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Neuronal cdc2-like kinase: A cdc2-related protein kinase with predominantly neuronal expression

(phosphorylation/intermediate filament/neurofilament/cell cycle)

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ABSTRACT **Recent studies have shown that there exists a family of protein kinases structurally and functionally related to the yeast cell cycle regulatory kinase cdc2 [Meyerson, M., Faha, B., Su, L.-K., Harlow, E. & Tsai, L.-H. (1991) *Cold Spring Harbor Symp. Quant. Biol.* 56, 177–186 and Meyerson, M., Enders, G. H., Wu, C.-L., Su, L.-K., Gorka, C., Nelson, C., Harlow, E. & Tsai, L.-H. (1992) *EMBO J.* 11, 2909–2917]. Two members of cdc2 family, $p34^{cdc2}$ (also named cdk1) and cdk2, have been identified in mammalian cells. cdk1 kinase regulates the progression from G_2 to M phase, and cdk2 kinase has been proposed to regulate the progression from G_1 to S phase. In this work, we have cloned and structurally characterized a third member of the cdc2 kinase family with 58% amino acid sequence identity to mouse cdk1 and 61% identity to human cdk2. We call this kinase neuronal cdc2-like kinase (nclk) because, in contrast to either cdk1 or cdk2, nclk is expressed at high levels in terminally differentiated neurons no longer in the cell cycle. Previous studies have shown [Hisanaga, S., Kusubata, M., Okumura, E. & Kishimoto, T. (1991) *J. Biol. Chem.* 266, 21798–21803 and Guan, R. J., Hall, F. L. & Cohlberg, J. A. (1992) *J. Neurochem.* 58, 1365–1371] that cdk1 kinase, but not other structurally defined protein kinases, could phosphorylate the repeated Lys-Ser-Pro (KSP) motifs found in mammalian high and middle molecular mass neurofilament subunits *in vitro*, but the precise molecular nature of the endogenous neuronal KSP kinase has remained undefined. The structural similarity of nclk to cdk1 kinase and its high level of expression in terminally differentiated neurons suggest that nclk may play a role in the phosphorylation of the neurofilament KSP repeats *in vivo*, a function distinct from cell cycle regulation.**

Members of the cdc2 family of protein kinases are best known for the pivotal role they play in the regulation of the eukaryotic cell cycle. The activity of $p34^{cdc2}$ (cdk1) kinase in dividing cells increases at the onset of M phase coincident with disassembly of the cell nucleus, generation of mitotic spindles, and chromosome condensation. Several proteins, including the nuclear lamins (1), histone H1, and $pp60^{c\text{-}src}$ (2), have been shown to be substrates for cdk1 phosphorylation *in vitro* and *in vivo*. Analysis of the phosphorylation site for these various substrates has revealed a consensus sequence that consists of Ser/Thr-Pro-Xaa-Zaa, where Xaa is generally a polar amino acid and Zaa is a basic residue (3).

The C-terminal tail domains of high and middle molecular mass neurofilament subunits (NF-H and NF-M, respectively) contain multiple repeats of a sequence conforming to the cdk1 phosphorylation motif Lys-Ser-Pro-Xaa-Lys (KSP-X-K) as well as a related motif, Lys-Ser-Pro-Xaa-Xaa-Xaa (KSP) (4). The region of NF-M and NF-H containing these repeats becomes highly phosphorylated in neurofilaments of terminally differentiated neurons. It is thought that phosphorylation at these sites stabilizes the neurofilament network, thus maintaining axon caliber and influencing axonal transport (4–6). Recently, Roder and Ingram (7) have biochemically defined two protein kinases (PK36 and PK40) from bovine brain that phosphorylate the KSP motif of NF-M, resulting in a shift of its apparent molecular mass on SDS/PAGE. The precise structural identity of these kinases, however, remains undefined.

A clue to the identity of the neurofilament KSP kinase was provided when cdk1 kinase, isolated from starfish oocytes (8) and mouse FM3A mammary carcinoma cells (9), was shown to phosphorylate the tail domain of the dephosphorylated form of NF-H at the KSP-X-K repeats. Phosphorylation at these sites, by cdk1, produces a shift of the apparent molecular mass of NF-H from 160 kDa to 200 kDa. Several other protein kinases (cAMP-dependent protein kinase, protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase II, and casein kinases I and II) phosphorylate both the phosphorylated and dephosphorylated form of NF-H, but cdk1 was the only kinase that is specific for the dephosphorylated form of NF-H, phosphorylating the KSP-X-K motifs contained within the tail domain (8).

