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# The Identification and Characterization of Expression of *Pftaire-1*, a Novel Cdk Family Member, Suggest Its Function in the Mouse Testis and Nervous System

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**ABSTRACT** We have isolated a murine cDNA encoding for a novel putative Cdk-related protein kinase, which has been named Pftaire-1, by screening a testis cDNA library for new serine/threonine kinases. Pftaire-1 showed 50% and 49% amino acid identity with Cdk5 and Pctaire-3, respectively, and contains the eleven subdomains characteristic of the protein kinases. By northern blot analysis we detected two transcripts of approximately 5.5 and 4.9 kb in size. These transcripts were expressed at low level in all murine tissues tested, except in the brain, testis and embryo, where high expression was detected. Cellular localization of the mRNAs by in situ hybridization analysis shows that *Pftaire-1* is expressed in late pachytene spermatocytes in the testis and in post mitotic neuronal cells both in the brain and the embryo, suggesting a role of *Pftaire-1* both in the process of meiosis as well as neuron differentiation and/or function. *Mol. Reprod. Dev. 50:18–29, 1998.*



**Key Words:** cell cycle; meiosis; serine/threonine kinases; brain development

## INTRODUCTION

The role of cyclins and their catalytic partners, the cyclin-dependent kinases (Cdks), in regulating the cell cycle in in vivo systems is only beginning to be elucidated. As part of our initial efforts to identify key cell cycle regulators of the germ line cells, we have examined the expression of various cyclins and Cdks in the testis and ovary (Wolgemuth et al., 1995). This analysis has revealed that several of the mitotic cyclins and Cdks are expressed in both mitotic and meiotic cells, in patterns consistent with a predicted role in proliferation and cell division (Chapman and Wolgemuth, 1992, 1993, 1994; Ravnik et al., 1995; Rhee and Wolgemuth, 1995; Ravnik and Wolgemuth, 1996). However, there were some surprising observations as well. For example, we have uncovered developmental stage-specific expression of the B-type cyclins (Chapman and Wolgemuth, 1992, 1993), identified a germ line-specific A-type cyclin (Sweeney et al., 1996), and have correlated the abundant expression of Cdk4 with differentiation, in nondividing Sertoli cells (Rhee and Wolgemuth, 1995).

The existence of cell cycle regulatory genes that function uniquely in meiosis has been documented at both the genetic and molecular levels in several powerful genetic model systems, including the yeasts *S. cerevisiae* and *S. pombe*, in *C. elegans*, and *Drosophila* (Nurse, 1994; Follette, 1997; Page and Orr-Weaver, 1997). We wanted to explore the idea that there are mammalian Cdk family members that are uniquely expressed and functioning in the germ line. To accomplish this, we screened cDNA libraries from mouse testis for novel Cdks. This search yielded a new Cdk family member, designated *Pftaire-1*, which is described in this study.

## MATERIALS AND METHODS

### Tissues, Chemicals, and Reagents

Normal tissues were obtained from Swiss Webster mice (Charles River, Wilmington, DE). The mouse mutant strains atrichosis (at; ATEB/Le a/a d/d + at/eb+) were obtained from The Jackson Laboratory (Bar Harbor, ME). Embryos were collected at the days noted (day 0.5 being the day the vaginal plug is detected) and dissected free of extraembryonic membranes.

The cDNA probes for *Cdc2, Cdk2, Cdk4, Cdk5, Pctaire-1,* and *Pctaire-3* have been described previously (Rhee and Wolgemuth, 1997). All radioactive nucleotides used in this study were obtained from NEN Life Science Products (Wilmington, DE). DNA probes for the library screening were labeled using the multiprime DNA labeling kit (Amersham, Arlington Heights, IL).

Valerie Besset and Kunsoo Rhee contributed equally to this work.

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### Library Screening and Plasmid Constructs

A mixture of *Cdc2, Cdk2, Cdk4, Cdk5, Pctaire-1,* and *Pctaire-3* cDNA probes were used to screen a testis cDNA library at low stringency (Rhee and Wolgemuth, 1997). Clones isolated from the screen were sequenced using an Applied Biosystem Model 373 DNA sequencer. Based on sequencing data, one of these clones 1D10, of 2.5 kb in length, appeared to encode for a new gene. This initial clone was isolated from the phage and used as a probe to screen the library again. Six positive plaques were recovered and confirmed to correspond by secondary and tertiary screening to the original clone. The largest clone (4.5 kb) was isolated from the phage, subcloned into pBluescriptSK$^+$ (Stratagene, La Jolla, CA) and sequenced in both directions. The cDNA corresponding to these clones has been designated *Pftaire-1* cDNA (see Results) in the constructs described below.

An epitope tag (YDVPDYA) was inserted at the carboxy terminal end of the predicted Pftaire-1 polypeptide sequence. An NcoI site was created just before the stop codon of the *Pftaire-1* coding sequence, using the Muta-Gene kit (Bio-Rad, Hercules, CA). This construct was then subcloned into the EcoRI/NcoI site of pBluescriptKS$^+$ carrying the HA epitope just downstream the NcoI site (pBluescriptKS$^+$ carrying the HA epitope was generously provided by Dr. J. Kitajewski).

A Pftaire-HA tagged dominant negative construct was generated by single point mutagenesis. The Asp$^{274}$ (D) residue, in the conserved region putatively involved in the phospho-transfer reaction, was substituted to an Asn (N) residue (van den Heuvel and Harlow, 1993).

Pftaire-HA and Pftaire DN-HA constructs were then subcloned in pLNC retrovirus vector (Miller and Rosman, 1989) (gift from Dr. J. Kitajewski) to use in transfection experiments.

### RNA Isolation and Northern Blot Analysis

Total RNA was isolated from mouse tissues by single-step acid guanidium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). A 0.8 kb cDNA corresponding to untranslated sequence of *Pftaire-1* cDNA (nucleotide 3210 to 4193 in Fig. 1 was used as a template to generate $^{32}$P-labeled antisense riboprobe. Electrophoresis and hybridization were performed as previously described (Rhee and Wolgemuth, 1995). Ethidium bromide staining of the ribosomal RNAs was used to confirm equal loading for each sample.

### In Situ Hybridization Analysis

Testes and day 14.5 embryos were fixed in 4% paraformaldehyde overnight at 4°C, rehydrated and embedded in paraffin before sectioning (Chapman and Wolgemuth, 1992).

For brain analysis, mice were sacrificed by cervical dislocation just before perfusion first with saline, and subsequently with 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde overnight at 4°C. Tissues were cryoprotected in 30% sucrose, embedded in OCT (Tissue-Tek, Elkhart, IN) and cut on a cryostat in 10 µm thick sections.

Sections were processed for in situ hybridization as previously described (Rhee and Wolgemuth, 1995).

*Pftaire-1, Cdk5,* and *Cdc2* cDNA were used as a template to generate $^{35}$S-sense or antisense-labeled riboprobe using T3 or T7 RNA polymerase (Promega, Madison, WI).

Autoradiography was performed with Kodak NT/B2 emulsion. After developing, slides were stained with hematoxylin and eosin, mounted, and viewed on a Leitz photomicroscope using dark-field illumination, unless stated otherwise.

