

# Cloning, central nervous system expression and chromosomal mapping of the mouse PAK-1 and PAK-3 genes

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## Abstract

Two cDNAs encoding PAK kinases were isolated from a mouse embryo library by screening with a PCR-generated probe derived from the kinase domain of a rat PAK kinase. These cDNAs, designated PAK-1 and PAK-3, encode mouse PAK kinases of 545 and 544 amino acids, respectively. Both proteins possess an N-terminal Cdc42/Rac interacting binding domain (CRIB) and a C-terminal serine/threonine kinase domain. Comparison of the two mouse PAK kinases revealed that the proteins show 87% amino acid identity. Northern analysis of a multiple mouse tissue blot with a PAK-1 probe detected a 3.0 kb transcript that was almost exclusively expressed in the brain and spinal cord compared to other tissues such as lung, liver and kidney. A similar pattern of central nervous system tissue expression of PAK-3 transcripts of 3.6 and 8 kb was also observed. Analysis of two multilocus genetic crosses localized *Pak1* and *Pak3* to a position on chromosome 7 and X, respectively. The high level of PAK-1 and PAK-3 kinase expression in the mouse brain and spinal cord suggests a potentially important role for these kinases in the control of the cellular architecture and/or signaling in the central nervous system. © 1999 Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Cdc42; Kinase signaling; Rac; Rho GTPases

## 1. Introduction

Rac and Cdc42 GTPases interact with their various effector proteins to control a number of signaling pathways, including cell polarization, actin polymerization, and kinase signaling (Van Aelst and D'Suza-Schorey, 1997). One of the first groups of Cdc42 and Rac effector proteins identified were the PAK kinase family of serine/threonine kinases (Manser et al., 1994). PAK kinases bind to both Cdc42 and Rac, but not the Rho GTPase in a GTP-dependent manner. Binding of Rac or Cdc42 to PAK kinases induces a conformational change, thereby activating their intrinsic kinase activity

(Manser et al., 1994, 1995; Martin et al., 1995; Teo et al., 1995; Diekmann et al., 1995).

Although at least three isoforms of PAK kinases ( $\alpha/A/1$ ,  $\beta/B/3$ ,  $\gamma/2$ ) exist in mammalian cells, their exact biological role in Cdc42 and Rac signaling pathways is not completely understood. PAK kinases appear to function in activating stress response signaling pathways, such as JNK and HOG kinase pathways (Bagrodia et al., 1995; Polverino et al., 1995; Zhang et al., 1995). The potential role of these kinases in actin polymerization, however, remains unresolved. Several groups have found that PAK kinases control actin polymerization (Sells et al., 1997; Manser et al., 1997), while other groups have not been able to substantiate these findings (Lamarche et al., 1996; Westwick et al., 1997). There is some evidence that PAK kinases may function in additional signaling pathways. For example, PAK- $\gamma$ /PAK-2 is activated by cell treatment with proteases such as thrombin (Teo et al., 1995). PAK is activated by caspase

Abbreviations: cDNA, DNA complementary to RNA; CRIB, Cdc42/Rac interactive binding; HIV, human immunodeficiency virus; mRNA, messenger RNA; PAK, p21 activated kinase.

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treatment and may be involved in membrane and morphological changes seen in apoptosis (Rudel and Bokoch, 1997). Sphingolipid and other lipid molecules have also been shown to activate PAK, independent of Rac and Cdc42 (Bokoch et al., 1998). Finally, there is strong evidence that the human immunodeficiency virus (HIV) Nef genes activate PAK and may be important in virulence (Sawai et al., 1996). Together these studies suggest an important role of PAK kinases in a Cdc42/Rac signaling, as well as other signaling pathways.

In order to understand more about the biological function of PAK kinases, we have cloned and sequenced two mouse PAK kinases, studied their mRNA distribution, and mapped their chromosomal location. We found that two mouse PAK isoforms, PAK-1 and PAK-3, are structurally quite similar, are highly expressed in the central nervous system, and map to mouse chromosome 7 and X, respectively.