Paradoxically, cdk1 kinase mRNA levels are down-regulated with terminal differentiation of rat neurons (10). In addition, experiments using different antibodies to detect cdk1 kinase in the brain have produced conflicting results. Draetta *et al.* (11) detected a protein in rat brain that cross-reacted with affinity-purified polyclonal antibody raised against fission yeast cdc2, whereas Hashimoto and Kishimoto (reported as an unpublished result in ref. 8) were unable to detect cdk1 protein in mouse brain with a monoclonal antibody to the conserved PSTAIRE amino acid sequence. Furthermore, Northern blot analysis performed in our laboratory showed very low cdk1 and cdk2 expression in adult rat brain mRNA. Based on these findings, we hypothesized that the endogenous neurofilament KSP kinase was related to, but structurally distinct from, the cell cycle cdk1 kinase and that the neurofilament KSP kinase would be expressed at high levels in differentiated postmitotic neurons. To test this hypothesis, a low-stringency screening procedure was used to screen an adult rat brain cDNA library by using a mouse cdk1 cDNA as a probe. A cDNA was identified that we have named *n*euronal *c*dc2-*l*ike *k*inase (nclk) based on its sequence similarity to cdk1 kinase and its predominantly neuronal expression.§

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Abbreviations: nclk, neuronal cdc2-like kinase; NF-H and NF-M, high and middle, respectively, molecular mass neurofilament subunits; E, embryonic day; cRNA, complementary RNA.

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§The sequence reported in this paper has been deposited in the GenBank data base [for nclk (1178 base pairs), accession no. L02121].

MATERIALS AND METHODS

Construction and Screening of Rat Brain cDNA Library. Total RNA was purified from rat brain, the polyadenylylated fraction was selected by oligo(dT)-cellulose chromatography, and a hexamer-primed cDNA library was constructed using established methods (12), except that the cDNA was size-fractionated by Sepharose 4B chromatography rather than polyacrylamide gel electrophoresis before ligation to the λgt10 vector. Approximately 1.2 × 10^6 plaques from the cDNA library were screened using a ^{32}P-labeled mouse cdk1 kinase cDNA as a probe (13), obtained by polymerase chain reaction (PCR) from mouse Swiss 3T3 cDNA. The cDNA probe was labeled by nick-translation as described (12). Filters were hybridized in 10% (wt/vol) dextran sulfate/40% (vol/vol) formamide/4× SSC (1× SSC is 150 mM NaCl/15 mM sodium citrate, pH 7.0)/20 mM Tris·HCl, pH 7.4/1× Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/herring sperm DNA (20 μg/ml) overnight at 37°C as described (12). Filters were washed for two 15-min periods at room temperature in 2× SSC/0.1% SDS followed by two additional 15-min washes at 37°C in 0.1× SSC/0.1% SDS. After autoradiography for 1–2 days, positive clones were plaque-purified, subcloned into pGEM-4 plasmid vector (Promega), and sequenced using the f-mol sequencing kit (Promega) as recommended by the supplier.

RNA Blot Analysis. Total or poly(A)$^+$ RNA was prepared from either tissues or cell lines, resolved by electrophoresis on agarose/formaldehyde gels, and blotted to nitrocellulose membranes by using standard methodology (12). After baking at 80°C, membranes were hybridized with a ^{32}P-labeled mouse cdk1, a rat cdk2, or a rat nclk cDNA fragment and washed at high stringency (65°C in 0.1× SSC/0.1% SDS, for two 15-min periods) as described (12).

