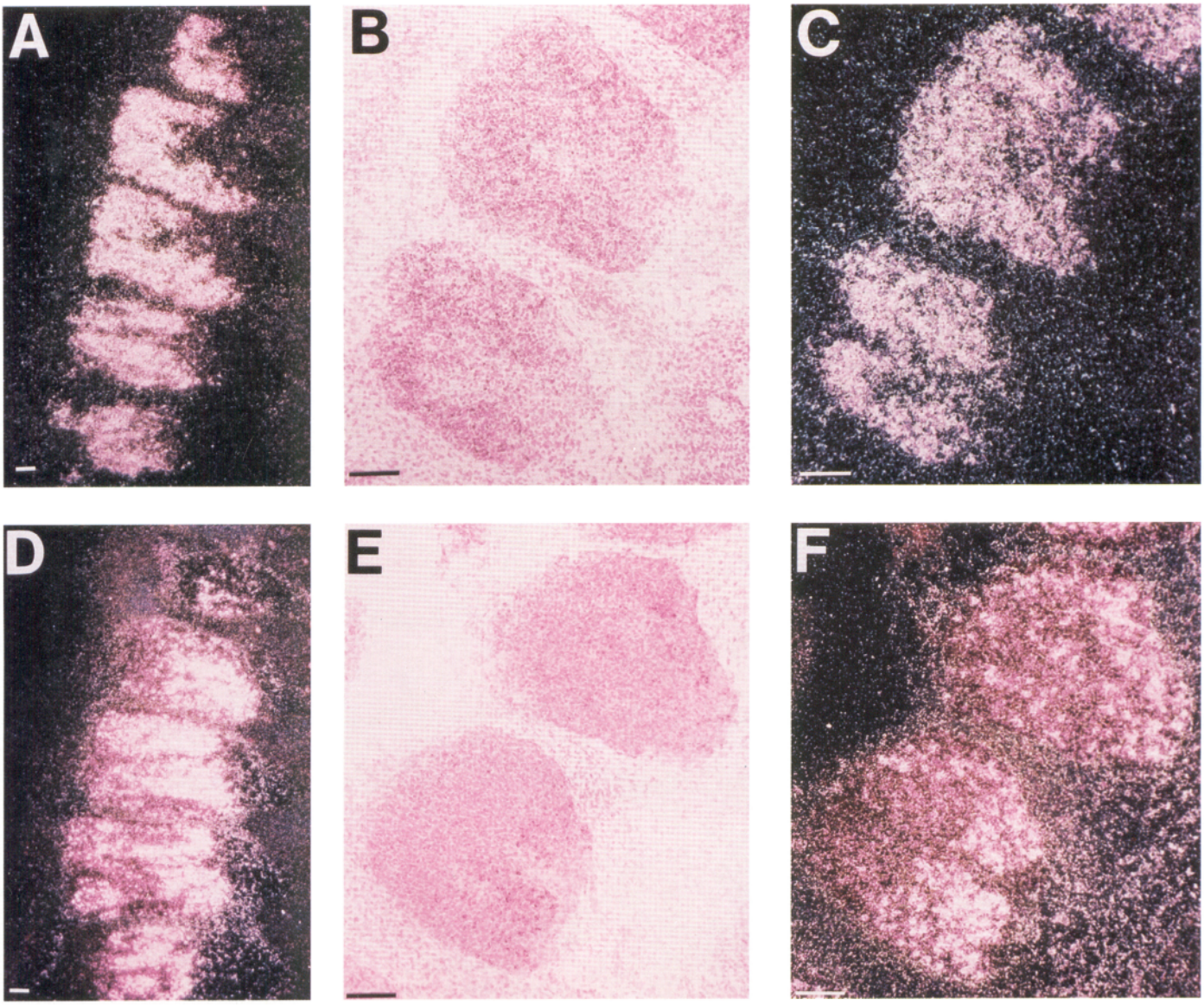

Figure 8. *In situ* hybridization of developing DRG with trkA and trkC. Emulsion autoradiography of developing DRG from human embryos hybridized with probes for trkA (*A*, *B*, and *C*) and trkC (*D*, *E*, and *F*). Ventral is to the right in all panels, and scale bars are 100 μ. *A* and *D* are dark-field photomicrographs of adjacent sections hybridized with probes for trkA and trkC in rostral DRG. *B* and *C* and *E* and *F* are bright-field and dark-field pairs of adjacent sections through lumbar DRG hybridized with trkA (*B* and *C*) or trkC (*E* and *F*). Note the reciprocal distribution of trkA and TrkC expressing cells, with trkA expressing cells being more abundant in the more dorsal aspect of the developing ganglia and trkC expressing cells more prevalent in the ventral aspect.

called immunoadhesins. Cells transiently transfected with these DNA constructs secreted protein that bound to protein A and migrated with an approximate molecular weight of 125 kDa on reducing SDS-polyacrylamide gels (data not shown). Trk-IgG chimeras could be easily purified to apparent homogeneity from conditioned media in a single round of affinity chromatography on a protein A column. Sequence analysis of these purified proteins verified the N-termini resulting from the predicted signal sequence cleavage site.

In order to test whether these chimeric proteins retained the binding specificity expected of the trk extracellular domain in a cellular environment, competitive displacement assays were done with iodinated neurotrophins. As shown in Figure 10, the trk-IgG chimeras did show specific binding to the expected neurotrophin(s). Chimeras containing trkA extracellular domain bound NGF with high affinity and NT3 and NT5 with much lower affinity. Chimeras containing trkB bound BDNF and NT5 with high affinity but only slightly better than NT3, and showed almost no detectable binding to NGF. Chimeras containing trkC were highly specific for NT3 over the other neurotrophins. The apparent affinity of the chimeras for their preferred ligand as determined in these competitive displacement assays is in the range of that determined for the high-affinity binding sites on cells expressing recombinant trk proteins. In one experiment, the IC_{50} values obtained for trkA were 62 pM for NGF and 20 nM for NT3, for trkB were 81 pM for BDNF, 200 pM for NT4/5 and 18 nM for NT3 and for trkC was 95 pM for NT3. The ratio

