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An Extended Family of Protein-Tyrosine Kinase Genes Differentially Expressed in the Vertebrate Nervous System

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Summary

We have used PCR to identify 13 novel protein-tyrosine kinase genes (tyro-1 to -13), six of which (tyro-1 to -6) are preferentially expressed in the developing vertebrate nervous system. The tyro-2 and tyro-9 genes encode kinase domains that exhibit strong amino acid sequence similarity to the equivalent regions of the receptors for EGF and FGF, respectively, and may encode novel receptors for these or related polypeptide ligands. The tyro-1 to -6 genes are all expressed during central nervous system neurogenesis and exhibit distinct and highly regionalized patterns of expression in the adult brain. Together with recent studies in invertebrates, these data are consistent with the hypothesis that protein-tyrosine kinases play a central role in neural development.

Introduction

Among the signal transduction molecules implicated in neural development, the receptor protein-tyrosine kinases (PTKs) are of particular interest. These proteins function as transmembrane receptors for polypeptide growth factors, and contain a tyrosine kinase as an integral part of their cytoplasmic domains (Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990). Binding of a polypeptide ligand to its corresponding cell surface receptor results in rapid activation of that receptor's intracellular tyrosine kinase, which in turn results in the tyrosine phosphorylation of the receptor itself and of multiple downstream target proteins (Hunter and Cooper, 1985; Hunter et al., 1990). For many receptor PTKs, growth factor binding ultimately triggers multiple rounds of cell division.

Molecular studies of mutations that affect cell differentiation have demonstrated that several of these receptor PTKs act as early determinants of cell fate. Loss-of-function mutations in the *sevenless* gene of Drosophila (Harris et al., 1976), for example, abolish the tyrosine kinase activity of a transmembrane receptor expressed in the developing ommatidia of the eye and result in the aberrant differentiation of the precursors to the number 7 photoreceptors (reviewed in Basler and Hafen, 1988b; Rubin, 1989). Rather than becoming number 7 photoreceptor cells, these precursors instead differentiate into nonneuronal cone cells, which form the lens. In marked contrast, the remaining complement of photoreceptors (numbers 1-6 and 8) differentiate normally.

Mutations in genes encoding other receptor PTKs have also been shown to affect cell differentiation. For example, mutations in the *torso* gene of Drosophila specifically disrupt the terminal differentiation of extreme anterior and posterior structures in the embryo (Sprenger et al., 1989), and mutations in the Drosophila *Ellipse* gene, which encodes a homolog of the mammalian epidermal growth factor (EGF) receptor, result in the developmental failure of multiple cell types in the eye (Baker and Rubin, 1989). In vertebrates, mutations in the mouse dominant white spotting locus (*W*), which encodes the c-*kit* receptor PTK, produce pleiotropic developmental effects that include disruption of the normal proliferation and differentiation of neural crest-derived melanocytes (Chabot et al., 1988; Geissler et al., 1988).

Parallel to these studies of the developmental role of receptor PTKs has been the demonstration that many of the ligands for these receptors influence the differentiation of neural cells in culture. Platelet-derived growth factor (PDGF), for example, has been shown to stimulate the proliferation and prevent the premature differentiation of oligodendrocyte/type-2 astrocyte glial progenitor cells in rat optic nerve cultures (Noble et al., 1988; Raff et al., 1988).

Similarly, both acidic and basic fibroblast growth factor (bFGF) have been shown to stimulate the neuronal differentiation of cultured rat pheochromocytoma (PC-12) cells (Togari et al., 1985; Wagner and D'Amore, 1986). bFGF has also been reported to prolong survival and stimulate neurite outgrowth in cultures of primary cortical and hippocampal neurons (Morrison et al., 1986; Walicke et al., 1986), to induce cell division, neuronal differentiation, and nerve growth factor (NGF) dependence in adrenal chromaffin cells (Stemple et al., 1988), and to function as a survival factor, both in vivo and in vitro, for neural crest-derived nonneuronal cells during the early development of sensory ganglia (Kalcheim, 1989). Recently, the product of the mouse mutant steel gene (*Sl*), which interacts genetically with *W*, has been identified as a growth factor ligand for the c-*kit* receptor (for review see Witte, 1990). Genetic and biochemical studies of the expression patterns of the *sevenless*, *torso*, and c-*kit* receptors suggest that specification of cell fates can be achieved through the spatially and temporally restricted expression of either the receptors or their ligands (Rubin, 1989; Tomlinson and Ready, 1987; Reinke and Zipursky, 1988; Banerjee and Zipursky, 1990; Stevens et al., 1990; Matsui et al., 1990).

Given these observations and our interest in cellular differentiation in the mammalian peripheral nervous system, we sought to define the repertoire of receptor PTK genes expressed in developing peripheral nerves. In this report, we present a polymerase chain reaction (PCR) analysis of subtracted sciatic nerve cDNAs that identifies 27 PTK genes expressed in the developing and mature peripheral and central

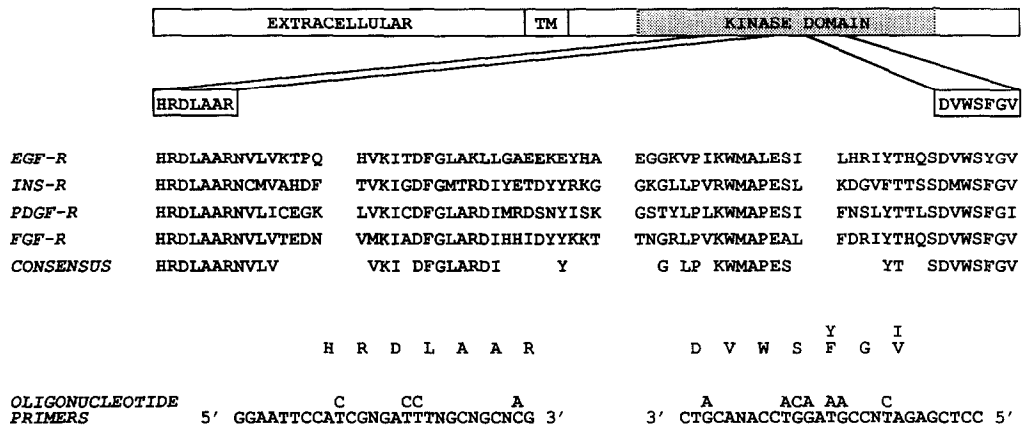


Figure 1. Sequence and Position of Oligonucleotides Used as Primers in PCR

The amino acid sequences HRDLAAR (upstream) and DVWS(F/Y)G(V/I) (downstream) were selected on the basis of their high degree of conservation in 14 PTK domain sequences (see Hanks et al., 1988). The sequences of four molecules are provided to illustrate the extent of similarity in this region: epidermal growth factor receptor (EGF-R; Ullrich et al., 1984), insulin receptor (INS-R; Ullrich et al., 1985), platelet-derived growth factor receptor (PDGF-R; Yarden et al., 1986), and the fibroblast growth factor receptor (FGF-R; Lee et al., 1989). The "consensus" sequence shown contains residues that are less well conserved across the panel of 14 sequences analyzed by Hanks et al. (1988). Degeneracies are as indicated, with N denoting all four deoxynucleotides. These primers are nearly fully degenerate, with the exception of the first arginine residue of the upstream primer and the serine residue of the downstream primer.

nervous systems of the rat. Thirteen of these 27 genes have not been described previously. Of these 13, 2 encode kinase domains that exhibit strong sequence similarity to those of known polypeptide growth factor receptors, and 6 are predominantly expressed by distinct sets of neural cells.