### Cell Culture and Transfection

All cells were cultured in Dulbecco's modified Eagle's medium (Cellgro, Herndon, VA) supplemented with 2.5% fetal bovine serum and 7.5% bovine calf serum at 37°C in a humidified atmosphere of 5% CO2.

pLNC/Pftaire-HA and pLNC/PftaireDN-HA constructs were first transfected in the ecotropic Bosc cell line (Pear et al., 1993) (provided by Dr. D. Baltimore) using the calcium phosphate method as previously described (Pear et al., 1993) and cultured 36 hr to allow the release of the viral particles into the media. RatB1A cells were then cultured for 5 hr with Bosc cells-conditioned media containing the viral particles. After this incubation time, the viral supernatant was replaced with complete media containing Geneticin (500 µg/ml) (Sigma, St. Louis, MO) as the selecting agent (Pear et al., 1993). After 2 weeks of selection, Geneticin-resistant cells were propagated, check for incorporation of the construct by immunoblotting with anti-HA antibody and then frozen for future use.

### Antibodies

A peptide corresponding to the 20-most carboxy terminal residues of the C terminal end of the deduced amino acid sequence of Pftaire-1 was synthesized, and used as an antigen for immunization of rabbits by MBL Ltd (Watertown, MA) (through the generosity of Drs. K. Tamai and T. Hara). The HA monoclonal antibody 12CA5 was purchased from Babco (Richmond, CA).

### Immunoprecipitation and Immunoblot Analysis

Stably transfected cells were washed with phosphate-buffered saline and scraped off in lysis buffer containing 50 mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM EGTA, 1 mM dithioethreitol, 1% nonidet-P40, 0.25% sodium deoxycholate, 1 µg/ml of apoprotinin, leupeptin and pepstatin and 1 mM phenylmethylsulfonyl fluoride. After 30 min incubation on ice, intact cells and debris were pelleted by centrifugation at 14,000*g* for 10 min, and protein concentration was determined in the supernatants with Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Tissues were homogenized in lysis buffer with a Dounce homogenizer and treated as described above.

For immunoprecipitation of Pftaire-1, 250 µg of protein were incubated with either anti-HA antibody (1:50)

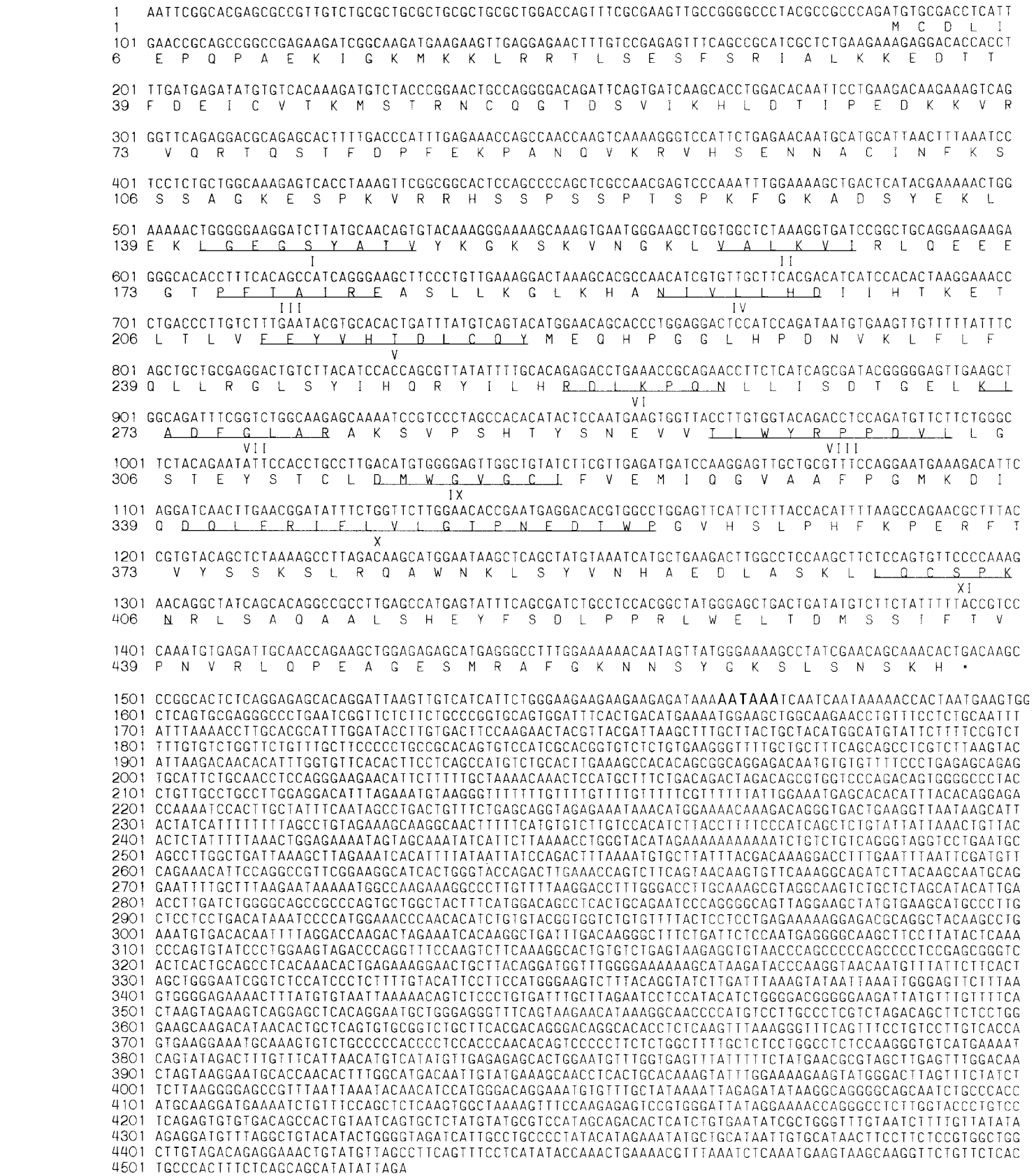

**Fig. 1.** Nucleotide and deduced amino acid sequence of murine *Pftaire-1*. The 11 kinase domains typically found in protein kinase sequences (Hanks et al., 1988) are underlined and numbered in Roman numerals. The putative polyadenylation signal is indicated in bold letters. The sequence have been deposited with GenBank (accession number AF033655).

or anti-Pft antibody (1:100) in the lysis buffer for 2 hr at 4°C, followed by incubation with 3 µg of protein A sepharose for 2 hr. Immune complexes were collected by centrifugation, washed three times with the lysis buffer, and resuspended in Laemmli buffer for electrophoresis (Laemmli, 1970).

For immunoblot analysis, proteins were mixed with Laemmli buffer and boiled for 5 min. Samples were resolved by SDS-PAGE and electroblotted onto a nitrocellulose membrane. The filter was blocked by soaking in Blotto (1× TBS, 0.5% Tween 20, 5% non-fat dried milk) or TBSB (1× TBS, 0.5% Tween 20, 2.5% BSA), depending on the antibody used, for 1 hr, incubated with the primary antibody diluted with Blotto (1:250 for anti-Pft Ab) or TBSB (1:100 for anti-HA Ab) for 3 hr, washed three times, and incubated with secondary antibody (1:5000, horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG; Boehringer-Mannheim, Indianapolis, IN) for 1 hr, and washed three time with TBST (1× TBS, 0.5% Tween 20). For detection, ECL Western blotting reagents (Amersham, Arlington Heights, IL) were used according to the manufacturer's recommendation.