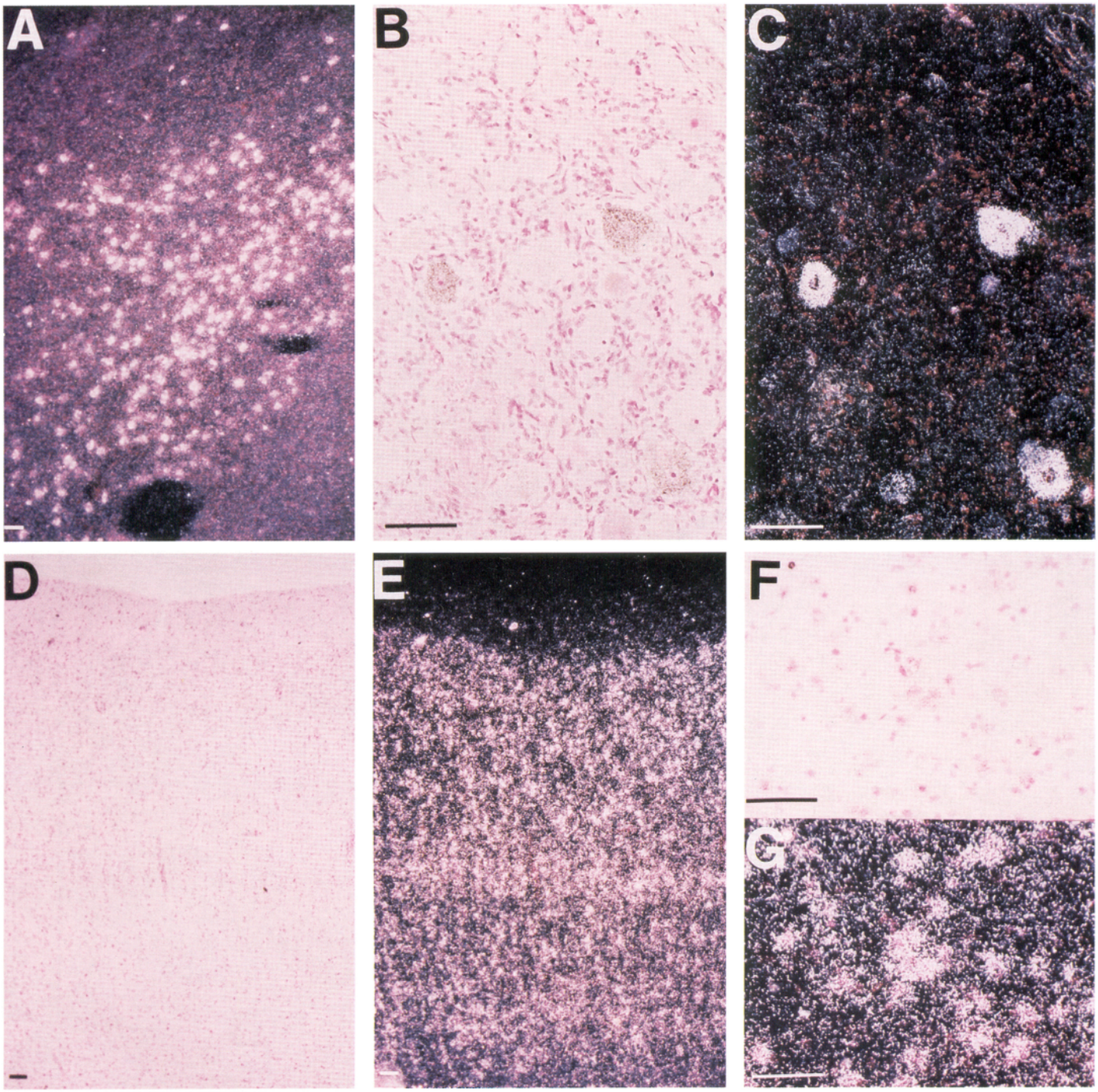

Figure 9. *In situ* hybridization analysis of expression in areas of the adult human nervous system. *A,* Dark-field photomicrograph of hybridization with trkA probe in nucleus basalis of Meynert. Bright-field (*B*) and dark-field (*C*) pair of paraffin section of adult DRG hybridized with TK-specific trkB. Note hybridization only over neurons, and that different neurons show different levels of hybridization. Bright-field (*D*) and dark-field (*E*) pair showing hybridization pattern of TK-containing trkC in parietal cortex. Note the more intense hybridization over layer four and almost complete lack of hybridization in layer I. Bright-field (*F*) and dark-field (*G*) pair of trkC in cortex showing hybridization is largely confined to large neuron-like cell bodies. All scale bars are 100 μ.

of specific to nonspecific binding is quite high in assays done with these reagents, usually at least 10 to 1 (see Fig. 10).

To check whether the trk-IgG chimeras are capable of blocking the biological activity of their cognate ligands, the neurotrophin-induced survival of peripheral neurons was assayed in the presence of the appropriate trk-IgG chimera. As can be seen in Figure 11, trkA-IgG is a potent inhibitor of NGF biological activity, trkB-IgG of BDNF, and trkC-IgG of NT3. In all cases, addition of excess neurotrophin is capable of overcoming this blockade, indicating that the trk-IgG chimeras are not generally toxic to the neurons.

Discussion

We report here the cloning and expression of the human homologs of trkB and C and a possible correction to the sequence of human trkA. Using a number of molecular biological ap-