***In Situ* Hybridization.** After sacrificing adult rats, the brain and embryos (at embryonic day 20, E20) were immediately removed, rinsed in 1× phosphate-buffer saline (PBS) at pH 7.4, and frozen on powdered dry ice. Sections (25 μm thickness) were cut using a cryostat and thaw-mounted on poly(L-lysine)-coated slides. Prior to hybridization, the sections were fixed for 1 h in 4% (wt/vol) paraformaldehyde/0.1 M sodium borate/10% (wt/vol) sucrose, rinsed for two 5-min periods in 1× PBS, treated with 0.5% Triton X-100/0.1 M Tris·HCl, pH 8.0/0.05 M EDTA for 30 min, rinsed with 0.1 M Tris·HCl, pH 8.0/0.05 M EDTA for 3 min, and finally rinsed with 2× SSC for 3 min. The sections were dehydrated by incubation in graded ethanol solutions (50%, 70%, 95%, and twice in 100%, 3 min per incubation) and dried under vacuum. ^{35}S-labeled antisense complementary RNA (cRNA) probe was synthesized from a pGEM-4 plasmid containing a 0.85-kilobase (kb) rat nclk cDNA fragment (Fig. 1, nucleotides 1–852) subcloned into the polylinker region between the SP6 and T7 RNA polymerase promoters. The plasmid was linearized with *Bam*HI and antisense cRNA was synthesized from the SP6 promoter. Hybridization was performed in 50% formamide/0.3 M NaCl/10 mM Tris·HCl, pH 8.0/1 mM EDTA/1× Denhardt's solution/10% dextran sulfate/yeast tRNA (0.5 mg/ml)/10 mM dithiothreitol overnight at 55°C with a probe concentration of 5 × 10^6 cpm/ml of hybridiza-