## RESULTS

### Cloning of Murine *Pftaire* cDNA

To isolate new members of the *Cdc2*-related protein kinase family that may be involved in spermatogenesis, murine testis cDNA libraries were screened at reduced stringency using a mixture of known murine Cdk cDNAs as probes (Rhee and Wolgemuth, 1997). Comparison of the sequence of several of the clones revealed that among the genes identified in this analysis were Cdk family genes that had not been used as probes (data not shown), such as *Pctaire-2* (Meyerson et al., 1992) and *Pitslre-1* (Bunnell et al., 1990), suggesting their expression in the testis. In addition, we isolated a cDNA designated 1D10 that appeared to code for a novel Cdk family member. To obtain full-length cDNA, the 1D10 clone was used to rescreen the adult testis cDNA library at high stringency.

The longest of the six clones corresponding to the 1D10 cDNA isolated, was 4.5 kb in size and included a prominent open reading frame (ORF) of 469 amino acids with a predicted relative molecular weight of 53 kDa (Fig. 1). The ORF began at nucleotide 86 and was preceded by the consensus sequence for translation, GCCA/GCC (Kozak, 1987). Clone 1D10 contained a phenylalanine instead of a serine residue in the highly conserved signature motif by which Cdks are classified, PSTAIRE; we have therefore named this new gene *Pftaire-1*. All subdomains characteristic of serine/threonine kinases (Hanks et al., 1988) were identified in the putative *Pftaire-1* polypeptide sequence, with the exception of a glutamate residue within subdomain VIII (Glu 173 of Cdc2) that was substituted with an Aspartate residue (Asp301). It is unlikely that such a substitution would result in the loss of kinase activity in this polypeptide, as a similar substitution has been observed in other active kinases such as Cdk5 and the Pctaires (Meyerson et al., 1992).

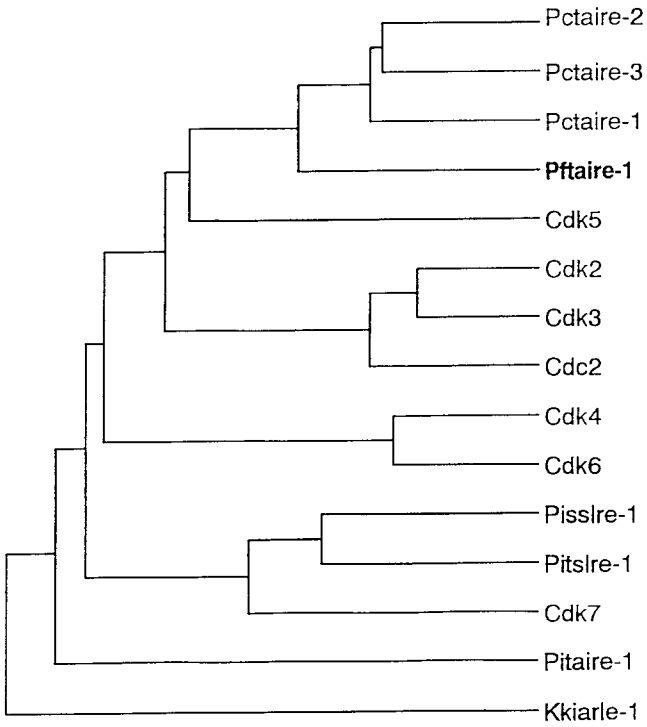


**Fig. 2.** Phylogenetic tree of the Cdc2 family of protein kinases. The alignment of the protein sequences was done using the Megalign program (DNAstar Inc.). All non-classical kinases were named according to their specific variation of amino acids corresponding to the conserved motif PSTAIRE first identified in Cdc2 (Lee and Nurse, 1987)

Specific residues within Cdk family member proteins whose phosphorylation status has been shown to be important for their kinase activity were identified in Pftaire-*1*, but with some modification. The corresponding target residues (Thr14 and Tyr15 in Cdc2) for the Wee-1, Myt-1, and Cdc25 phosphatases were changed to Ser145 and Tyr146 in Pftaire and the target sequence for the Cdk7 kinase (Thr161 in Cdc2) was changed to Ser289 (Fig. 1). Since most Ser/Thr kinases and phosphatases do not discriminate Ser and Thr in their target sites, it is likely that these substitutions would not affect the regulatory mechanisms of Pftaire's activity.

The amino acid sequence of *Pftaire-1* was compared with those of other Cdk family members and their relationship plotted (Fig. 2). The analysis revealed that the highest homology of *Pftaire-1* was with murine Cdk5 (50%), followed by Pctaire-3 (49%). Recently, a *Drosophila* Pftaire kinase has been reported (Sauer et al., 1996). As these authors were not aware of the existence of the mammalian Pftaire-1, they proposed that *Drosophila* Pftaire was the fly counterpart of mammalian Pctaire kinases (Sauer et al., 1996). However, our comparison of the predicted amino acid sequence of *Drosophila* Pftaire with mouse Pftaire-1 revealed 75% homology and only 58% homology with