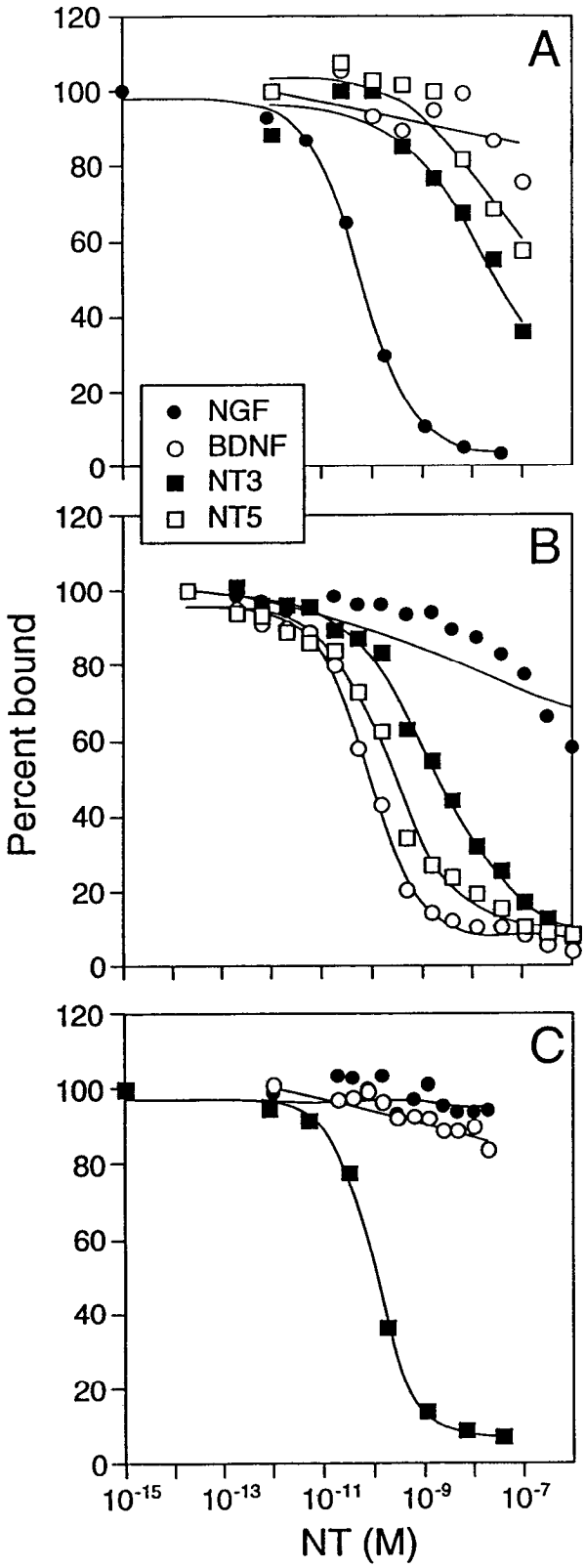

Figure 10. Competitive displacement of neurotrophins bound to trk-IgG. Radiolabeled neurotrophins (25–35 pM) were bound to trk-IgG in the presence of increasing concentrations of various unlabeled neurotrophins. *A*, Labeled NGF binding to trkA-IgG. *B*, Labeled BDNF bound to trkB-IgG. *C*, Labeled NT3 bound to trkC-IgG. Displacement was with cold NGF (●), cold BDNF (○), cold NT3 (■), or cold NT5 (□).