## 2. Material and methods

### 2.1. Isolation and characterization of mouse PAK clones

A PAK probe was obtained using PCR with human fibroblast cDNA as template and degenerate oligonucleotides based on published amino acid sequences (amino acids 488–540) of rat PAK (Manser et al., 1994). DNA sequencing confirmed that this was a human PAK-2 cDNA. This PAK probe was labeled with  $^{32}\text{P}$  by the random prime method (Boehringer Mannheim, Indianapolis), purified and used as probe to screen a mouse 14 day embryo  $\lambda$ -ZAP cDNA library. The DNA library screening hybridization conditions were 68°C for 18 h in 6×SSC, 5×Denhart's reagent, 0.5% SDS and 100 µg/ml of denatured salmon sperm DNA. After washing at 68°C in 1×SSC and 0.1% SDS, the filters were air-dried and exposed to X-ray film with intensifying screens. Following three rounds of screening, ten positive clones were plaque-purified and then rescued as plasmids. From these ten positive clones, three different sets of PAK clones (PAK-1, PAK-2 and PAK-3) were identified by restriction enzyme analysis and DNA sequencing. The longest PAK-1 and PAK-3 clones contained 2.2 kb and 2.4 kb inserts, respectively. However, PAK-3 was missing the 5'-end of the coding sequence. Approximately 100 bp of additional 5'-end PAK-3 sequence, including the translation start site, was obtained by rescreening the 14 day embryo cDNA library with a PCR probe generated from the initial PAK-3 clone. PAK clones were sequenced by the nucleotide method with Sequenase (United States Biochemicals, Cleveland, OH) using sequence-deduced oligonucleotide primers. In some cases DNA sequencing was performed using a cycle sequencing kit (Applied

Biosystems) and an automated sequencer (Applied Biosystems 373A). All database searches were performed using the GCG version 7.0 software package.

### 2.2. Northern analysis of PAK-1 and PAK-3

Total RNA was prepared from a number of different mouse tissues and brain regions by the method of Chomczynski and Sacchi (1987). Total RNA (10 µg per lane) was separated by electrophoresis on 1.2% agarose formaldehyde gels, and transferred to nitrocellulose by capillary transfer. A paired set of blots was used to evaluate PAK-1 and PAK-3 mRNA expression. The PCR-derived probe used for mouse PAK-1 corresponded to the 550 bp region from the 5' untranslated region and 5'-end of the gene (from nt 1 to 556 from GenBank entry AF082077). The PCR-derived probe for mouse PAK-3 corresponded to a unique 267 bp fragment within the coding sequence of the gene (from nt 356 to 623 of GenBank AF082297). Probes were labeled with  $^{32}\text{P}$  by nick-translation and hybridized under stringent conditions [42°C, 50% (v/v) formamide, 5×SSPE (1×SSPE = 150 mM NaCl, 10 mM phosphate, pH 7.4, 1 mM EDTA), 0.1% (w/v) SDS, 0.1 mg/ml yeast tRNA, 5×Denhardt's, 1% (w/v) dextran sulfate]. Following washing at 60°C with 2×SSPE, 0.1% SDS, the blots were exposed to X-ray film overnight.

### 2.3. Chromosomal mapping of PAK-1 and PAK-3

Two sets of multilocus genetic crosses were analyzed by Southern blotting for inheritance of restriction size variants of the mouse PAK-1 (*Pak1*) and PAK-3 (*Pak3*): (*NFS/N* or *C58/J* × *Mus musculus musculus*) × *M. m. musculus* and (*NFS/N* × *M. spretus*) × *M. spretus* or (*C58/J* × *M. musculus*) × *M. m. musculus*, and (*NFS/N* × *M. spretus*) × *C58/J J* (Kozak and Buckler, 1997). DNAs from the progeny of these crosses have been typed for over 1200 markers which map to all 19 autosomes and the X chromosome. The same 550 bp and 267 bp PCR fragments used in Northern analysis were also used to chromosomally map PAK-1 and PAK-3, respectively. Mapping data were stored and analyzed using the program LOCUS developed by C.E. Buckler (NIAID, Bethesda, MD). Percentage recombination and standard errors between specific loci were calculated from the number of recombinants (Green, 1981). Loci were ordered by minimizing the number of recombinants.