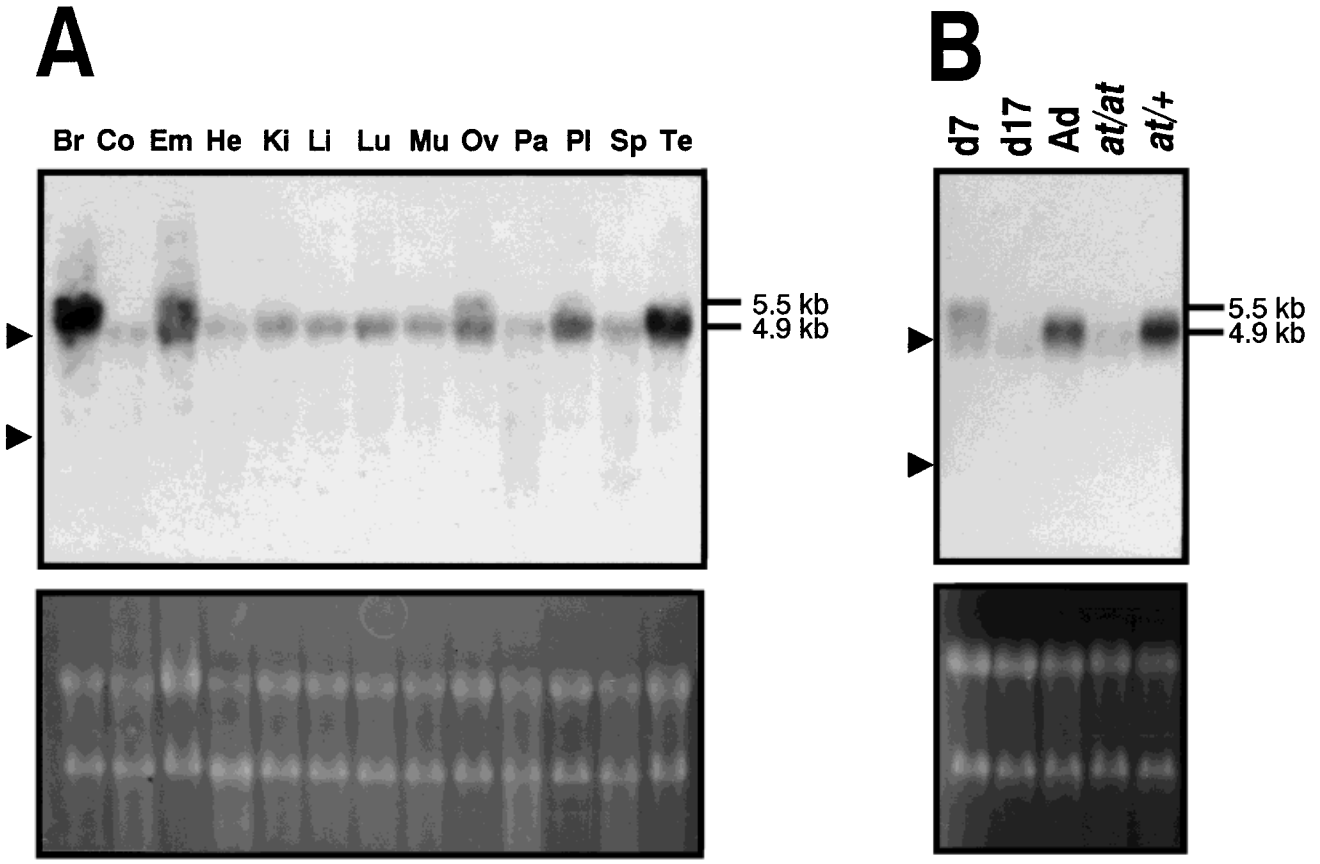


**Fig. 3.** Northern blot hybridization analysis of *Pftaire-1* mRNA expression. (**A**) *Pftaire-1* mRNA distribution in murine tissues. Total RNA (10 µg per lane) was extracted from the following mouse tissues. Br: brain, Co: colon, Em: embryo 12.5 days, He: heart, Ki: kidney, Li: liver, Lu: lung, Mu: muscle, Ov: ovary, Pa: pancreas, Pl: placenta, Sp: spleen, Te: testis. Upper panel: hybridization with an antisense $^{32}$P-labeled *Pftaire-1* riboprobe. Exposure time was 4 days. Lower panel: ethidium bromide staining of the gel. (**B**) Developmentally regulated expression of *Pftaire-1* mRNA in the testis. Total RNA (10 µg per lane) was extracted from wild type mouse testes at different stage of development or from mutant *atrichosis* sterile mice. 7d: 7day-old testis, 17d: 17day-old testis, Ad: adult testis, at/at: testis from homozygous mice, at/+: testis from heterozygous mice. Upper panel: hybridization with an antisense $^{32}$P-labeled *Pftaire-1* riboprobe. Lower panel: ethidium bromide staining of the gel. On the left of the panel, the size of the two *Pftaire-1* transcripts, as determined by the relative mobilities of ribosomal RNAs, 18S and 28S (indicated with arrows), is shown. Exposure time was 4 days.

mouse Pctaire-1. Furthermore, sequences in the conserved domains were diverged similarly in the mouse and *Drosophila Pftaire* genes. Therefore, it is likely that the *Drosophila Pftaire* is the homologue of the murine *Pftaire-1* gene rather than the *Pctaire* genes.

### Northern Blot Hybridization Analysis

To determine the tissue distribution of expression of *Pftaire-1*, Northern blot hybridization analysis was performed on RNA isolated from adult mouse tissues and the mid-gestation embryo. The analysis revealed that *Pftaire-1* yielded two transcripts of ~5.5 and 4.9 kb in size (Fig. 3A). The 4.9 kb transcript was relatively ubiquitously expressed, being detected at low levels in all the tissue examined, except in the brain, embryo, and testis, where it was much more abundant. The 5.5 kb transcript was more restricted in its expression, being detected most clearly in ovary and brain among adult tissues and in the embryo.

As the *Pftaire-1* cDNA was isolated from a mouse testis library and the levels of expression were abundant in this tissue, we were particularly interested to determine its cellular site of expression in this organ. Germ cell-versus somatic cell-expression can be investigated through the use of mutant strains of mice which are virtually devoid of germ cells, but which have the normal complement of somatic cells, including Leydig, Sertoli, and peritubular cells (Mintz and Russell, 1957). RNA was isolated from testes from animals which were either homozygous (and are sterile) or heterozygous (normal) for the *atriochosis* (*at*) allele and examined by Northern blot hybridization analysis (Fig. 3B). Little, if any, expression of *Pftaire-1* was detected in the germ cell-deficient animals, indicating that the germ cells are likely a major site of expression of *Pftaire-1*, at least in the adult animal.

The characteristic progression of spermatogenic differentiation in the postnatal mouse testis allows for the enrichment or elimination of particular germ cell types depending upon the age of the testis (Bellve et al.,

1977). For example, testes from 7-day-old mice (d7) consist largely of Sertoli cells (73%–84%) and spermatogonia (16%–27%), both of which are active mitotically. By day 17 (d17) of postnatal development, a proportion of the germ cells have reached prophase of meiosis, and there are also mitotic spermatogonia. The Sertoli cells at this stage comprise ~29%–39% of the total testicular cells and have stopped dividing (Bellve et al., 1977). Both d7 and d17 testes lack post-meiotic cells. The two *Pftaire-1* transcripts were detected at low levels in d7 testes, barely detected in d17, and then detected at much higher levels in the adult testis. The distribution was not uniform, however, in that the larger transcript was more abundant in the d7 testes whereas the slightly smaller transcript was the predominant one in the adult testis samples (Fig. 3B). The larger transcript is therefore likely to be expressed in Sertoli cells which are still mitotically active (d7 testis) and the smaller transcript in spermatocytes in late meiotic prophase or after meiosis is completed (adult testis).

### In Situ Hybridization Analysis

To determine more precisely the testicular cell types expressing *Pftaire-1*, in situ hybridization analysis with radioactively labeled probes was performed on histological sections of testes from immature and adult animals. In postnatal d7 testis, specific localization was detected in the cytoplasm of Sertoli cells in the lumen of the tubules (Fig. 4A), supporting the Sertoli cell expression of the smaller *Pftaire-1* transcript. No specific signal was detected in the testis of 17-day-old mice (Fig. 4B). In adult testis, *Pftaire-1* expression was observed to be most abundant in subsets of tubules associated with particular stages, namely tubular stages 10–12 (Oakberg, 1956), of the cycling seminiferous epithelium (Fig. 4C). Examination of the sections at higher magnification revealed that specific signals were localized in late pachytene/diplotene spermatocytes, cells about to undergo the first meiotic division.

Although our initial focus was on the expression of *Pftaire-1* in the testis, the fact that it was abundantly expressed in the adult brain prompted our examination of its cellular distribution in this tissue as well (Fig. 5). Specific signal was detected in the cortex, the hippocampus, dentate gyrus, amygdala cortex, and the parasubiculum, all of which are part of the limbic system. In the cerebellum, specific expression was restricted to the Purkinje cell layer. *Pftaire-1* expression was also abundant in mamillary bodies which are extensions of the hippocampus and in the habenulae, which are part of the epithalamus (data not shown).