Results

Isolation of Clones

We used PCR to amplify PTK-related sequences located between the degenerate oligonucleotide primer sequences shown in Figure 1. These primers correspond to the amino acid sequences HRDLAAR (upstream) and DVWS(F/Y)G(I/V) (downstream), which flank a highly conserved region of the kinase domain shared by receptor PTKs (Hanks et al., 1988). The upstream primer was chosen to exclude members of the *src* family of cytoplasmic tyrosine kinases. The downstream primer was chosen such that a second highly conserved amino acid sequence diagnostic of PTKs – P(I/V)(K/R)W(T/M)APE – would be contained within amplified PCR products. The DNA substrates used for amplification were subtracted sciatic nerve cDNAs, enriched either for transcripts expressed in sciatic nerve or for transcripts shared between sciatic nerve and brain. Three different PCR substrates were used. The preparation of each of these substrates, and the hybridization and extension parameters used for their amplification, are described in Experimental Procedures. Amplified products were size fractionated on polyacrylamide gels, and appropriately sized products (~220 bp) were subcloned into pBluescript (Stratagene). Nucleotide sequences of plasmid DNAs isolated from multiple independent transformants were then determined using double-stranded dideoxy sequencing.

Sequence analysis of 168 PCR product subclones yielded 155 with significant similarity to the tyrosine kinase family. Figure 2 lists the 27 distinct kinase domain sequences contained in this set, which includes those of the *abl*, *arg*, and *fer* cytoplasmic (nonreceptor) kinases, as well as those of the receptors for EGF (Ullrich et al., 1984), PDGF-A (Matsui et al., 1989; Lee et al., 1990), PDGF-B (Yarden et al., 1986; Claesson-Welsh et al., 1988; Gronwald et al., 1988), bFGF (Lee et al., 1989; Reid et al., 1990; Safran et al., 1990), colony-stimulating factor 1 (CSF-1; Coussens et al., 1986; Rothwell and Rohrschneider, 1987), and insulin-like growth factor 1 (IGF-1; Ullrich et al., 1986). The high percentage of isolates encoding tyrosine kinases (92%) and the large number of different kinase clones obtained probably reflect the highly degenerate primers and low temperature annealing and extension parameters we used for PCR amplification (see Experimental Procedures), as well as the stringent size criteria we used in the subcloning and sequencing of PCR products.

Thirteen Novel PTK Sequences Are Identified

Of the 27 different kinases identified in this nervous system survey, 13 (tyro-1 through tyro-13) are novel. For the previously identified kinases, several of our rat isolates differ by 1 or 2 amino acids from the kinase domain sequences reported for other species. Nucleotide sequence comparisons suggest that these differences are accounted for by species variation and do not represent the amplification of novel kinase cDNAs. The novel isolates tyro-1, tyro-9, and tyro-11 were each obtained only a single time. We cannot

KINASE SUB-FAMILY		DEDUCED AMINO ACID SEQUENCES FOR PUTATIVE TYROSINE KINASES				
		VI	VII	VIII	IX	INCIDENCE
abl	abl	NCLVGENH	LVKVADFGLSRLMTGDTYTAH	AGAKFPIKWTAPESL	AYNKFSIKS	6
	arg	NCLVGENH	VVKVADFGLSRLMTGDTYTAH	AGAKFPIKWTAPESL	AYNTFSIKS	3
	fes/fps*	NCLVTEKN	VLKISDFGMSREEADGVYAASG	GLRQVPVKWTAPEAL	NYGRYSSES	
	fer	NCLVGENN	TLKISDFGMSRQEDGGVYSSS	GLKQIPIKWTAPEAL	NYGRYSSES	2
src	Dsrc28*	NCLVGSEN	VVKVADFGLARYVLDDQYTSSG	GTKFPIKWAPPEVL	NYTRFSSKS	
	tyro-8	NCLVDSDL	SVKVSDFGMTRYVLDDQYVSSV	GTKFPVKWSAPEVF	HYFKTSSKS	2
tyro-13	tyro-13	NVLVSEDN	VAKVSDFGLTKEASSTQ	DTGKLPVKWTAPEAL	REKKFSTKS	11
eph/eck/elk	eph*	NILVNQNL	CCKVSDFGLTRLL DDFDGTYET	QGGKIPIRWTAPEAI	AHRIFTTAS	
	eck	NILVNSNL	VCKVSDFGLSRVLEDDPEATYTT	SGGKIPIRWTAPEAI	SYRKFTSAS	5
	tyro-1	NILVNSNL	VCKVSDFGMSRVLEDDPEAAYTT	RGGKIPIRWTAPEAI	AYRKFTSAS	1
	tyro-4	NILINSNL	VCKVSDFGLSRVLEDDPEAAYTT	RGGKIPIRWTSPEAI	AYRKFTSAS	4
	elk	NILVNSNL	VCKVSDFGLSRYLQDDTSDPTYTSS	LGGKIPVRWTAPEAI	AYRKFTSAS	1
	tyro-5	NILVNSNL	VCKVSDFGLSRFLEDDTSDPTYTSA	LGGKIPIRWTAPEAI	QYRKFTSAS	(3)
	tyro-6	NILVNSNL	VCKVSDFGLSRFLEDDPSDPTYTSS	LGGKIPIRWTAPEAI	AYRKFTSAS	3
	tyro-11	NILVNSNL	VCKVSDFGLSRFLEENSSDPTYTSS	LGGKIPIRWTAPEAI	AFRKFTSAS	(1)
EGF-R	EGF-R	NVLVKTPQ	HVKITDFGLAKLLGAEEKEYHA	EGGKVPIKWMALESI	LHRIYTHQS	3
	neu	NVLVKSPN	HVKITDFGLARLLDIDETEYHA	DGGKVPIKWMALESI	LRRRFTHQS	10
	tyro-2	NVLVKSPN	HVKITDFGLARLLEGDEKEYNA	DGGKMPIKWMALECI	HYRKFTHQS	8
FGF-R	bFGF-R	NVLVTEDN	VMKIADFGLARDIHHIDYYKKT	TNGRLPVKWMAPEAL	FDRIYTHQS	4
	bek	NVLVTENN	VMKIADFGLARDINNIDYYKKT	TNGRLPVKWMAPEAL	FDRVYTHQS	2
	tyro-9	NVLVTEDD	VMKIADFGLARGVHHIDYYKKT	SNGRLPVKWMAPEAL	FDRVYTHQS	1
PDGF-R	PDGF-A R	NVLLAQGK	IVKICDFGLARDIMHDSNYVSK	GSTFLPVKWMAPESI	FDNLYTTLS	3
	PDGF-B R	NMLICEGK	LVKICDFGLARDIMRDSNYISK	GSTFLPLKWMAPESI	FNSLYTTLS	1
	CSF-1 R	NVLLTSGH	VAKIGDFGLARDIMNDSNYVVK	GNARLPVKWMAPESI	FDCVYTVQS	20
	flt	NILLSENN	VVKICDFGLARDIYKNPDYVRR	GDTRLPLKWMAPESI	FDKVYSTKS	5
tyro-3	tyro-3	NCMLAEDM	TVCVADFGLSRKIYSGDYYRQG	CASKLPVKWLALESL	ADNLYTVHS	3
	tyro-7	NCMLNENM	SVCVADFGLSKKIYNGDYYRQG	PFAKMPVKWIAIESL	ADRVYTSKS	4
	tyro-12	NCMLRDDM	TVCVADFGLSKKIYSGDYYRQG	RIAKMPVKWIAIESL	ADRVYTSKS	(3)
Insulin-R	trk*	NCLVGQGL	VVKIGDFGMSRDIYSTDYYRVG	GRTMLPIRWMPPESI	LYRKFTTES	
	trkB*	NCLVGENL	LVKIGDFGMSRDVYSTDYYRVG	GHTMLPIRWMPPESI	MYRKFTTES	
	IGF1R	NCMVAEDF	TVKIGDFGMTRDIYETDYYRKG	GKGLLPVRWMSPESL	KDGVFTTHS	2
	tyro-10	NCLVGKNY	TIKIADFGMSRNLYSGDYYRIQ	GRAVLPIRWMSWESI	LLGKFTTAS	(6)