## 3. Results

### 3.1. Cloning of mouse PAK-1 and PAK-3

A portion of a human PAK cDNA was amplified and used as probe to screen a 14 day whole embryo

mouse cDNA library. The primary screen revealed over 35 positive clones from the screening of  $3 \times 10^8$  plaques. Following three rounds of screening, three different sets of PAK clones were identified by restriction enzyme analysis and DNA sequencing. Two of the genes, designated PAK-1 and PAK-3, were sequenced over their entire coding region (GenBank Accession Nos. AF082077 and AF082297). PAK-1 represents a novel PAK isoform in the mouse (Fig. 1), while /PAK-3 was cloned previously by Bagrodia et al. (1995). Northern analysis suggests that these clones do not represent full-length cDNA sequences and are missing additional 5'- and 3'- untranslated sequences. The PAK-1 cDNA encodes a 545 amino-acid residue protein (Fig. 1). There is only one in-frame methionine that matches the optimal consensus as defined by Kozak (1989). PAK-1 has a predicted molecular mass of 60.7 kDa and a  $pI=5.53$ . PAK-3 cDNA encodes for a 544 amino-acid residue protein (Fig. 2). PAK-3 has a predicted molecular mass of 60.8 kDa and a  $pI=5.32$ . Mouse PAK-1 is nearly identical (98%) to both rat and human PAK-A/PAK-1 proteins and thus represents the mouse homologue.

Finally sequence analysis of a third distinct partial mouse cDNA (designated PAK-2) containing part of the catalytic domain showed identity to the rat and human PAK- $\gamma$ /PAK-2 (Martin et al., 1995). These results confirm that there are three PAK kinase isoforms in the mouse.

### 3.2. Sequence homology of mouse PAK-1 and PAK-3

Overall, PAK-1 and PAK-3 encode proteins that are identical over 72% of their length (Fig. 2). In addition to the high conservation of both the kinase and CRIB domain, both forms also contain proline-rich motifs which may represent potential SH3 binding sites. Both PAK-1 and PAK-3 show a high degree of homology to PAK kinases from other species including those from human, rat, *Drosophila* and *Caenorhabditis elegans* (data not shown). Although PAK-1 and PAK-3 appear to be quite similar, there are regions that are different. For example, a region consisting of 15 acidic amino acid residues (amino-acid residues 172–186) exists within the PAK-3 isoform, while a smaller acidic stretch occurs in