To determine if the nervous system was also a site of expression of *Pftaire-1* in the embryo, in situ hybridization analysis was performed on sections of midgestation (day 14) embryos. As shown in Figure 6A, *Pftaire-1* expression was mainly, if not exclusively, detected in the nervous system. In the developing brain, expression was found in structures such as the striatum, the roof of the midbrain, and the neopallial cortex, which is the future cerebral cortex. Expression of *Pftaire-1* is not restricted to the central nervous system, however, as specific signal was also readily detected in the gray matter of the spinal cord, the dorsal root ganglia, and in all the major ganglia, of which the trigeminal and vestibulocochear ganglia can be seen in Figure 6A. *Pftaire-1* expression was also observed in the primary cranial nerves such as the optic and maxillary nerves (Fig. 6A).

To determine if this pattern of embryonic expression is unique to *Pftaire-1*, or is typical of Cdks in general, we examined the expression of *Cdc2*, the prototype Cdk, and also the expression of *Cdk5*, which is a closely related Cdk family member (see Fig. 2). A comparison of the resulting hybridization patterns revealed striking differences among the three Cdk family members. *Cdc2* was highly expressed in proliferating tissues, such as cartilage and tooth primordia but not in spinal cord or in ganglia (Fig. 6B). Closer examination revealed that Cdc2 was also expressed in the proliferative layer of the developing central nervous system whereas *Pftaire-1* was restricted to the marginal layer where differentiating neurons are located. The pattern of *Cdk5* expression more closely resembled that of *Pftaire-1* (Fig. 6A and C), suggesting that both may be involved in the development of the nervous system. There are differences, however, which will be presented in detail in a separate study (Besset et al., in preparation).

### Detection of Pftaire-1 Protein

Polyclonal anti-peptide antibodies specific for the carboxy-terminal 20 aa of murine Pftaire-1 were generated in rabbits. The carboxy terminal end of Pftaire-1 has very little homology with the other members of the Cdk family and therefore was a good candidate for raising an antibody specific against Pftaire-1. To assess the ability of these antibodies (anti-Pft) to recognize the Pftaire-1 protein, ratB1A cells were transfected with constructs containing hemaglutinin (HA) epitope-tagged full length *Pftaire-1* cDNA driven by the cytomegalovirus (CMV) promoter (Pft-HA/pLNC) or with vector alone (pLNC). Constructs in which Pftaire-1 function would be putatively altered in a dominant negative manner for future biochemical studies were also prepared (PftDN-HA/pLNC) and used in the transfection studies. Lysates were prepared and immunoprecipitated with either anti-HA antibody (right panel) or anti-Pft antibody (left panel) and then examined by immunoblot analysis with either anti-HA or anti-Pft antibodies (Fig. 7). Anti-Pft antisera do not detect any specific proteins in cellular extracts transfected with vector alone and precipitated with anti-HA (right panel, lane 1). In extracts of cells transfected with Pft-HA/pLNC or PftDN-HA/pLNC, a single band migrating with a relative molecular weight of 54 kDa was observed, a size consistent with its predicted size of 53 kDa and the presence of the epitope tag. This band was detected whether anti-HA or anti-Pft antibodies were used in the immunoprecipitation and with both antibodies in the immunoblot detection, confirming the specificity of the anti-Pft antibodies for Pftaire-1 protein. The

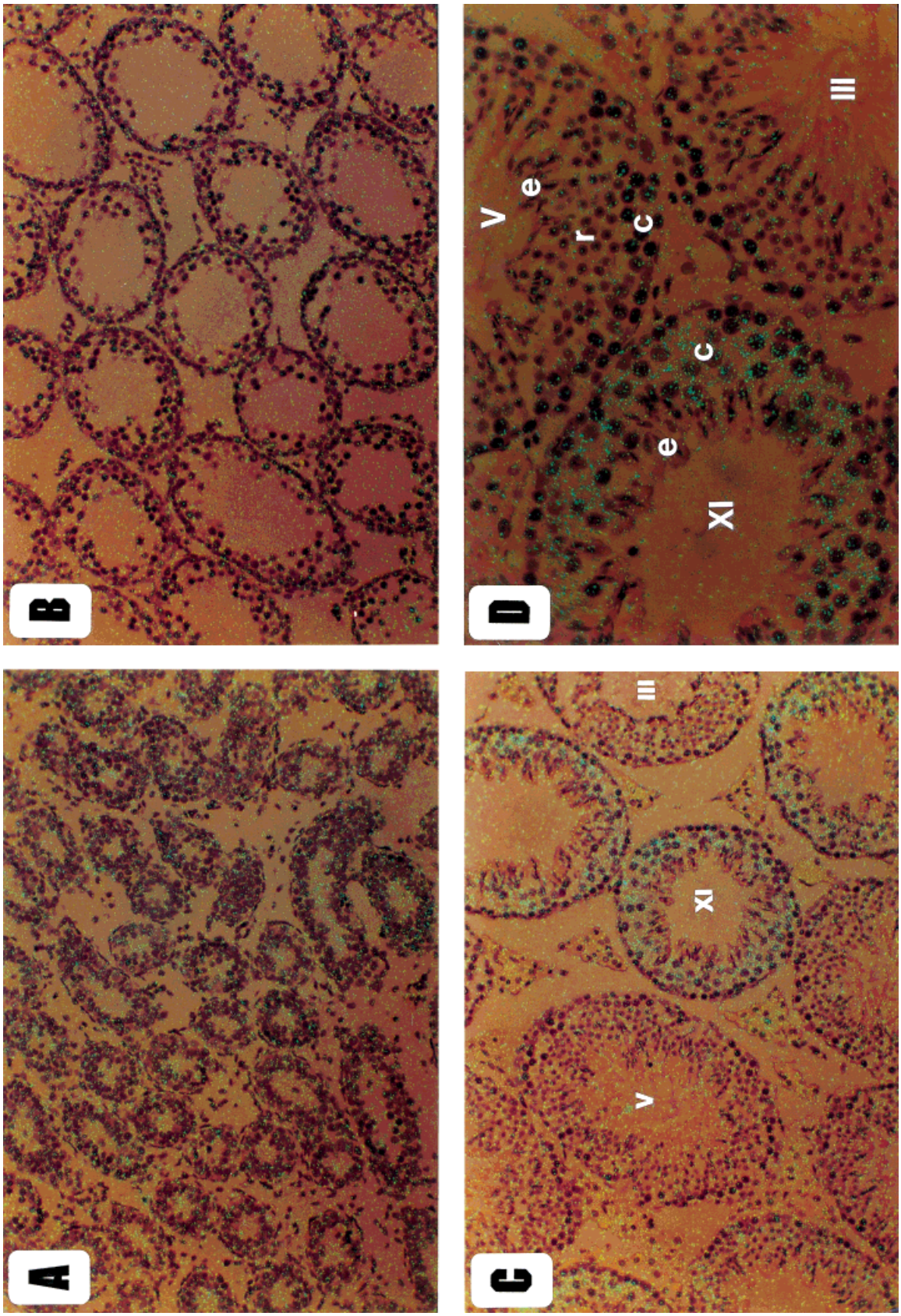


**Fig. 4.** Cellular localization of *Pftaire-1* mRNA in mouse testes by in situ hybridization. Paraffin-sectioned testis samples were prepared from 7-day-old (**A**), 17 day-old (**B**) or adult (**C,D**) mice. Hybridization was with a $^{35}$S-labeled antisense *Pftaire-1* riboprobe and exposure was for 15 days. The photomicrographs in this figure were taken using epiluminescence optics with a low bright-field background, so that signals were visualized with a bright green color. The stages of the cycle of the seminiferous epithelium (Oakberg, 1956) are designated by Roman numerals in C and D. Spermatocytes (c), round spermatids (r), and elongating spermatids (e) are designated by letters.