Figure 2. Deduced Amino Acid Sequences of PCR Product Subclones

Amino acid sequences were deduced from the nucleotide sequences of the 27 different PTK domain cDNAs encountered in this survey. Deduced amino acid sequences corresponding to the oligonucleotide primers used for PCR amplification are not included. Kinase domain sequences are segmented according to the subdomains defined by Hanks et al. (1988). After each sequence is a number indicating the number of times it was identified. Numbers listed parenthetically correspond to clones uniquely obtained from amplification of the BD substrate (see Experimental Procedures). The segregation of kinase domain subfamilies is based solely on amino acid sequence conservation (see Results and Experimental Procedures); sequences denoted by an asterisk were not encountered in this survey but have been included to facilitate comparisons. References are as follows: *abl* (human; Shtivelman et al., 1986), *arg* (human; Kruh et al., 1986), *fes* (human; Roebroek et al., 1985), *fer* (human; Hao et al., 1989), *Dsrc28* (Drosophila; Gregory et al., 1987), *eph* (human; Hirai et al., 1987), *eck* (human; Lindberg and Hunter, 1990), *elk* (rat; Letwin et al., 1988), EGF-R (human; Ullrich et al., 1984), *neu* (rat; Bargmann et al., 1986), bFGF-R (chicken; Lee et al., 1989), *bek* (mouse; Kornbluth et al., 1988), PDGF-A R (human; Matsui et al., 1989) (rat; Lee et al., 1990), PDGF-B R (human; Claesson-Welsh et al., 1988; Gronwald et al., 1988), CSF-1R (human; Coussens et al., 1986) (mouse; Rothwell and Rohrschneider, 1987), *flt* (human; Shibuya et al., 1990), *trk* (human; Martin-Zanca et al., 1986), *trkB* (mouse; Klein et al., 1989), and IGF1-R (human; Ullrich et al., 1986).

therefore exclude the possibility that the nucleotide sequences of these clones contain rare incorporation errors due to the infidelity of the Taq polymerase. We do note, however, that in the two cases where the previously reported cDNAs sequences were from rat—for *elk* and *neu*—our PCR nucleotide sequences are identical.

The kinase domain sequences of tyro-1 through tyro-13 have been grouped by similarity to the equivalent sequences of known PTKs (Figure 2). The indicated subfamilies were defined with reference to a computer-generated phylogenetic tree, constructed as described in Experimental Procedures. Tyro-1 and tyro-4, for example, are related to the epithelial cell kinase (*eck*) (Lindberg and Hunter, 1990), tyro-2 to the EGF receptor and the *neu* proto-oncogene (Bargmann et al., 1986), tyro-5, tyro-6, and tyro-11 to the *elk* kinase (Letwin et al., 1988), tyro-9 to the bFGF receptor, and tyro-10 to *trk* and *trkB* (Martin-Zanca et al., 1986; Klein et al., 1989). Although they exhibit similarity to the insulin receptor, tyro-3, tyro-7, and tyro-12 are listed as a novel subfamily since they are more closely related to each other than to any previously described kinase. The *eck*- and *elk*-related sequences are listed in separate subsets, but it is important to note the high degree of similarity between these subfamilies. The sequences of *fes*, *trk*, *trkB*, and *Dsrc28* (each marked with an asterisk) are included in Figure 2 only for comparison, since they were not encountered in our survey.

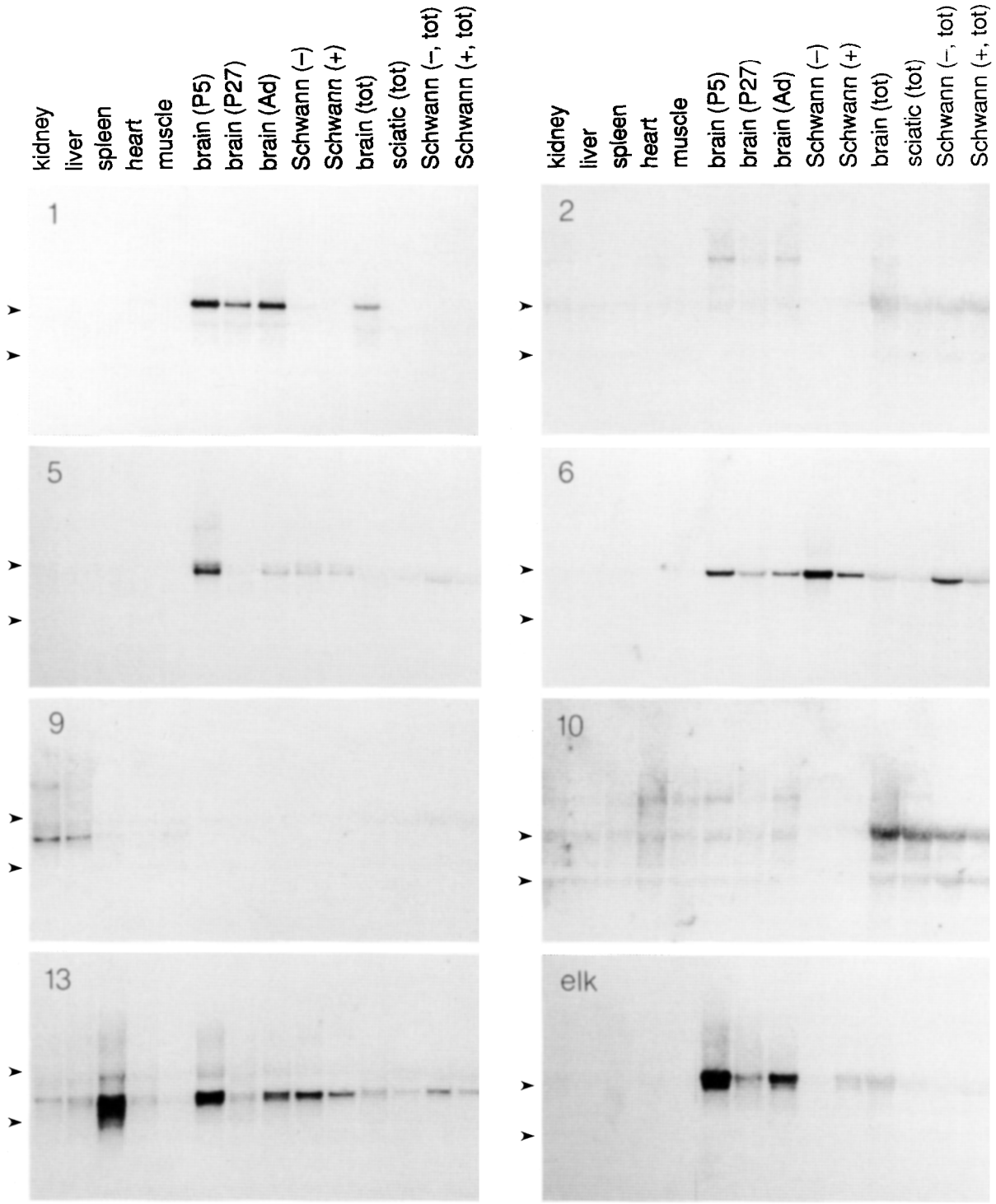
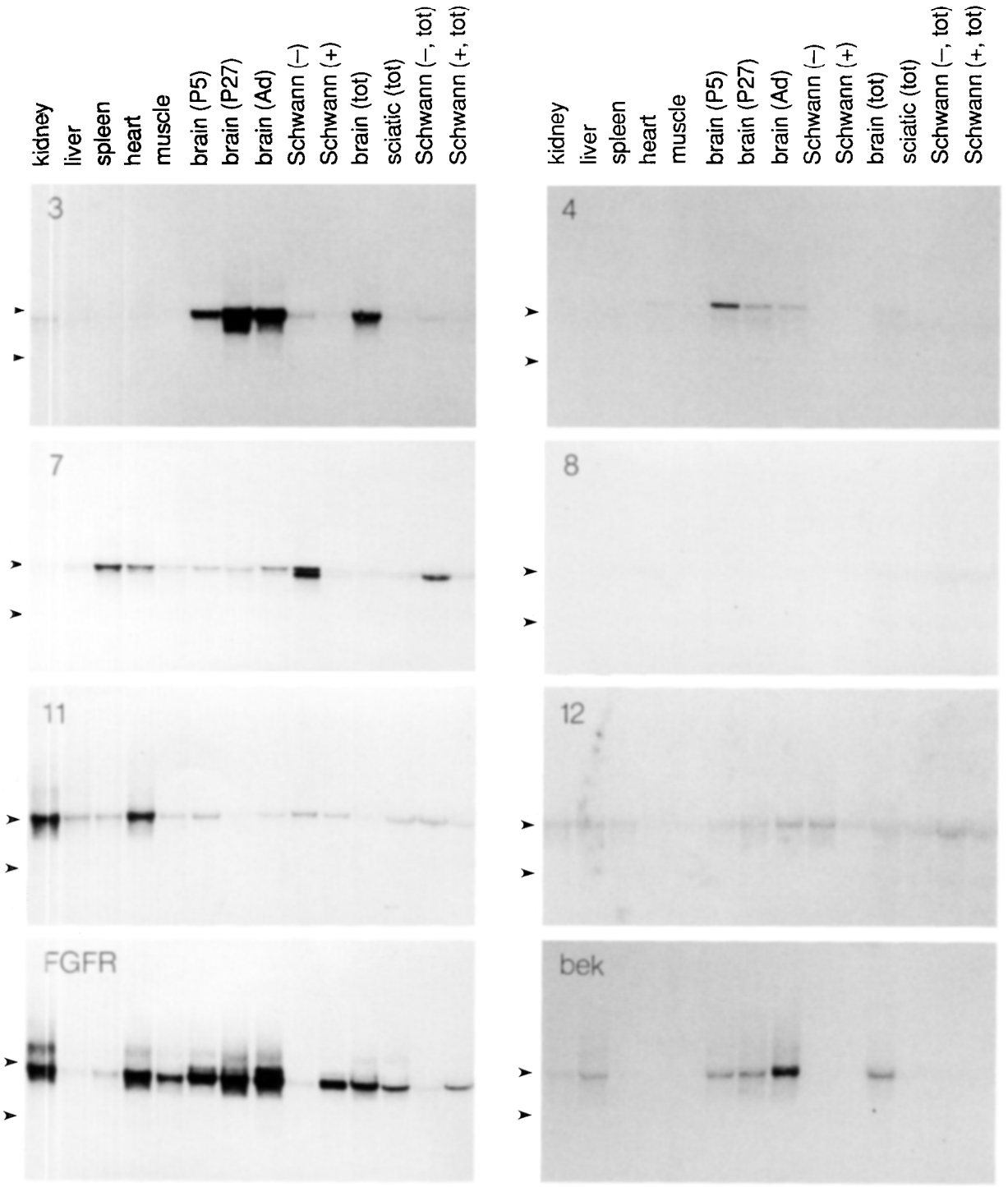