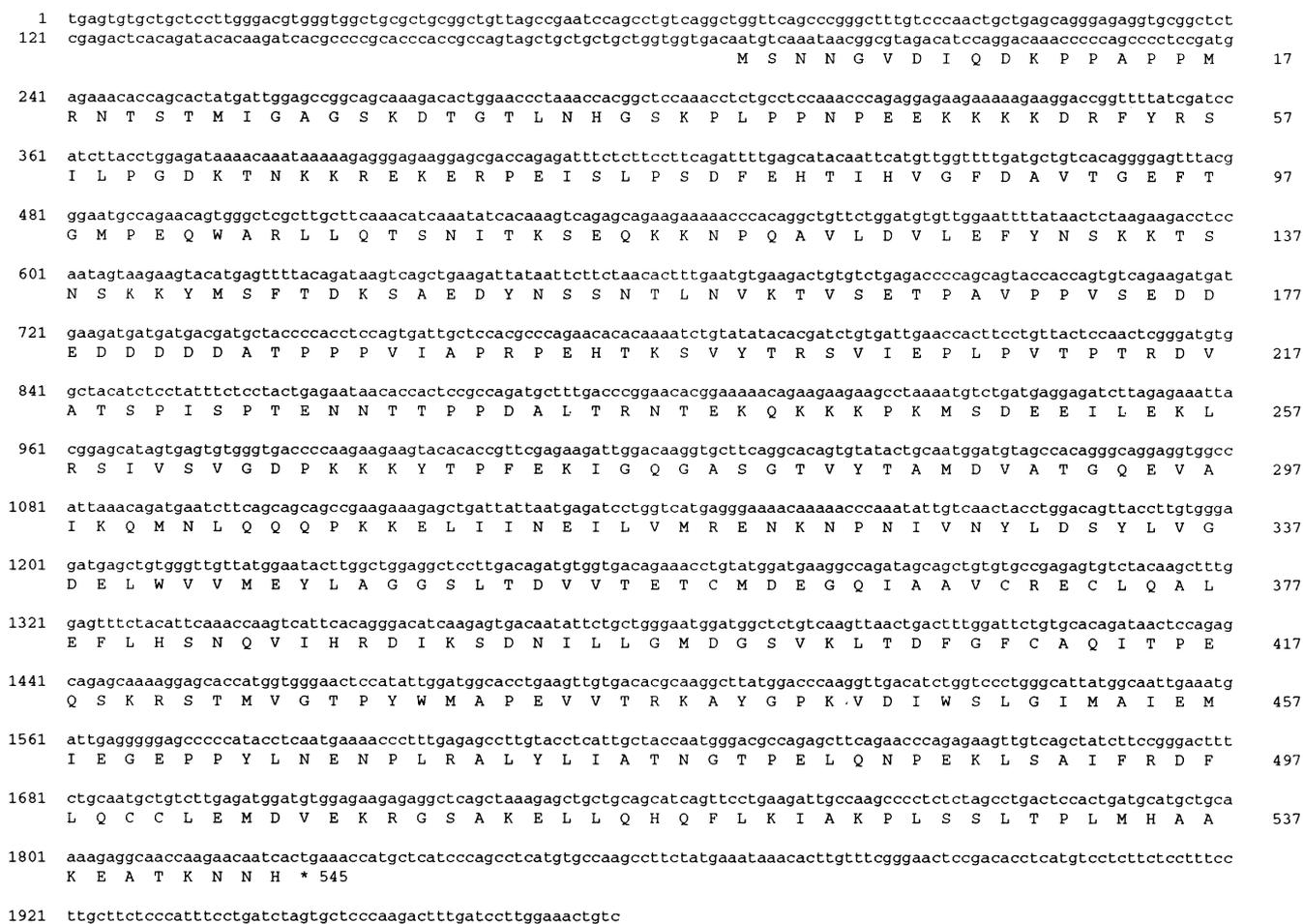


Fig. 1. Nucleotide and predicted amino-acid sequence of mouse PAK-1. Nucleotides are numbered on the left; amino-acid residues are numbered on the right. This sequence has GenBank Accession No. AF082077.

mPAK-1	1	MSNNGVDIQDKPPAPPMPRNTSTMIGAGSKDTGTLNHGSKPLPPNPPEKKKKDRFYRSILP	60
mPAK-3	1	MS-DSLNEEKPPAPPLRMNSN-----NRDSSALNHSSKPLPMAPEE-KNKKARLRSIFP	53
mPAK-1	61	G--DKTNKKREKERPELISLPSDFEHTIHVGFDAVTGGEFTGMPEQWARLLQTSNITKSEQK	118
mPAK-3	54	GGGDKTNKKKREKERPELISLPSDFEHTIHVGFDAVTGGEFTGIPEQWARLLQTSNITKLEQK	113
mPAK-1	119	KNPQAVLDVLEFYNSKKTNSKKYMSFT--DKSAEDYNSNTLNVKTVSETPAVPPVSED	176
mPAK-3	114	KNPQAVLDVLFYDSKETVNNQKYSFTSGDKSAHGVIAAHQSNKTASEPPLAPPVSEE	173
mPAK-1	177	EDDDDDDAT----PPPVIAPRPEHTKSVYTRSVEIPL--PVTPTRDVATSPISPTEENNT	230
mPAK-3	174	EDEEEEEEDDNEPPPVIAPRPEHTKSIYTRSVVESIASPAAPNKE-DIPPSAENANSTT	232
mPAK-1	231	PPDALTRNTEKQKKPKMSDEEILEKLRSIVSVGDPKKKYTPFEKIGQGASGTVYVTAMDV	290
mPAK-3	233	----LYRNTDRQRKSKMTDEEILEKLRSIVSVGDPKKKYTRLEKIGQGASGTVYVTALDI	288
mPAK-1	291	ATGQEVVAIKQMNLQQPKKELINEILVMRENKPNIVNYLDSYLVGDELWVVMYELAGG	350
mPAK-3	289	ATGQEVVAIKQMNLQQPKKELINEILVMRENKPNIVNYLDSYLVGDELWVVMYELAGG	348
mPAK-1	351	SLTDVVTETCMDEGQIAAVCRECLQALEFLHSNQVIHRDIKSDNILLGMDGSVKLTDFGF	410
mPAK-3	349	SLTDVVTETCMDVGQIAAVCRECLQALDFLHSNQVIHRDIKSDNILLGMDGSVKLTDFGF	408
mPAK-1	411	CAQITPEQSKRSTMVGTPTYWMAPEVVTRKAYGPKVDIWSLGIAMAIEMIEGEPYLNENPL	470
mPAK-3	409	CAQITPEQSKRSTMVGTPTYWMAPEVVTRKAYGPKVDIWSLGIAMAIEMVEGEPYLNENPL	468
mPAK-1	471	RALYLIATNGTPELQNPKEKLSAIFRDFLNRCLEMDVEKRGSAKELLQHQLKIAKPLSSL	530
mPAK-3	469	RALYLIATNGTPELQNPERSAVFHDFLNRCLEMDVDRRGSAAKELLQHPFLKLAKPLSSL	528
mPAK-1	531	TPLIAAAKEATKNN-H	545
mPAK-3	529	TPLIAAAKEAIKNSSR	544