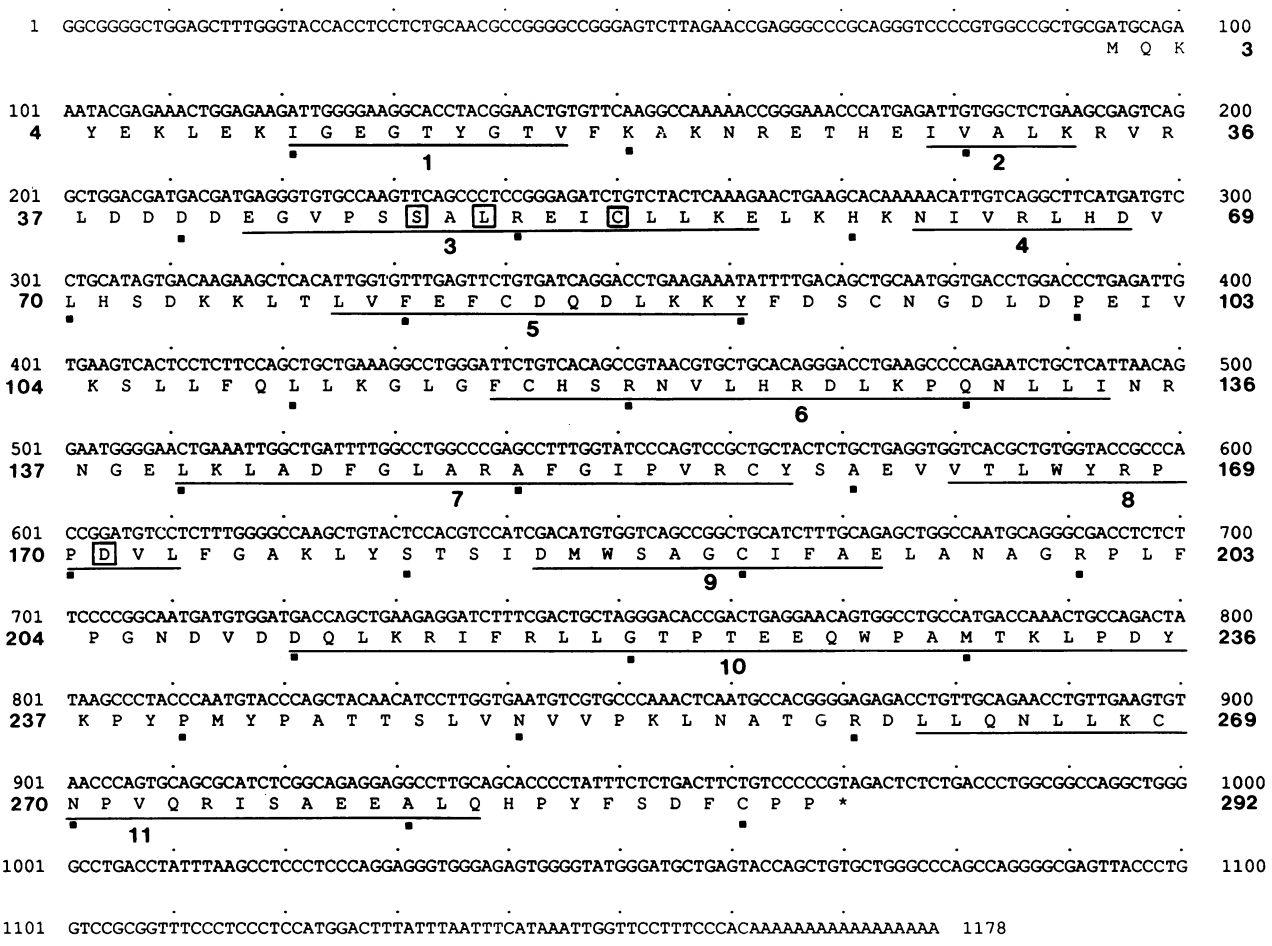

FIG. 1. Nucleotide and derived amino acid sequence of nclk. The 11 kinase domains typically found in protein kinase sequences (14) are underlined and numbered. Conservative amino acid substitutions within domains 3 (PSTAIRE sequence) and 8 are boxed. Solid squares indicate every 10th amino acid residue.

tion buffer. The slides were washed for four 5-min periods in 4× SSC, incubated with RNase A (20 μg/ml in 500 mM NaCl/10 mM Tris·HCl, pH 8.0/1 mM EDTA) at 37°C for 30 min, and washed at room temperature with solutions containing 1 mM dithiothreitol and progressively lower concentrations of SSC, beginning with 2× SSC and ending with 0.5× SSC. A final high-stringency wash was performed in a solution containing 0.1× SSC and 1 mM dithiothreitol at 55°C for 30 min. The slides were dehydrated in 50%, 70%, 95%, and 100% ethanol and exposed to β-max film (Amersham) at room temperature for 1–2 days. To obtain higher resolution, liquid emulsion autoradiography was performed as previously described (15), except NTB3 emulsion (Kodak) was used.

RESULTS AND DISCUSSION

A rat brain cDNA library was screened at low stringency with a mouse cdk1 cDNA as a probe. Four distinct clones were identified and plaque-purified, and partial nucleotide sequence for each was obtained. Two of the clones did not share any significant similarity to mouse cdk1 kinase in a long open reading frame (<35% nucleotide sequence identity). A third clone appears to be the rat homolog of human cdk2 cloned by Tsai *et al.* (16), sharing ≈83% nucleotide sequence identity. Preliminary RNA blot analysis using this rat cdk2 cDNA as a probe indicated that like cdk1, the rat cdk2 mRNA is expressed at very low levels in the adult rat brain (see Fig. 3*A*). The fourth cDNA contained a 1.8-kb insert derived from an mRNA that is expressed at high levels in rat brain and was, therefore, selected for further evaluation.

The 1.8-kb clone contained an internal *Eco*RI restriction site that divides the cDNA into 1.0- and 0.8-kb fragments. The 0.8-kb fragment appears to be a 5′ untranslated sequence with little similarity to cdk1 kinase. The 1-kb fragment contained a long open reading frame that begins at the initiation methionine and encodes 10 of the 11 domains characteristic of protein kinases (14), including the ATP binding site. A 3′-RACE (*r*apid *a*mplification of *c*DNA *e*nds) reaction (17) was performed to isolate overlapping cDNA clones containing the remaining coding region and 3′ untranslated sequence. This procedure used a gene-specific sense primer (GAGGGTGTGCCAAGTTCAGCC, nucleotides 217–237, Fig. 1) and an antisense $(dT)_{17}$ adaptor (17). Nucleotide sequence analysis of three cDNA clones generated by 3′-RACE identified the 11th kinase domain (14) and completed the coding region.