Figure 3. Tissue Expression Profile of Novel PTK mRNAs

Poly(A)$^+$ (left 10 lanes) or total RNA (tot, right 4 lanes) from the indicated rat tissues was analyzed for expression of PTK mRNAs. All tissues were taken from animals 27 days postnatal, except where otherwise indicated. Sciatic nerves (sciatic) were obtained from 7- to 8-day-old rats. Rat Schwann cells were cultured in either the presence (+) or absence (–) of 20 μM forskolin for 48 hr prior to harvesting. All lanes contain either 2.5 μg of poly(A)$^+$ RNA or 10 μg of total RNA, except for the cultured Schwann cell poly(A)$^+$ lanes, which contain 1.0 μg each. The relative migration of 18S and 28S ribosomal RNAs, as determined by methylene blue staining, is indicated by the arrowheads. Filters 1–13 show hybridization with ^{32}P-radiolabeled cDNA probes to tyro-1 through tyro-13. Also shown for comparison is the hybridization observed using our isolates of *elk*, the bFGF receptor (FGFR), and the *bek* FGF receptor. Exposure times are as follows: 34 hr (1, 5, 6, 7, 11), 41 hr (3, 4, FGFR), 120 hr (2, 9, 10, bek), 158 hr (8, 13, elk), 8 days (12).



Six Novel Clones Are Expressed Predominantly or Exclusively in Neural Cells

We characterized the expression pattern of the 13 novel kinase clones by first examining the relative levels of mRNA present in a variety of neonatal and adult rat tissues. Radiolabeled cDNA probes for each of these clones, as well as probes prepared from our isolates of the bFGF receptor, *bek*, and *elk* kinases, were hybridized to a set of eight parallel Northern blots containing RNA isolated from kidney, liver, spleen, heart, skeletal muscle, brain, sciatic nerve, and cultured Schwann cells. RNA was isolated from Schwann cells cultured in both the presence and absence of the adenylate cyclase activator forskolin, since at least one receptor PTK gene (that encoding the PDGF-B receptor) exhibits cell-specific cAMP induction in these cells (Weinmaster and Lemke, 1990). Individual blots were in some cases reutilized for as many as four rounds of hybridization. Relative exposure times are indicated in the legend to Figure 3.