Fig. 2. Homology between mouse PAK-1 and PAK-3. The amino-acid sequence comparison is shown between the two PAK isoforms using the program BESTFIT. Identical amino-acid residues are denoted by a dashed line; + denotes semiconservative amino-acid substitutions. The Cdc42 Rac interactive Binding (CRIB) domain is boxed. Roman numerals indicate conserved kinase subdomains. The nucleotide and amino-acid sequences of PAK-1 and PAK-3 have GenBank Accession Nos. AF082077 and AF082297, respectively.

the PAK-1 (Fig. 2). It is possible that these differences may influence how different PAK isoforms interact with various substrates or localize to different regions within the cell.

3.3. Tissue distribution of mouse PAK-1 and PAK-3

Northern hybridization analysis on RNA extracted from various mouse tissues and brain regions was performed to examine the tissue distribution of PAK-1 and PAK-3. Because of the high similarity between the two proteins, cDNA probes were utilized from distinct regions of the two PAK isoforms to avoid cross-hybridization. Northern analysis (Fig. 3) revealed that the PAK-1 probe hybridized to a single transcript of 3.0 kb. PAK-1 was not expressed significantly in peripheral tissues such as lung, liver, kidney or heart, as compared to various brain regions. Among the brain regions tested, PAK-1 was expressed at a very high level in the thalamus, cerebellum, midbrain and pons medulla.

In the brain and spinal cord, the PAK-3 probe hybridized to two transcripts of 3.6 and approx. 8 kb in

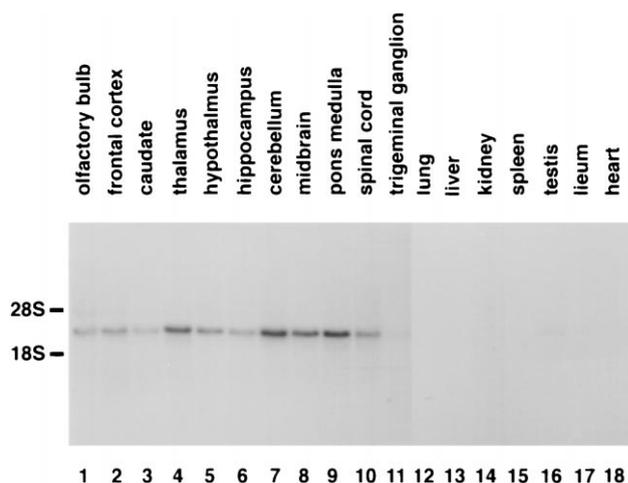


Fig. 3. Northern analysis of mouse PAK-1 mRNA. Total RNA from mouse tissues and brain regions were analyzed for PAK-1 mRNA expression. The position of the 28S and 18S ribosomal RNA are shown. One transcript of 3.0 kb was detected.