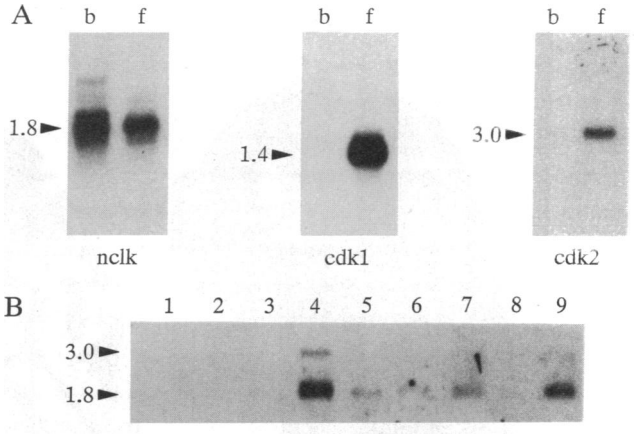

FIG. 3. Northern blot analysis of nclk mRNA expression. (*A*) Comparison of nclk, cdk1, and cdk2 poly(A)$^+$ mRNA (1 μg per lane) expression in rat brain (lanes b) and mouse Swiss 3T3 fibroblast cells (lanes f). Unlike either cdk1 or cdk2, nclk mRNA is expressed at a high level in adult rat brain. The size of each mRNA is indicated to the left (units = kilobases) determined by the relative mobilities of 18S (2.0 kb) and 28S (4.9 kb) rRNAs. (*B*) Northern blot of poly(A)$^+$ mRNA (1 μg per lane) from the following adult rat tissues. Lanes: 1, heart; 2, skeletal muscle; 3, spleen; 4, brain; 5, jejunum/ileum, 6, duodenum; 7, thymus; 8, esophagus; 9, colon. The blot was probed with a ^{32}P-labeled nclk cDNA fragment.

Fig. 1 shows the nucleotide and derived amino acid sequence for the nclk cDNA. The open reading frame encodes a protein of 292 amino acid residues with a calculated molecular mass of 33 kDa. Overall, nclk has ≈58% amino acid sequence identity with mouse cdk1 kinase (Fig. 2) with the highest level of identity corresponding to the 11 domains characteristic of protein kinases (14). For comparison, human cdk1 shares 65% amino acid sequence identity with human cdk2. The putative ATP binding site of nclk (GEGTYG, domain 1, Fig. 1), like cdk1 kinase, contains a tyrosine residue at position 15. Phosphorylation of this tyrosine on cdk1 kinase inhibits its activation before DNA synthesis is completed. Nclk also contains a PSTAIRE-like domain (PSSALRE) with three conservative substitutions that are

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rat nclk   1 MQKYEKLEKIGEGTYGTVFKAKNRETHEIVALKRVRLDDDDEGVPSSALR 50
mouse cdc2 1 MEDYIKIEKIGEGTYGVVYKGRHRVTGQIVAMKKIRLESEEEGVPSTAIR 50

          51 EICLLKELKHKNIVRLHDVLHSDKKLTLVFEFCDQDLKKYFDSCNGD..L 98
          51 EISLLKELRHPNIVSLQDVLMQDSRLYLIFEFLSMDLKKYLDSIPPGQFM 100

          99 DPEIVKSLLFQLLKGLGFCHSRNVLHRDLKPQNLLINRNGELKLADFGLA 148
         101 DSSLVKSYLHQILQGIVFCHSRRVLHRDLKPQNLLIDDKGTIKLADFGLA 150

         149 RAFGIPVRCYSAEVVTLWYRPPDVLFGAKLYSTSIDMWSAGCIFAELANA 198
         151 RAFGIPIRVYTHEVLTLWYRSPEVLLGSARYSTPVDIWSIGTIFAELA.T 199

         199 GRPLFPGNDVDDQLKRIFRLLGTPTEEQWPAMTKLPDYK.PYPMYPATTS 247
         200 KKPLFHGDSEIDQLFRIFRALGTPNNEVWPEVESLQDYKNTFPKWKP.GS 248

         248 LVNVVPKLNATGRDLLQNLLKCNPVQRISAEEALQHPYFSDFCPP.... 292
         249 LASHVKNLDENGLDLLSKMLVYDPTKRISGKMALKHPYFDDLDNQIKKM 297
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FIG. 2. Comparison of the amino acid sequences of rat nclk and mouse cdk1 kinase. Vertical lines indicate identical amino acid residues. The overall amino acid identity between rat nclk and mouse cdk1 kinase is 58%. By comparison nclk has 61% amino acid sequence identity with human cdk2 kinase and 57% identity with human cdk1 kinase.