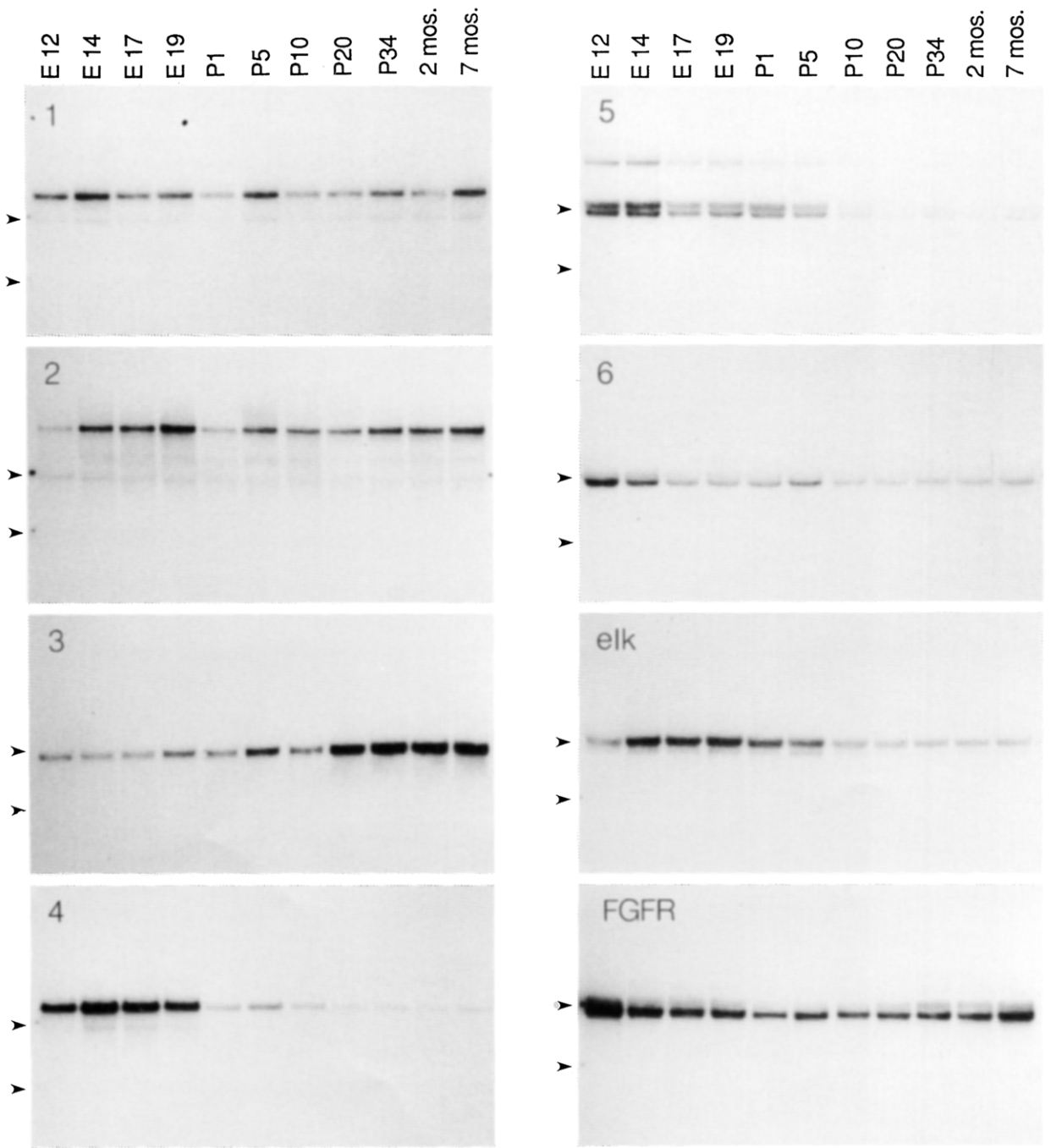


Figure 4. Developmental Expression Profile of Neural PTK mRNAs

Poly(A)$^+$ RNA (2 μg) from rat brains obtained from animals of the indicated ages (E12 to 7 months postnatal) was analyzed for the expression of PTK mRNAs. Filters 1–6 show hybridization obtained with ^{32}P-radiolabled cDNA probes to tyro-1 through tyro-6. Also shown are the hybridization profiles obtained using our isolates of *elk* and the bFGF receptor (FGFR). The relative migration of 18S and 28S ribosomal RNAs, as determined by methylene blue staining, is indicated by the arrowheads. Exposure times are as follows: 15 hr (1, 3, 5, elk, FGFR), 22 hr (4, 6), 50 hr (2).

The results of this analysis (Figure 3) demonstrate that 6 of the 13 novel kinase genes (tyro-1 through tyro-6), together with the *elk* gene, are preferentially expressed by cells of the nervous system. For example, tyro-1, a novel member of the *eck* kinase subfamily, exhibited strong hybridization to brain mRNA, a faint signal in Schwann cells, and very faint signals in kidney and heart. Tyro-4, also a novel member of the *eck* subfamily, exhibited more modest hybridization to two mRNAs in postnatal day 5 (P5) brain, with lower signals evident in older brains as well as kidney and heart. The novel EGF receptor homolog tyro-2 identi-

fied a high molecular weight mRNA in brain that could also be detected in kidney and heart. We cannot exclude the possibility that the very low tyro-1, tyro-2, and tyro-4 hybridization signals observed in kidney and heart are due to neural contamination from the adrenal gland and cardiac ganglia, respectively. Tyro-3, a member of the novel kinase subfamily with similarity to the insulin receptor, showed intense hybridization to brain mRNA, with very faint signals in perhaps all other tissues.

Members of the same receptor-configured kinase subfamily occasionally exhibited very different patterns of expression. Within the *elk* subfamily, for example, *elk* itself and the related kinases tyro-5 and tyro-6 were exclusively or predominantly expressed in neural tissues. *elk* strongly hybridized to two mRNA species in brain and Schwann cells, tyro-5 exhibited strong hybridization to P5 brain mRNA with reduced signals present in later stage brains and in Schwann cells, and tyro-6 gave a strong hybridization signal in cultured Schwann cells, weaker signals in brain, and very faint but detectable signals in other tissues. In contrast, expression of the *elk*-related kinase tyro-11 was predominant in heart and kidney, but expressed at lower levels in neural tissue. The distinct hybridization patterns observed between members of this closely related subfamily indicate that despite significant similarity at the nucleotide level, cross-hybridization is not readily detected when hybridizations are carried out at high stringency. Tyro-5 and tyro-6, the most closely related of the PTK domains we analyzed, exhibit 84.2% nucleotide identity over the kinase domain, but their hybridization profiles can be readily distinguished (Figure 3, compare profiles 5 and 6).

Among those novel kinases not restricted to neural cells, tyro-9, a member of the FGF receptor subfamily, exhibited a pattern of expression that was distinct from that of either the bFGF receptor or *bek*. Most strongly expressed in kidney and liver, it exhibited only weak hybridization signals with brain mRNA. At two extremes of expression, tyro-12 yielded weak hybridization signals in all tissues, with expression being somewhat lower in heart and muscle, but tyro-8 (distantly related to *Dsrc28*) yielded only an extremely faint signal in spleen and heart.

Schwann cell expression of certain kinase genes was strongly regulated by cAMP (Figure 3). As for the PDGF receptor gene (Weinmaster and Lemke, 1990), expression of the *elk* and FGF receptor genes was significantly up-regulated by 48 hr treatment with forskolin. Since cAMP induction of the PDGF receptor appears to account for the synergistic effect on Schwann cell proliferation achieved with combined application of PDGF and forskolin (Weinmaster and Lemke, 1990), cAMP induction of the FGF receptor may also explain the similar synergistic effect observed for the combination of FGF and forskolin (Davis and Stroobant, 1990). Importantly, cAMP induction was not observed for most of the receptor PTKs expressed by Schwann cells; the tyro-1, tyro-3, tyro-6, tyro-7, tyro-12, and tyro-13 mRNAs were down-regulated in the presence of forskolin, and expression of the tyro-5 and tyro-11 genes was not affected by the drug.

Several receptor PTKs exhibited relatively modest signals in sciatic nerve compared with cultured Schwann cells or other tissues. This is probably a function of both the cellular heterogeneity of the nerve, which contains a substantial number of fibroblasts and endothelial cells, and the great sensitivity of PCR amplification.

The Novel Neural PTK Genes Are Expressed during Embryogenesis and Are Developmentally Regulated

Since many of the determinative events in mammalian neural development occur near the midpoint of embryogenesis, we investigated whether any of the novel neural kinase genes were expressed embryonically. To assess their developmental expression, we probed a set of Northern blots containing mRNA isolated from the brains of rats ranging in age from embryonic day 12 (E12) to adult. For comparison, we included the bFGF receptor and *elk* in this survey, the results of which are presented in Figure 4. For each of the novel kinase genes, expression was observed in the developing central nervous system at E12, a time at which multiple influences on both neural cell proliferation and differentiation are known to be exercised. Indeed, although detected in adult brain, three of the novel kinase genes were maximally expressed embryonically. mRNA encoding the *elk*-related kinase tyro-6, for example, was most abundantly expressed at E12; expression gradually fell until P10 and was relatively constant thereafter. Similarly, mRNA encoding the closely related kinase tyro-5 was maximally expressed at E14; expression fell sharply after P5 to a much lower steady-state level in the adult brain. The gene encoding the *eck*-related kinase tyro-4 exhibited a similar, though even more dramatic regulation, with a peak in expression at E14/17, a sharp drop at birth, and a low steady-state level after P10.

In contrast to the pronounced drop in expression for tyro-4 and tyro-5, expression of mRNA encoding the *eck*-like kinase tyro-1, while exhibiting some temporal fluctuation, was relatively constant throughout neural development. A similar, though less variable developmental profile, was observed for mRNA encoding the bFGF receptor. Although maximal expression was observed at E12, bFGF receptor mRNA levels fell only modestly during the course of brain development and remained high in adult animals. Of the novel kinase genes analyzed in Figure 4, only tyro-3 exhibited a significant increase in expression during late neural development, with appreciably higher mRNA levels (relative to E12) evident after P20.