size (Fig. 4). Similar results were observed in the rat (Manser et al., 1995), using a probe derived from a different region. The highest level of PAK-3 mRNA

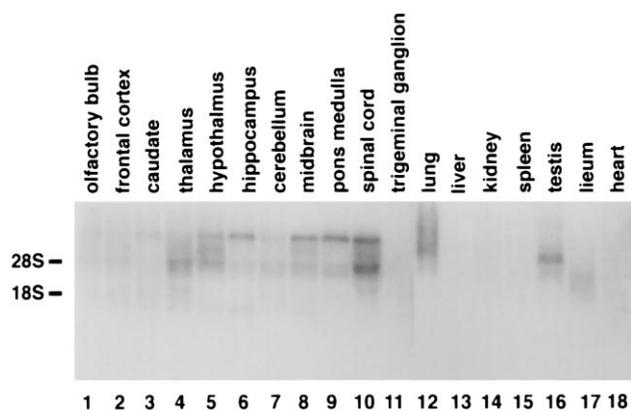


Fig. 4. Northern analysis of PAK-3 mRNA. Total RNA from mouse tissues and brain regions were analyzed for PAK-3 mRNA expression. The position of the 28S and 18S ribosomal RNA are shown. Two transcripts of 3.6 and 8 kb were detected.

expression was seen in the spinal cord. The PAK-3 probe, like PAK-1, showed little hybridization to RNA from peripheral tissues, although some hybridization was seen in the lung and testis. The testis and lung transcripts were of molecular mass different from that seen in central nervous system tissues, and thus may represent cross-hybridization of the probe with other genes, or alternatively spliced forms of the PAK-3 transcript. Interestingly, differential expression of the two PAK-3 transcripts was seen in various tissues. For example, the thalamus express mostly the 3.6 kb form, whereas hippocampus, midbrain, and pons medulla express mainly the 8 kb form.

### 3.4. Chromosomal mapping of the mouse Pak1 and Pak3 genes

*PvuII* digestion identified major PAK-3 fragments of 14.5 and 8.2 kb in NFS/N and 15.5 and 8.2 kb in *M. spretus* and *M. m. musculus*. For PAK-1, *HpaI* produced fragments of 23, 18.7 and 13.5 kb in *M. m. musculus* and 18.7, 13.5, and 6.0 in NFS/N. *PvuII* digestion produced PAK-1 fragments of 8.5 and 6.6 kb in *M. spretus* and 6.9 and 7.2 in NFS/N. The inheritance of these fragments was typed in both genetic crosses and the genes encoding PAK-1 and PAK-3, *Pak1* and *Pak3* were mapped to mouse chromosome 7 and X, respectively (Fig. 5).

### 4. Discussion

Here we describe the cloning, characterization and chromosomal location of two mouse PAK genes. The interest in PAK kinases stems from the fact that these kinases bind and are activated by Cdc42 and Rac (Manser et al., 1994; Martin et al., 1995; Diekmann et al., 1995) and thus function in Cdc42/Rac signaling pathways. Cloning of mouse PAK-1 revealed that this gene is highly conserved at both the nucleotide and amino-acid level. The mouse PAK-3 gene has been cloned previously (Bagrodia et al., 1995). We have used these kinases for recombinant protein production to functionally dissect the biochemical properties of these kinases (Diekmann et al., 1994; Burbelo et al., 1995).