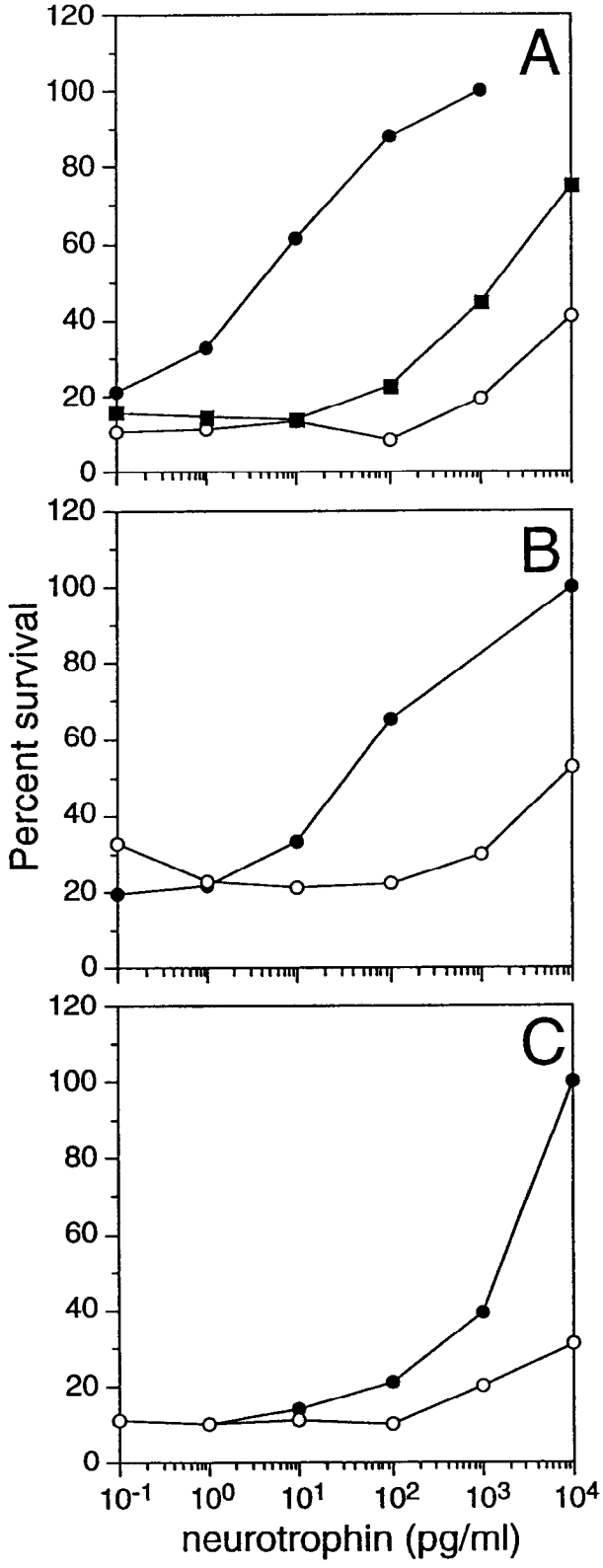

Figure 11. Neurotrophin bioactivity is blocked by trk immunoadhesins. Neurotrophin bioactivity was assessed by measuring the survival of chick dorsal root (*A* and *B*) or sympathetic (*C*) ganglion neurons in the absence or presence of trk immunoadhesins. *A* shows the blockade of NGF activity by trkA-IgG, *B* shows the blockade of BDNF activity by trkB-IgG, and *C* shows the blockade of NT3 activity by trkC-IgG. Trk immunoadhesins were not present (●), present at 1 μg/ml (○) or at 0.1 μg/ml (■).

proaches, we have determined the nucleotide and deduced amino acid sequence for several different forms of each molecule. Previous work described the nucleotide and deduced amino acid sequence of trkB from the mouse and rat, and trkC from rat and pig. The human sequence for trkA has been reported; however, there is a discrepancy between the original sequence and that reported here. Due to the sequencing of multiple reactions, and the conservation of this cysteine at position 300 in rat trkA (Meakin et al., 1992) and all other known trks (see Fig. 3), it seems likely that the original sequence is in error, although a formal possibility still exists that this is a polymorphism. This cysteine may be an important residue, as it would lie in a position to participate in a disulfide bond which helps to stabilize the second Ig-like domain of the extracellular region. This second Ig domain is interesting, as it is the most highly conserved extracellular region and so, therefore, is likely to be structurally important and perhaps contribute to binding (see below).

The human sequences of the trks are highly conserved compared to the other mammals, especially in the TK domain. In particular, all of the consensus motifs that are thought to define the trk family and differentiate it from other receptor tyrosine kinases are conserved. By examining the degree of similarity between the different trks in a single species and the same trk in different species, certain generalizations may be drawn. The comparison of the three human trks to each other and the equivalent trk from the rat is shown for the different domains (Schneider and Schweiger, 1991) in Figure 3. Each domain of the trks is quite conserved between human and rat, with trkB and trkC being almost identical between these two mammalian species. Each individual domain of trk B and trkC is at least 85% similar between rat and human. Although its overall degree of similarity between human and rat is quite high, trkA shows regions of significant sequence divergence. In particular, in the extracellular domain, only the leucine rich and second Ig-like domain have greater than 85% similarity. This may have implications for the localization of the neurotrophin binding domain(s) of the trks. Like trkB and trkC, the transmembrane and intracellular domains of trkA are highly conserved between rat and human.