Each Neural Kinase Gene Exhibits a Regionalized Pattern of Expression in the Brain

To determine whether any of the novel neural kinases exhibited cell type–restricted expression in the verte-

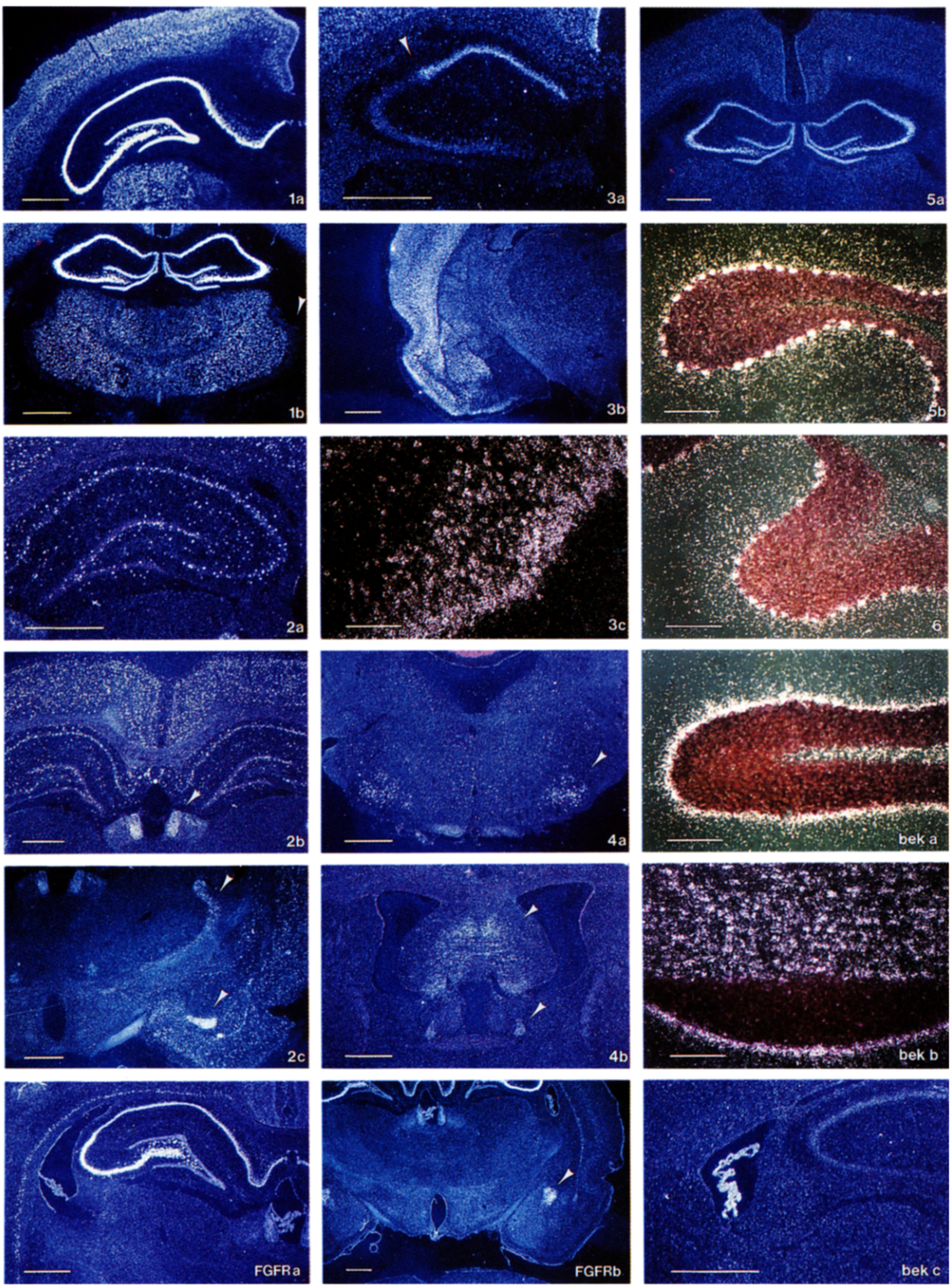


Figure 5. In Situ Localization of Novel PTK Transcripts in the Brain

Dark-field micrographs of selected brain regions are shown. The novel PTK clones tyro-1 to -6 are indicated by number. (1a) Tyro-1 mRNA in all fields of hippocampus and in neocortex, especially layer 3. (1b) Tyro-1 mRNA present in thalamus, but only very weakly

brate central nervous system, we prepared radiolabeled antisense RNA probes for each of the clones and hybridized these probes in situ to 30 μm brain sections prepared from 33-day-old and adult male rats. For comparison, we included antisense probes prepared from our isolates of the bFGF receptor and the related FGF receptor *bek*.

The results of this analysis are illustrated in the representative in situ hybridization profiles of Figure 5. Although these profiles represent only a selective sampling of the brain (we have not performed an exhaustive survey), they nonetheless demonstrate that expression of each of the novel neural kinases is highly regionalized. Tyro-1 mRNA was the most widely expressed in adult brain. Tyro-1 probes exhibited exceptionally strong and continuous hybridization in all fields of the hippocampus and the dentate gyrus (Figure 5, profile 1a). Strong tyro-1 hybridization was also evident in the thalamus, exclusive of the reticular nucleus (profile 1b), and throughout the neocortex, with a diffuse band present in layer 3 (profile 1a). Strong hybridization was also seen in the Purkinje cell layer, the inferior olive, and lateral nucleus of the cerebellum, but not in the cerebellar granule cell layers (data not shown).

In contrast, the tyro-2 gene exhibited a much more restricted pattern of expression. Hybridization was again evident throughout all fields of the hippocampus and the dentate gyrus, but signals were restricted to occasional (~1 in 10) cells (Figure 5, profile 2a). This striking, punctate pattern of hippocampal hybridization was not seen for any other PTK gene. A similarly restricted pattern of tyro-2 hybridization was also observed throughout neocortex (profile 2b). Stronger and more continuous hybridization was evident in the medial habenula (profile 2b) and in the reticular nucleus of the thalamus, but in contrast to tyro-1, no signal above background was observed in the remainder of the thalamus (Figure 5, profile 2c). The strongest tyro-2 hybridization signal in the brain was observed in an intercalated nucleus of the amygdala (profile 2c). No signal was evident in the Purkinje cell layer in the cerebellum. As is discussed below, the hybridization pattern we have observed for tyro-2 is largely consistent with its expression by a subset of γ-amino-n-butyric acid (GABA)-ergic neurons.

In situ hybridization signals corresponding to tyro-3 mRNA presented an equally striking pattern. In the hippocampus, strong hybridization was observed in the CA1 field. However, upon crossing the border from CA1 to the shorter CA2 field (Figure 5, profile 3a), we observed an abrupt drop in the tyro-3 hybridization signal. The tyro-3 signal remained much reduced in CA3 (relative to CA1), and we observed no signal at all in the dentate gyrus. Tyro-3 therefore provides an excellent molecular marker for the CA1/CA2 transition, previously defined on the basis of hippocampal cell size and circuitry. Robust tyro-3 hybridization was also evident in large cells throughout neocortex, with the strongest signals being observed in deeper layers (Figures 5, profiles 3b and 3c). In the cerebellum, strong hybridization was observed to granule cells but not to Purkinje cells, a pattern that was the opposite of that observed for tyro-1 (data not shown).

Consistent with their developmental expression profiles (Figure 4), tyro-4, tyro-5, and tyro-6 exhibited the most restricted patterns of expression in adult brain. Distinct hybridization to tyro-4 was evident in the facial nucleus of the pons (Figure 5, profile 4a), with more modest signals present in the bed nucleus of the anterior commissure and the triangular nucleus of the septum (profile 4b). The tyro-5 gene was expressed weakly in cortex, at a modest level in all fields of the hippocampus (profile 5a), and in a subset of Purkinje cells in the cerebellum (Figure 5, profile 5b). The tyro-6 gene showed a similar pattern of expression, giving a signal in Purkinje cells (profile 6) and weak signals in the hippocampus (data not shown). Although tyro-5 and tyro-6 exhibited no detectable cross-hybridization in our Northern blot analyses, we cannot exclude the possibility of cross-hybridization in these in situ profiles, due to the greater stability of RNA–RNA hybrids. We are currently performing in situ hybridizations of tyro-4, tyro-5, and tyro-6 probes to embryonic rat brain sections, where expression of these genes is more abundant.