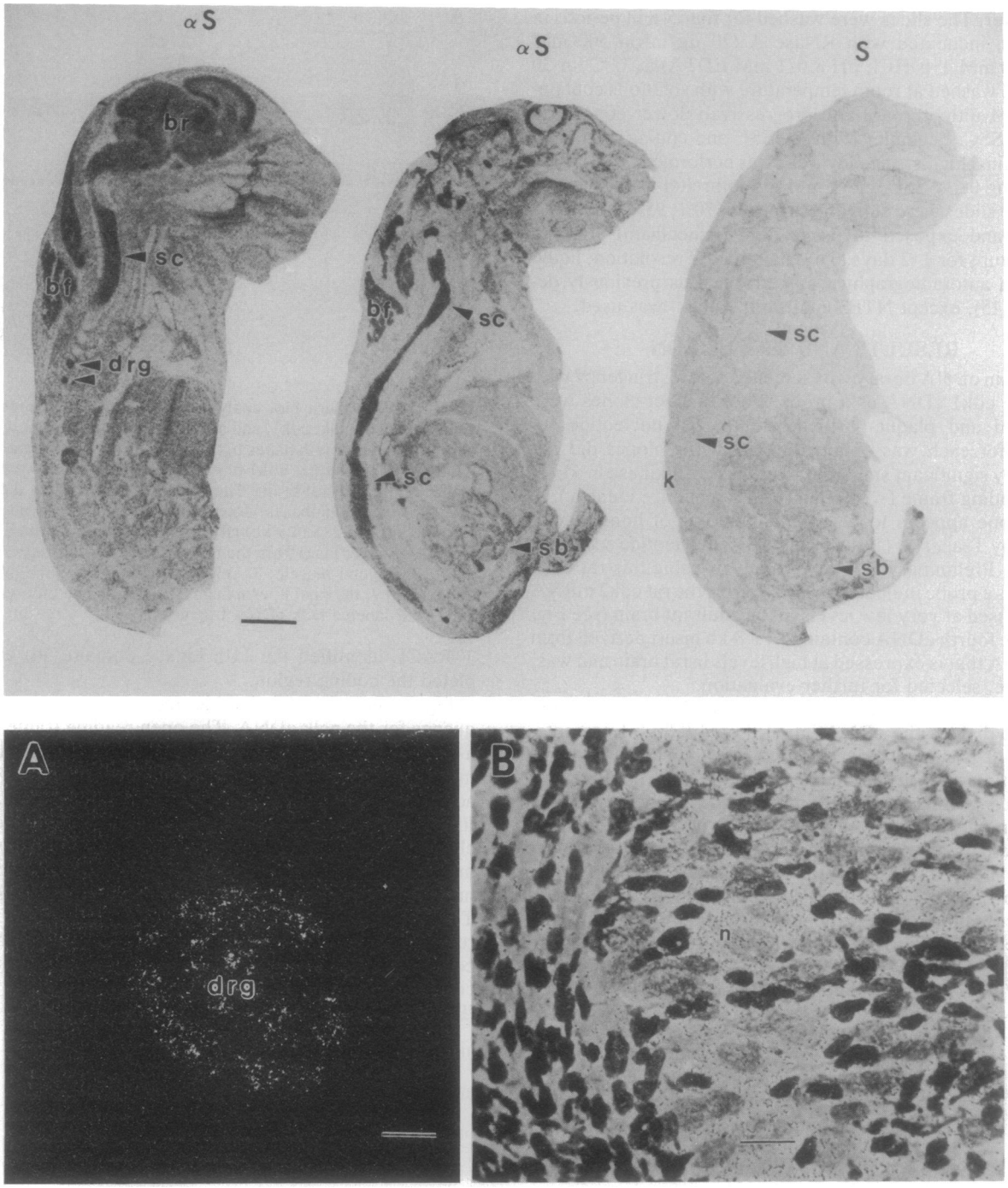

FIG. 4. (*Upper*) Tissue distribution of nclk in E20 rat embryo. Autoradiogram shows the distribution of nclk in 25-μm cryostat sections from an E20 rat embryo. The left and middle sections were hybridized with an antisense ^{35}S-labeled cRNA probe (αS). The predominant expression of nclk is in tissues of the nervous system, such as the brain (br), spinal cord (sc), and dorsal root ganglion (drg), and the highly innervated brown fat (bf). A lower level of hybridization can be seen in the small bowel (sb). The control section on the right is a section adjacent to the middle section and was hybridized with a sense ^{35}S-labeled cRNA probe (S). (Bar = 0.27 cm.) (*Lower*) Localization of nclk mRNA to neurons of the dorsal root ganglion (drg). (*A*) Dark-field image of the lower dorsal root ganglion from the E20 embryo shown above. (Bar = 13.3 μm.) (*B*) Bright-field image of the same dorsal root ganglion. The silver grain exposure, corresponding to nclk mRNA, can be clearly seen over the cytoplasm and nuclei of the large "light" neurons (n). (Bar = 6.7 μm.)