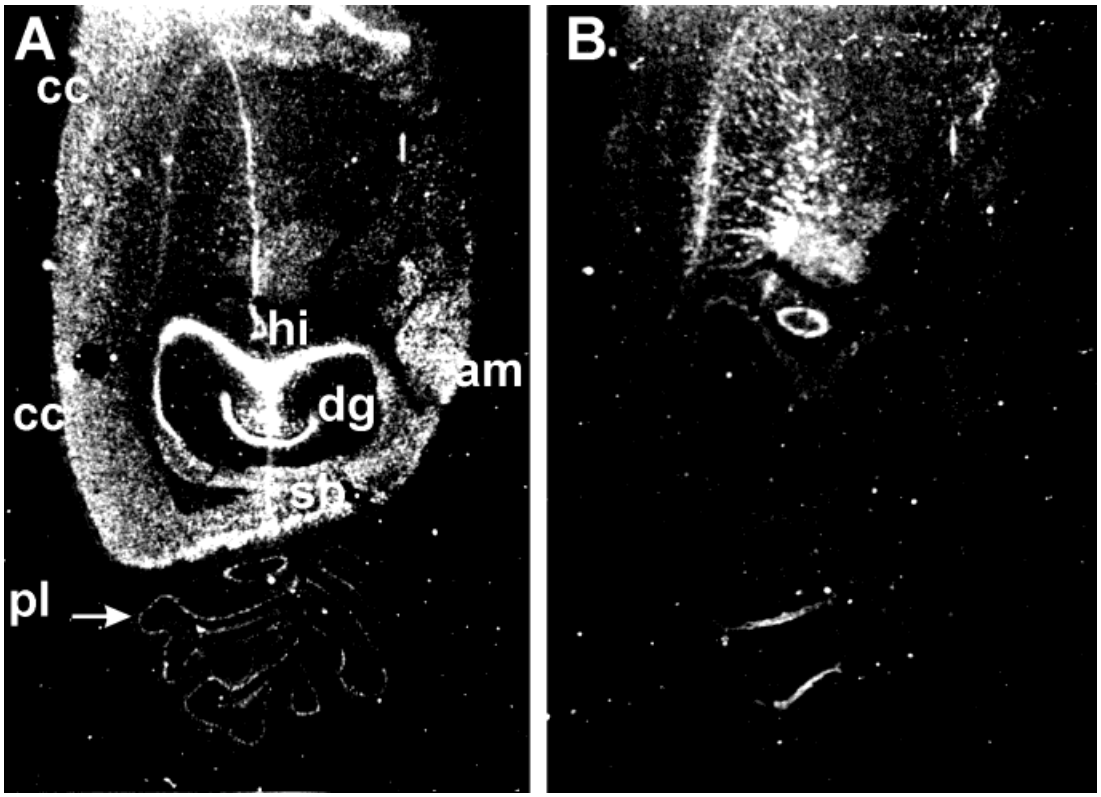


**Fig. 5.** Expression of *Pftaire-1* mRNA in the adult mouse brain. 10 µm transverse frozen sections of adult mouse brain were hybridized with an antisense (**A**) or sense $^{35}$S-labeled *Pftaire-1* riboprobe (**B**). Exposure time was for 10 days. *Pftaire-1* signal was strongly expressed in the following structures: pl, Purkinje layer; sb, parasubiculum; dg, dentate gyrus; hi, hippocampus; am, amygdala cortex; cc, cerebral cortex.

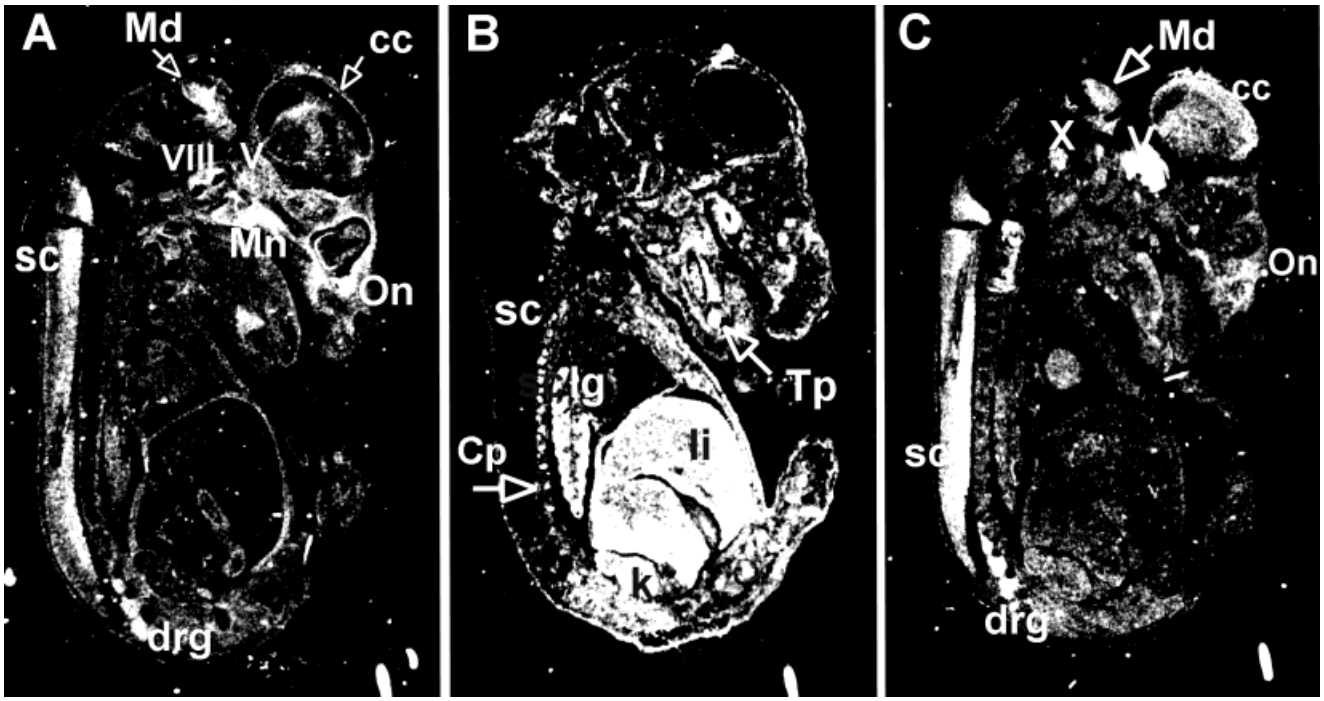


**Fig. 6.** Expression of *Pftaire-1* mRNA in 14.5 day embryo compared with that of *Cdc2* and *Cdk5*. 8 µm sagittal paraffin sections of 14.5 day embryos were hybridized with an antisense $^{35}$S-labeled *Pftaire-1* (**A**), *Cdc2* (**B**), or *Cdk5* (**C**) riboprobe. Exposure times were 7 days for *Cdc*2 and 10 days for *Pftaire-1* and *Cdk*5 slides. The predominant site of expression of *Pftaire-1* and *Cdk5* is in tissues of the nervous system: sc, spinal cord; drg, dorsal root ganglia; on, optic nerve; mn, maxillary nerve; md, midbrain; cc, future cerebral cortex; V, trigeminal ganglion; VIII, vestibulocochlear ganglion; X, vagal ganglion. In contrast, *Cdc2* was expressed abundantly in proliferating tissues: cp, cartilage primordium; lg, lung; k, kidney; li, liver; tp, tooth primordium.