The two FGF receptor genes that we examined, those encoding the bFGF receptor and *bek*, exhibited very different patterns of expression in the brain. mRNA encoding the bFGF receptor was expressed at high levels in hippocampal neurons, but exhibited a field distribution that was nearly the inverse of tyro-3,

in the reticular nucleus of the thalamus (arrowhead). (2a) Tyro-2 mRNA expressed by a subset of cells in all fields of the hippocampus. (2b) Tyro-2 mRNA present in neocortex, hippocampus, and the medial habenula (arrowhead). (2c) Tyro-2 mRNA present in the reticular nucleus of the thalamus (upper arrowhead) and an intercalated nucleus of the amygdala (lower arrowhead). (3a) Tyro-3 mRNA predominant in CA1 field of hippocampus, extremely weak in the dentate gyrus. The border between CA1 and CA2 is indicated by the arrowhead. (3b) Tyro-3 mRNA in neocortex. (3c) Tyro-3 mRNA in large cells in cortex (higher magnification). (4a) Tyro-4 mRNA in facial nuclei (arrowhead). (4b) Tyro-4 mRNA in triangular nucleus of the septum (upper arrowhead) and in the bed nucleus of the anterior commissure (lower arrowhead). (5a) Modest expression of tyro-5 mRNA in all fields of hippocampus and in neocortex. (5b) Tyro-5 mRNA in Purkinje cells in cerebellum (higher magnification). (6) Tyro-6 mRNA in Purkinje cells in cerebellum (higher magnification). (FGFRa) bFGFR mRNA in all fields of hippocampus, especially CA3, in the medial habenula and the choroid plexus, and in neocortex; note the band of cells deep in layer 6. (FGFRb) Strong bFGFR mRNA hybridization in the central nucleus of the amygdala (arrowhead). (bek a) *bek* mRNA in cells coincident with the Bergmann glia of the cerebellum (higher magnification). (bek b) *bek* mRNA expressed strongly in white matter glia of cerebellum (higher magnification). (bek c) Strong *bek* mRNA expression in the choroid plexus, but low expression in the hippocampus. Brain sections were obtained from P33 male rats in profiles 1b, 3a–3c, 4a, 5a and 5b, 6, and bek a. Sections were prepared from adult male rats in profiles 1a, 2a–2c, 4b, FGFRa and FGFRb, and bek b and bek c. In lower magnification photos, the scale bar represents 1 mm. In higher magnification photos, the scale bar represents 200 μm. Dipped slides were exposed for 2 weeks.

i.e., expression was reduced in CA1 relative to CA2 and CA3 (Figure 5, compare profiles FGFRa and 3a). mRNA levels in the dentate gyrus were lowest of all. We also observed expression of bFGF receptor mRNA in the choroid plexus and in the central nucleus of the amygdala and in a narrow band of cells in layer 6 of neocortex (profile FGFRb), a region not seen in the previous work of Wanaka et al. (1990). In contrast, expression of *bek* mRNA was largely confined to nonneuronal cells. High level expression was observed in the choroid plexus (Figure 5, profile bek c), and in the white matter glia of the cerebellum and the pons (profile bek b). Diffusely localized hybridization to a layer of cells that may be Bergmann glia was also apparent in the cerebellum (profile bek a). The cerebellar expression pattern of *bek* was clearly distinct from the patterns observed for tyro-5 and tyro-6, which marked Purkinje cells but exhibited no hybridization to white matter glia (Figure 5, compare profiles 5b and 6 with profile bek a).

Discussion

New PTKs

The identification of tyro-1 through tyro-13 significantly extends the repertoire of mammalian PTKs. Although we have deduced the amino acid sequence for only a small portion of these proteins, the presence of highly conserved features of the kinase domains of individual receptor PTK subfamilies suggests that the tyro-1 through tyro-12 genes are likely to encode cell surface receptors. Based on the conservation of diagnostic residues, the kinase domain sequences of each of these genes can be segregated into PTK subfamilies whose members include known cell surface receptors. The 54 residue portion of the kinase domain of tyro-9, for example, exhibits strong similarity to the equivalent domains of proteins contained in the FGF receptor subfamily, differing by 5 amino acids from the chicken bFGF receptor tyrosine kinase domain, 7 amino acids from the rat *bek* domain, and 10 amino acids from the human *flg* domain (Ruta et al., 1988). Conserved motifs evident in this particular subfamily include the sequences VMKIAD at the beginning of kinase domain segment VII, IDYYKKT at the end of this segment, and ALFDR at the boundary between segments VIII and IX.

Five of the novel kinases are related to the *eph/eck/elk* subfamily of kinases. Expression of four of these five (tyro-1, tyro-4, tyro-5, tyro-6) is predominantly neural, as is that of *elk*, suggesting that this large subfamily may be of particular importance to the differentiation of neural tissue. Tyro-11 and *eck* mRNAs are expressed in the brain but are also expressed in other tissues. Despite strong similarities at the amino acid level, our results demonstrate that these genes display distinct temporal and spatial patterns of expression. The extracellular domains of *eph* and *eck* share a set of 20 similarly spaced cysteines (Lindberg and Hunter, 1990), different from those seen in either the EGF receptor or insulin receptor subfamilies, and we are determining whether this set is conserved throughout the subfamily. The ligands for *elk*, *eph*, and *eck* have yet to be identified.

The kinase domains of tyro-3, tyro-7, and tyro-12 display the segment VII motif $Y(N)_3YY$ that is characteristic of the insulin receptor subfamily (Hanks et al., 1988), but have been grouped separately in Figure 2 because they are otherwise much more closely related to each other than to any previously reported kinase. While all of the known members of the insulin receptor subfamily are receptor configured, they display divergent extracellular structures; elucidation of the full sequence of tyro-3, tyro-7, and tyro-12 may therefore define novel extracellular domains. Of the novel PTK domains identified in our survey, the tyro-13 domain is 4 amino acids shorter than any other. Curiously, it lacks a tyrosine residue, positioned near the end of domain segment VII, which in other kinases has been shown to be a site of receptor autophosphorylation (Hunter and Cooper, 1985) implicated in the regulation of kinase activity. Although we have grouped it separately, amino acid sequence comparisons indicate that the tyro-13 domain shows similarity to those of the cytoplasmic kinases *abl* and *fes/fps* and suggest that this domain may not be part of a transmembrane receptor.

Neural Kinases and Neural Phenotype

Of the six novel PTKs that are predominantly expressed in neural cells, all are present in the embryonic brain and most are maximally expressed at a time when the proliferation and differentiation of neural precursors are known to occur. Together with their restricted distribution in the adult brain, this early expression is at least consistent with a determinative role in neural development. As noted above, the best evidence that receptor PTKs can play a role in the determination of specific neural phenotypes is provided by loss-of-function mutations in the *sevenless* gene of Drosophila. Although expression of this gene is localized to the eye, it is not restricted to the number 7 photoreceptor: the *sevenless* protein can be detected transiently in 11 cells in the developing ommatidia, including the number 1, 3, 4, 6, and 7 photoreceptors, two cells of unknown fate, and four cells that ultimately produce the lens (Tomlinson et al., 1987; Bowtell et al., 1989). This expression pattern notwithstanding, the loss of *sevenless* kinase activity results in the aberrant differentiation of only the precursor to the number 7 photoreceptor (Hafen et al., 1987; Basler and Hafen, 1988a). It has been suggested that this cell has unique access to a ligand that allows it to adopt its normal fate (Tomlinson et al., 1987; Reinke and Zipursky, 1988; Banerjee and Zipursky, 1990).

Direct extension of these findings to vertebrate nervous systems is dependent, first, on the demonstration that expression of the genes encoding receptor PTKs and/or their ligands is regionalized, and, second, on the association of a particular receptor PTK with a

specific neural phenotype. The in situ hybridization profiles of Figure 5 demonstrate that, for tyro-1 through tyro-6, the first of these criteria is met. Similarly, dramatic restrictions have been observed for the receptor PTKs *trk* and *trkB* (Klein et al., 1989, 1990a, 1990b; Martin-Zanca et al., 1990).

With regard to the second criterion, the restricted expression pattern of the tyro-2 gene in the adult brain raises an interesting possibility. Tyro-2 represents a novel member of the erbB/EGF receptor subfamily of kinases, exhibiting a 9 amino acid difference with the kinase domain of the *neu* oncogene (erbB2), a 14 amino acid difference with that of the EGF receptor, and a 23 amino acid difference with that of erbB3 (Kraus et al., 1989). As noted above, tyro-2 mRNA is expressed strongly in the reticular nucleus of the thalamus, where it has been established that virtually all neurons are GABA-ergic (Houser et al., 1980). Conversely, no tyro-2 hybridization can be detected in the remainder of the thalamus, which is not thought to contain significant numbers of GABA-ergic cells. In addition, the tyro-2 hybridization pattern observed in the hippocampus and neocortex bears a striking resemblance to the staining pattern observed with antibodies to glutamic acid decarboxylase (GAD) (Mugnaini and Oertel, 1985), the terminal enzyme in GABA biosynthesis, and tyro-2 and GAD are each present at unusually high levels in the intercalated cells of the amygdala.