When similarity comparisons of different trks in the human are examined, it is readily apparent that the TK domain is the most highly conserved across the different trks. Of the extracellular domains, it is, again, the second Ig-like domain, along with the second cysteine-rich domain that are most similar between the different human trks.

While the primary sequences of the trks are quite conserved between humans and other mammals, there were species differences observed in splicing patterns. In the rodent, trkB encodes at least two different truncated forms and Northern blots probed for trkB transcripts exhibit a complex pattern. We failed to find evidence for the existence of the t2 form of trkB in the human despite considerable effort and observed a much simpler trkB transcript pattern. While we cannot rule out the existence of a homolog of this form in the human, a t2 equivalent seems unlikely to be expressed as abundantly as in the rodent. Since the different forms of truncated trkB are differentially expressed in various cell types in the rodent (Armanini et al., 1993, and in preparation), it will be interesting to see how the distribution of the single form of trkB seen in the human corresponds to the distribution of the various forms seen in the rodent.

One of the proposed roles for the truncated forms of the trks is to act as a dominant negative influence on signal transduction by neurotrophin in the expressing cell (Jing et al., 1992). This is consistent with the relative lack of efficacy of neurotrophin signalling seen in tissue from the adult brain when stimulated by neurotrophins (Knüsel et al., 1994), as the ratio of truncated to nontruncated forms of the trks is quite high in the adult (see Fig. 6). If this is the main role for truncated trks, then the apparent absence of t2 in the human is all the more interesting, as it has been shown that, in the rodent, t2 is primarily expressed in neurons (Armanini et al., 1993). If t2 is, indeed, not expressed in humans, and this results in a lower level of truncated trkB expressed in neurons, then the proposed dominant negative effect might not be as important in human neurons as in the rodent.

There are also differences between human and other mammalian transcripts of trk C. In the human extracellular domain, there is apparent alternate splicing giving rise to two forms (with and without an insert of nine amino acids). This apparent deletion site aligns with the previously characterized insertion site in trkA. As yet, no functional differences in binding or signal transduction have been described between the two splice forms in the rat trkA where the insert is six amino acids (Barker et al., 1993). Whatever the biological role for the differently spliced forms, they are species specific, as no evidence of alternate splicing at this site was seen in trkC outside the human (Lamballe et al., 1991; Tsoulfas et al., 1993; Valenzuela et al., 1993).

We also found examples of various forms of human trkC differing in the intracellular part of the molecule. We observed the presence of a truncated form of trkC that does not contain the consensus tyrosine kinase domain. Unlike trkB, where the truncated forms have a very short cytoplasmic tail, the added cytoplasmic portion of truncated human trkC is 83 residues long. In addition, there is a very high degree of conservation among species in this region, suggesting that it may have an important function, perhaps serving as a signal to specify subcellular localization.

As has been described for rat trkC, there are forms of human trkC that contain an insert in the TK domain. Unlike the rat, where there are possible inserts of 14 and 25 or 39 amino acids, there appears to be only a 14 amino acid insert possible at this site in the human. It is likely that these inserts play an important role in modulating the signalling cascade induced by ligand binding to trkC. Using PC12 cells expressing various forms of trkC, it has been shown that expression of trkC with no insert in the TK domain confers the ability to respond to NT3 with neurite outgrowth as well as NT3-induced autophosphorylation. Cells expressing trkC with a TK insert are capable of ligand-induced autophosphorylation, but do not respond to NT3 with neurite outgrowth (Tsoulfas et al., 1993; Valenzuela et al., 1993). There are no differences yet described between the 14, 25, and 39 amino acid inserts in this regard, but there are many downstream sequelae to neurotrophin binding, and very few have been examined to date. This processing is tissue specific, as no evidence of the insert-containing form was observed in human testis.