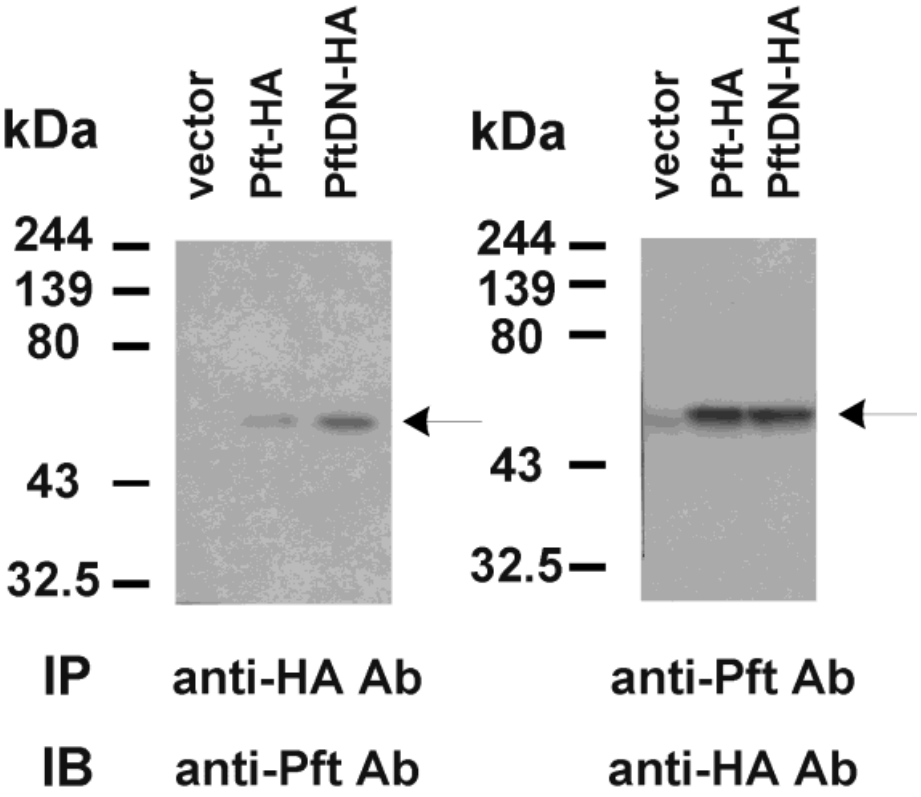


**Fig. 7.** Specificity of anti-Pft polyclonal antibody. RatB1A cells stably expressing either pLNC vector, pLNC-Pft-HA, or pLNC-PftDN-HA were established as described in Materials and Methods and used as the source of cellular lysates. Protein extracts from the three cell lines were immunoprecipitated with anti-HA antibody or anti-Pft antibody, separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Pft antibody or anti-HA antibody respectively. Pftaire-1 protein is indicated by arrows and molecular weight standards are indicated on the left of the panels.

band weakly detected by the anti-HA antibody in the extract of cells immunoprecipitated with anti-Pft antibody (left panel, lane 1) might be due to residual cross reaction between the horseradish peroxidase-conjugated goat anti-mouse immunoglobulin IgG used for detection and the rabbit IgG present in the immuno complex.

The anti-Pft antibody was then used for immunoblot analysis of various murine tissues and cell lines in which *Pftaire-1* had been shown to be expressed at the RNA level. Anti-Pft recognized a protein of ~53 kDa in the tissue lysates (Fig. 8); however, the expression pattern at the protein level differed somewhat from that of the mRNA profile. Detectable levels of Pftaire-1 protein in whole tissue lysates were only observed in brain and kidney (Fig. 8A), although testis and embryo had been shown to also express high levels of *Pftaire-1* mRNA on northern blot analysis (Fig. 3) and in which mRNA was readily localized by in situ hybridization analysis (Figs. 4 and 6). In the brain extracts, an additional band of ~39 kDa was reproducibly detected. This band is likely related to Pftaire-1 since it was competed by incubation of the antibodies with the C-terminal peptide used in generating the antisera (data not shown). The immunoblot depicted in Figure 8B shows that among the several cell lines examined, only the neuronal cell line PC12 exhibited the 53 kDa protein.

## DISCUSSION

In this study we report the isolation and characterization of a cDNA encoding a new member of the Cdk family, that was tentatively named *Pftaire-1*. Northern blot analysis revealed that low levels of *Pftaire-1* mRNA were ubiquitously expressed in murine tissues, but high levels of expression were found in testis, brain and embryo. These tissues are very different in terms of proliferative activity. The testis, for example, is composed of actively dividing cells, the spermatogonia, as well as fully differentiated cells, such as spermatids and spermatozoa. Previous studies have shown that different members of the Cdk family are distributed in very specific patterns among these cell types (Rhee and Wolgemuth, 1995).

In contrast, most of the cells in adult brain are fully differentiated and the level of cellular proliferation, as measured by MPF kinase activity, is barely detectable (Tsai et al., 1993). We therefore were interested to determine which kinds of cells were expressing *Pftaire-1* in these tissues.

In situ hybridization analysis of adult brain showed robust expression of *Pftaire-1* signal, with particularly

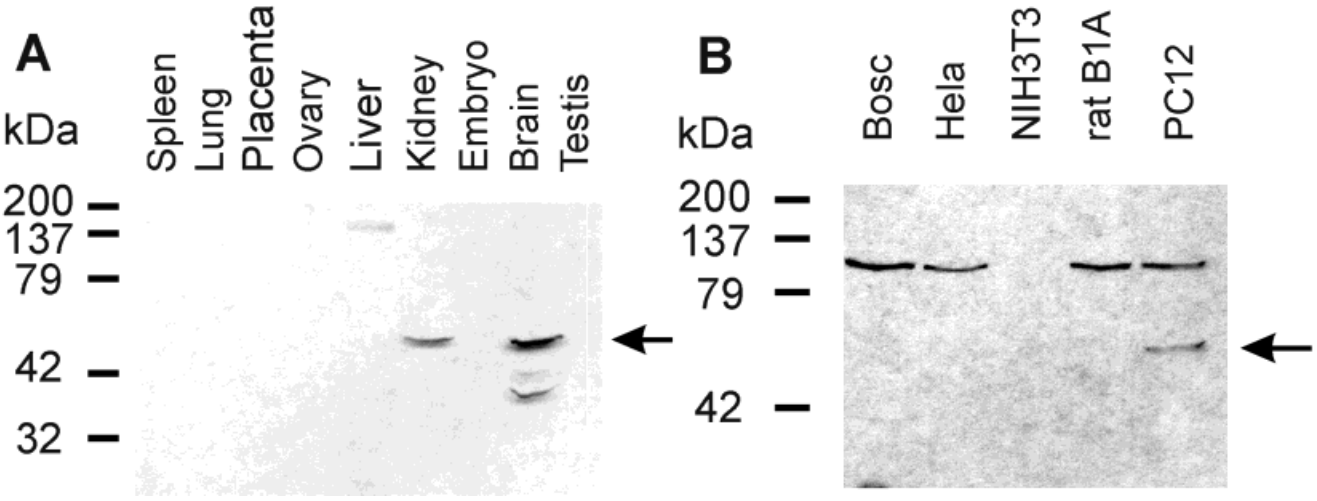


**Fig. 8.** Immunoblot analysis of Pftaire-1 expression in mouse tissues and cell lines. Protein extracts (50 µg) from murine tissues (**A**) or cell lines (**B**) were separated by SDS-PAGE, transferred to nitrocellulose and reacted with anti-Pft antibody. The arrow indicates the position of the Pftaire-1 protein. The sizes of the protein markers are indicated on the left side of the panels.