While these multiple concordances are suggestive, we have also observed clear tyro-2 expression in the medial habenula, a structure that is thought to contain few GABA-ergic cells (Mugnaini and Oertel, 1985), and a paucity of expression in Purkinje cells, where copious GAD expression has been observed. Thus if tyro-2 expression is associated with specific GABA-ergic neurons, this expression (like that of *sevenless*) cannot by itself be determinative. We are currently using in situ hybridization analyses to determine if the expression of tyro-2 and GAD mRNA is coincident at the level of single cells.

Although we have focused on the developmental role that receptor PTKs might play in cell determination, it should be noted that several previous studies have implicated PTKs and tyrosine phosphorylation in the regulation of later events. For example, a role in the regulation of neurotransmitter release has been suggested by the observation of high levels of tyrosine phosphorylation associated with synaptic vesicles (Pang et al., 1988). PTKs have also been tied to the development and maturation of synapses. Expression of *c-src* is particularly abundant in nerve growth cone membranes (Maness et al., 1988), and loss-of-function mutations in the Drosophila *abl* gene, when coupled with similar mutations in the fasiclin I gene, result in pronounced deficiencies in axon guidance and outgrowth (Elkins et al., 1990). In addition, PTKs have been implicated in the functional modulation of neurotransmitter receptors. Desensitization of the nicotinic acetylcholine receptor is brought about by extensive tyrosine phosphorylation of its β, γ, and δ subunits (Hopfield et al., 1988), and tyrosine phosphorylation of the receptor has also been associated with synapse formation at the neuromuscular junction (Qu et al., 1990). Together with the expression profiles presented above, these results suggest that PTKs may play regulatory roles in both the early development and subsequent synaptic organization of the nervous system.

Experimental Procedures

PCR

The DNA substrates used for amplification were sciatic nerve cDNA populations prepared for use in the construction of subtracted cDNA libraries. Three different subtracted cDNAs were produced. The first two, UN and TWI, were enriched for transcripts expressed predominantly in Schwann cells. The third, BD, was enriched for transcripts shared between Schwann cells and myelinating stage (P17–23) brain. Two initial hybridizations were performed. Both samples contained 500 ng of single-stranded sciatic nerve cDNA mixed with the following poly(A)-selected RNAs: 10 μg of muscle, 7.5 μg of liver, and 5 μg of kidney. Both samples also contained a series of RNAs synthesized in vitro; these encoded portions of the sense strand of the following Schwann cell transcripts: NGF receptor, glial fibrillary acidic protein, proteolipid protein, protein zero, myelin basic protein, and CNPase. The first sample contained, in addition, 10 μg of poly(A)-selected RNA from rat brain cerebellum (P19) and cortex (P3). Each hybridization was allowed to proceed to approximately R_0t 2000. Following hybridization, these samples were bound to hydroxylapatite (0.12 M phosphate buffer, 65°C). For the first sample, material not binding to hydroxylapatite was collected and converted to a double-stranded form. This material was designated UN (unbound). For the second sample, cDNA not binding to the column was further hybridized with 40 μg of poly(A)-selected RNA from rat cerebellum (equal mix of P17 and P23) until R_0t 800. This mixture was reapplied to hydroxylapatite. The unbound material was collected and converted to a double-stranded form and designated TWI (twice unbound). The material that bound to the HAP column was then eluted and also converted to a double-stranded DNA form. This fraction was called BD (bound).

Approximately 2–4 ng of the UN and TWI subtracted cDNAs and 1 ng of the BD cDNA were used in each of the amplifications, which were conducted using reagents and instructions provided by US Biochemical Corp. The final concentration of magnesium ion was increased to 2.1 mM. Thirty-nine cycles of amplification were performed on a water-cooled vtwb Model 1 cycler (San Diego, CA). Amplification parameters included an initial 1 min denaturation step at 94°C, a 5 min annealing at 37°C, a 5 min extension at 65°C, and a 0.3 min denaturation at 94°C. Approximately 4 μg of each of the degenerate primers (Figure 1) was included in each amplification. We believe that the unusually low annealing temperatures employed in these amplifications favor polymerase extension from stably hybridized oligonucleotide primers, resulting in a broader and less-biased amplified population than those obtained with previous protocols (Wilks, 1989).

Subcloning of Amplified DNAs and DNA Sequencing

Amplified DNAs were size fractionated on 5% nondenaturing acrylamide gels. The gels were stained with ethidium bromide (1 μg/ml), and amplified bands of ~220 bp were excised. These bands were eluted overnight into 0.5 M ammonium acetate, 1 mM EDTA, and 0.2% SDS, and eluted DNA was then precipitated with 10 μg of tRNA carrier. Recovered PCR products were blunt ended using T4 DNA polymerase and phosphorylated using T4 polynucleotide kinase. Approximately 40 ng of insert was then ligated with 200 ng of dephosphorylated SmaI- and EcoRV-

digested pBluescript plasmid. One-tenth of each ligation was used to transform MC1061 bacteria.

The DNA sequence of both strands of each PCR product subclone was determined from alkaline lysis miniprep DNA, using the dideoxy chain termination method. In those cases in which clones having apparently identical inserts were isolated multiple times, the sequence of complementary strands was derived from independent clones.

Computer-Assisted Amino Acid Sequence Comparisons

A phylogenetic tree (data not shown) was constructed from an analysis of our 13 novel partial PTK sequences along with a set of 55 additional PTKs, according to the methods of Fitch and Margoliash (1967) as implemented by the programs of Feng and Doolittle (1987). The resulting closely related sequence clusters were used to organize the kinase subfamilies presented in Figure 2.

Northern Analyses

Total RNA from various tissues was prepared by the method of Chomczynski and Sacchi (1987). One additional phenol–chloroform extraction was performed prior to nucleic acid precipitation. Poly(A)-selected RNA samples were purified by either column chromatography or in batch using oligo(dT)-cellulose type III (Collaborative Research). RNA samples were denatured in 50% formamide, 2.2 M formaldehyde, and MOPS at 65°C for 10 min, electrophoresed in 1.0% agarose, 2.2 M formaldehyde, and MOPS, transferred to Nytran filters (Schleicher & Schuell), and baked at 80°C for 2 hr as previously described (Monuki et al., 1989). Probes for blot hybridizations were prepared using [α-^{32}P]dCTP and a random hexamer priming kit, according to instructions provided by the manufacturer (Bethesda Research Laboratories). In all cases, final wash stringency for Northern blots was set at 0.2× SSC, 0.2% SDS, 65°C.

In Situ Hybridization

In situ hybridization was performed according to Simmons et al. (1989), with minor modifications. We used 30 μm paraformaldehyde-fixed brain sections from either adult or 33-day-old rats. Antisense probes from PCR product subclones were prepared using 125 μCi of [^{35}S]UTP (1250 Ci/mmol; New England Nuclear) in a 10 μl transcription reaction, with reagents obtained from Stratagene (La Jolla, CA). Hybridizations were performed at 55°C for 22 hr using approximately 75 μl of 5 × 10^6 cpm/ml probe per slide. RNAase A digestions were performed in buffer prewarmed to 37°C. The final wash stringency was 0.1× SSC at 60°C for 35 min. Emulsion-dipped slides were exposed for 2 weeks prior to developing. Slides were counterstained with thionin.

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Note Added in Proof

A recent report by Partanen et al. (1990) (Proc. Natl. Acad. Sci. USA *87*, 8913–8917) describes the partial sequence of a putative PTK (JTK2) that may represent the human homolog of the tyro-9 sequence presented here.