Analysis of the transcripts for trkB using Northern blots showed a relatively simple pattern compared to that seen in the rodent (Middlemas et al., 1991). This is consistent with the idea that there is only a single main truncated form of trk B in the human. Analysis of the trkC showed a more complex pattern of transcript sizes, in keeping with the greater number of forms detected during sequence analysis of the clones. Evidence was seen for a transcript hybridizing with the kinase probe but not with the

extracellular probe, as has been described in rat trkC (Valenzuela et al., 1993). In analyzing different tissues, the primary location of trkB and trkC expression was in the nervous system and, specifically, in regions of the CNS. Although the levels of expression were low compared to those found in various regions of the brain, there is expression of trkB and trkC in a wide variety of tissues outside the nervous system. Some of the expression seen in certain tissues might be due to expression on elements of the nervous system sparsely scattered through the tissue. For example, the expression of trkC in the small intestine may turn out to be due in whole or in part to expression by the neurons of the enteric nervous system. Final elucidation of this will have to await a detailed *in situ* hybridization analysis of tissues outside the nervous system.

The *in situ* hybridization analysis of the expression of the trks in the human nervous system confirmed that the general expression pattern is similar to that seen in other mammals, yet there were subtle differences seen between the human tissues and those from rodents. An example of this is the unexpected finding of the apparent polarization of the developing human DRG, with trkA cells predominant in the dorsal and trkC expressing cells predominant in the ventral area of the developing ganglia. This is reminiscent of the polarization of cell size seen in the developing avian DRG. This polarization of trk expression was not apparent in sections from the adult human DRG or in rat embryos hybridized with rat trkA and trkC probes (data not shown). This finding underscores the importance of not relying entirely on rodent model systems in order to understand normal human development and pathological conditions. The ability to perform *in situ* hybridization studies on human tissue should provide a foundation for further studies designed to examine the expression of the differently spliced forms of the human trks in detail in certain areas of normal and pathological tissues. In this regard, given the difficulty of obtaining human tissue, it is encouraging that the *in situ* hybridization was performed on tissues handled in a variety of ways postmortem. Sections were cut unfixed, fixed and frozen, and fixed and paraffin-embedded. All of these methods yielded useful results.

Expression of the soluble trk extracellular domain fusion proteins proved particularly useful for the evaluation of binding characteristics of neurotrophins. These proteins are produced at high levels in cell culture supernatant and are simple to purify using protein A. Indeed, purification is not necessary for many uses, as we have successfully performed binding assays by immobilizing trk-IgG proteins directly from total cell supernatants onto 96-well plates previously coated with Fab fragments of goat anti-human Fc. The binding data presented here demonstrates that the trk-IgG fusions bind neurotrophins with a selectivity and affinity similar to that seen by expression of the whole receptor in cells. The binding assays as reported here are very simple to do in large numbers, have excellent reproducibility and low background, and retain the specificity of the native trks. These qualities have proven quite valuable in analyzing the binding characteristics of mutant neurotrophins (Winslow et al., 1993; Laramee et al., 1994).

In addition to their utility in analyzing the binding of neurotrophins, the trk-IgG chimeras are potent inhibitors of the biological activity of their cognate neurotrophin. All of the experiments shown here have been performed in *in vitro* systems, but preliminary experiments indicate that trkA-IgG is capable of inhibiting NGF activity *in vivo* as well (data not shown). This will fill an unmet need, as it has been difficult to raise good blocking antisera to BDNF, NT3, and NT4/5. A potential drawback to using these reagents to block endogenous ligands in a biological system is that they are no more ligand specific than the trk receptors themselves. Blockade of an endogenous activity with trkB, for example, will not distinguish between that activity being due to BDNF or NT4/5. However, given the lack of alternatives for blocking BDNF and NT4/5, and the good specificity of trkA and trkC, these reagents should prove of use.

With the information in hand about the forms of trk present in humans, it should now be possible to begin to investigate the expression of these forms in the normal and diseased state. Knowledge of the expression levels of all forms of each trk will be crucial, as the different forms can display different and sometimes counteracting signal transduction properties in response to neurotrophins. In addition, the availability of soluble forms of the human trks should, by allowing the blocking of endogenous bioactivity, accelerate the investigation of the biology of neurotrophins *in vivo*.

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