high expression found in several structures of the limbic system. Interestingly, in the embryo *Pftaire-1* signal was only found in the nervous system, both central and peripheral. In comparison with Cdc2 expression, *Pftaire-1* was expressed in the marginal layer of the developing brain while Cdc2 was expressed in the proliferative layer. During embryonic and early postnatal development of the nervous system, neurons first go through a state of active mitotic division in the proliferative layer, then, as they exit the cell cycle, they migrate to the marginal layer and start differentiating (Purves and Litchman, 1985). The specific expression of *Pftaire-1* in the marginal layer of the embryonic brain and in the pyramidal cells of the hippocampus as well as the Purkinje cells of the cerebellum in the adult brain, suggested that this new Cdk plays a role in post-mitotic neurons. In the adult testis, however, highest expression was found specifically in late stages of meiotic prophase spermatocytes, suggesting that *Pftaire-1* might be responsible for meiosis-specific cell-cycle events.

It would therefore appear that *Pftaire-1* could be involved in different cellular functions depending on the tissue studied. Such dual functions are not without precedent among the Cdc2-related protein kinases. The most well studied is Cdk5, which has been shown *in vitro* to be capable of interacting with cyclin D (Xiong et al., 1992), but whose function is also related to neuronal cell differentiation (Nikolic et al., 1996) as well as apoptosis (Lee et al., 1997; Zhang et al., 1997). *Pctaire-1* is a Cdk family member which is also closely related to *Pftaire-1*, and like *Pftaire-1* and *Cdk5*, is expressed at high levels in brain and testis (Okuda et al., 1992; Tsai et al., 1993; Rhee and Wolgemuth, 1995). By a two hybrid-screening assay, *Pctaire-1* has recently been shown to associate with 14-3-3 proteins (Sladeczek et al., 1997), suggesting that *Pctaire-1* may be a new player in the cascade of proteins kinases involved in signal transduction.

As an approach to begin to test the putative function of *Pftaire-1*, we have generated stable ratB1A cell lines overexpressing normal Pftaire-1 protein or a mutant of the protein, Pftaire-DN, containing a single point mutation in a region critical for the putative kinase activity (van den Heuvel and Harlow, 1993). These cell lines were used in the present study to assess the specificity of the antibody made against the C-terminal end of *Pftaire-1*. A 54 kDa protein was detected in transfected cells, in agreement with the calculated molecular weight of the open reading frame of *Pftaire-1* cDNA. These cell lines were also used to examine the potential kinase activity of *Pftaire-1* toward different substrates. Although several substrates, including histone H1, casein, p56 Rb protein, neurofilaments and myelin basic protein have been examined to date, none of them have been found to be phosphorylated by *Pftaire-1* (data not shown). This observation is analogous to properties of *Cdk5*, whose kinase activity is seen only when it is complexed with p35 (Lew et al., 1994; Tsai et al., 1994), a tissue-specific, non–cyclin like regulatory partner. These observations further suggest that *Pftaire-1* may also need a specialized regulatory partner in order to exhibit any kinase activity. In experiments in which constructs of PftHA and either p35 or p39, regulatory partners for Cdk5 (Tang et al., 1995; Tsai et al., 1994), were cotransfected in the SW1 cell line (Sun et al., 1996), no kinase activity was detected toward neurofilaments (D. Sun and R. Liem, personal communication). In the last two years, new partners of Cdk5 have been cloned (Shetty et al., 1995; Tang et al., 1995). It will be therefore of interest to test the ability of these proteins to associate and possibly activate *Pftaire-1*. Alternatively, *Pftaire-1* may require as yet unidentified part-

ners in order to display kinase activity, or may phosphorylate specific substrates not yet tested.

It is curious that we could not detect any band corresponding to Pftaire-1 protein in the testis by immunoblotting, although the level of *Pftaire-1* RNA in testis was one of the most abundant of all the tissues tested and it was expressed in a specific pattern in late pachytene spermatocytes by in situ analysis. This lack of detection of the protein could be explained if only a small percentage of the cells expressing *Pftaire-1* mRNA in the testis were actively translating it. Post-transcriptional and translational regulatory events have been well documented in the testis (Braun et al., 1995; Schafer et al., 1995). Protamines are one of the best studied example of these regulations. Although the *protamine 1* gene is first transcribed in round spermatids, translation does not occur before the elongating spermatid stage (Braun et al., 1989). The 3' and 5' untranslated regions of several mRNAs in mammalian testis have been shown to play a crucial role in translational control, and proteins that bind to specific sequence of these regions have been described and postulated to play an important role in translational repression (Kwon et al., 1991; Fajardo et al., 1994; Kreysing et al., 1994; Gu and Hecht, 1996). It will be of interest to determine whether *Pftaire-1* mRNA is under such translational control during the process of spermatogenesis.

When wild type and dominant negative constructs of *Pftaire-1* were transfected stably in tissue culture cells, no obvious phenotype, such as a delay in cell cycle progression, was observed (data not shown). These results suggest that the biological functions of *Pftaire-1* are likely to be revealed only in specialized physiological situations. Such tissue-restricted function has been shown for Cdk5, with Cdk5 (–/–) mice exhibiting lesions uniquely in the central nervous system (Ohshima et al., 1996) and dominant-negative Cdk5 expression resulting in disruption of somitic muscle patterning in *Xenopus* embryos (Philpott et al.,1997).

While this manuscript was in preparation, a paper appeared by Lazarro et al. (1997), describing the cloning of a new Cdc2-related protein kinase, which was called Pftaire, from a 18-day-old mouse brain cDNA library. Comparison between the open reading frames reported by Lazarro et al. (1997) and that reported in the present study revealed three single amino acid differences between the two cDNAs and a 46 amino acid extension at the amino terminus of the cDNA we cloned. There are also differences in the size of the transcripts detected: only one transcript of 5 kb was reported by Lazarro et al. (1997) whereas we have detected two transcripts of 4.9 and 5.5 kb for *Pftaire-1*. Although both studies reported high level of *Pftaire-1* expression in the brain, the pattern of expression of *Pftaire-1* transcripts observed in the other tissues was quite different. We found *Pftaire-1* expressed at low levels in nearly every tissue except embryo and testis, where the expression was high, while Lazarro et al. (1997) did not report any *Pftaire-1* mRNA expression in non-neuronal tissues.

Nevertheless, both cDNAs code for proteins that are highly related, if not identical, and both are expressed in patterns suggesting an important role in the development and functioning of the nervous system and possibly the germ line as well.

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