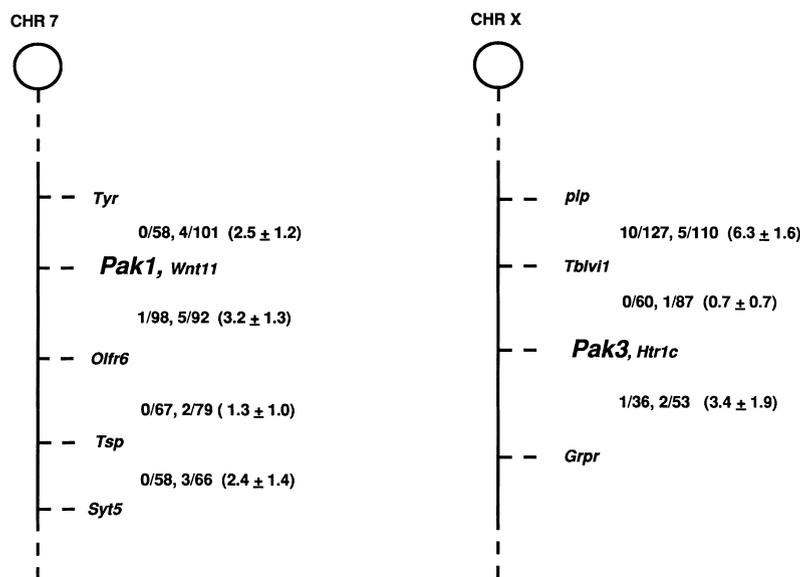


Fig. 5. *Pak1* and *Pak3* map to mouse chromosome 7 and X. Recombination fractions are given to the right for each locus pair, with the first fraction representing results from the *M. m. musculus* crosses and the second from the *M. spretus* crosses. Percent recombination and standard errors are given in parentheses and were determined according to Green, 1981. Human map locations for homologues of the underlined genes are given to the left of the map.

The distribution of the mRNA of the two mouse PAK isoforms was studied in multiple tissue Northern blots. Our Northern analysis data, together with those of Manser et al. (1995) support an important role of the PAK-1 and PAK-3 kinases in neuronal tissue. Consistent with these mRNA results, Manser et al. (1995) reported relatively high levels of rat PAK-1/PAK-A and PAK-3/PAK-B protein in neurons versus other cell types such as glial cells. This pattern of distribution is in sharp contrast to that seen for PAK- $\gamma$ /PAK2 which shows ubiquitous distribution (Teo et al., 1995; Martin et al., 1995). The restricted distribution of these isoforms is important, because it suggests that there may be unique PAK substrates in neuronal cells. Thus, expression of PAK-1 and PAK-3 in non-neuronal cells, where these PAK isoforms are not normally expressed, should be interpreted with caution.

Since PAK kinases may represent candidate genes for a number of genetic diseases, we mapped the chromosomal localization of both *Pak1* and *Pak3*. *Pak1* mapped to mouse chromosome 7 and is in agreement with the mapping of human *Pak1* to a region of conserved synteny on chromosome 11q13 (G28601 and G26649). Mouse *Pak3* mapped to the X chromosome. Mutations in Rho GTPases and their regulators and effectors have been shown to be important in some human diseases of the central nervous system. For example, the Rho GTPase activating protein, oligophrenin-1, is mutated in some forms of nonspecific X-linked mental retardation (Billuart et al., 1998). Thus, the regional assignment of the *Pak1* and *Pak3* genes to these regions suggests that they may be candidate gene for several neurological diseases. While our work was in progress, human *Pak3* was localized to the region of Xq22.2–Xq24 and found to be mutated in nonsyndromic X-linked mental retardation (Allen et al., 1998). These data further support an important role of PAK-1 and PAK-3 in human brain development.

Although a high level of PAK-1 and PAK-3 expression was observed in the central nervous system, its exact function is not known. The high level of PAK kinase expression in the central nervous system is consistent with a large body of data that supports an important role for the Rho GTPases and their effectors in neuronal cell growth, morphogenesis and cell motility. In *C. elegans*, the Rac guanine exchange factor (UNC-73) has been shown to play an important role in neuronal growth cone migration (Steven et al., 1998). Another Rho guanine exchange factor, *Still Life*, may regulate synaptic differentiation of neurons (Sone et al., 1997). Along these lines, some data already exist supporting an important role of PAK kinases in neurons. For example, PAK kinases have been implicated in Ras-dependent Schwann cell transformation (Tang et al., 1998). A potential role of PAK involved in growth cone extension is suggested by a study showing that PAK-1

targeted to the plasma membrane induces neurite outgrowth (Daniels et al., 1998). Finally, future efforts may determine the specific effects of these genes by using these cDNA clones to obtain genomic clones for the purpose of generating knockout mice. It is expected that these knockout mice may be useful as mouse models of central nervous system disorders.

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