boxed (domain 3, Fig. 1). All cdk1 kinases and human cdk2 kinase contain the conserved EGVPSTAIREISLLKE sequence (PSTAIRE) ≈25 amino acid residues downstream from the ATP binding site (18) and a glutamic acid at position 171 (domain 8) (14). The nclk cDNA has three conservative amino acid substitutions within the region analogous to the cdk1/cdk2 PSTAIRE sequence (amino acid 47 is serine rather than threonine, residue 49 is a leucine rather than isoleucine, and residue 53 is a cysteine rather than a serine) (Fig. 1, domain 3). nclk contains an aspartic acid rather than glutamic acid at position 171.

The significance of these modifications for either the functional properties of nclk, including its potential interaction with cyclin(s), or its reactivity with anti-PSTAIRE antibodies remains to be determined. Ducommun *et al.* (19) have shown by the method of scanning mutagenesis that the N-terminal third of human cdk1 kinase, including the ATP-binding domain and portions of the PSTAIRE region are

required for cyclin A and B binding. Presently, six mammalian cyclins have been characterized. Three (cyclins A, B1, and B2) have been shown to associate with cdk1 and/or cdk2 (20). Three others (cyclins C, D, and E) will rescue *cln*$^-$ yeast mutants but have not been shown to directly activate any of the known cdc2 family members (21). If conservation of the PSTAIRE sequence is essential for association of cdk1 or cdk2 with cyclin A and B, one would predict that nclk interaction with these cyclins may differ from that of cdk1 and cdk2 kinases. In addition, the substitutions within the PSTAIRE domain of nclk may explain the conflicting results observed when brain proteins were studied with anti-cdk1 antibodies.

Perhaps the most compelling evidence suggesting that nclk may be a neurofilament KSP kinase can be seen in mRNA distribution studies. Northern blot analysis of rat brain and mouse Swiss 3T3 fibroblast cell poly(A)$^+$ mRNA show that nclk is expressed at high levels in adult rat brain where as cdk1 and cdk2 are expressed at very low levels (Fig. 3*A*). Also, Northern blot analysis of mRNA from various rat tissues shows that nclk expression is highest in the brain (Fig. 3*B*, lane 4). Heart (lane 1), skeletal muscle (lane 2), and spleen (lane 3), as well as lung, kidney, and testis (data not shown), show no expression of nclk mRNA. Several other tissues do show detectable levels of expression of nclk mRNA, however, at levels lower than observed in brain. These tissues include the jejunum/ileum (lane 5), duodenum (lane 6), thymus (lane 7), and colon (lane 9).

An overview of the pattern of nclk mRNA expression in rat is provided by *in situ* hybridization analysis of sections encompassing the entire animal. (Fig. 4 *Upper*). We selected E20 for this study because terminal differentiation of the cerebral cortex is essentially completed (22) and cdk1 kinase mRNA expression is down-regulated by E20 (10). The autoradiograms shown in Fig. 4 *Upper* are from an E20 rat (25 μm thick). The left and middle sections were hybridized with nclk antisense ^{35}S-labeled cRNA. These sections show strong hybridization signals in the tissues of the nervous system, such as the brain, spinal cord, and dorsal root ganglia. In addition there is also a high level of mRNA expression in the interscapular brown fat deposits. Interestingly, these deposits are highly innervated with sympathetic terminals from the five intercostal nerve fibers bundle (23). nclk mRNA can also be seen in the region of the small bowel consistent with the Northern blot data (Fig. 3*B*). As expected, no expression over background is observed in a section hybridized to the sense cRNA control probe (Fig. 4 *Upper right*).

The distribution described above indicates that nclk is expressed at highest levels in nervous tissue of the adult and E20 rats. If nclk is the neurofilament KSP kinase, one would expect to find expression in neurons, the cells that specifically express neurofilament protein. A higher magnification analysis of the dorsal root ganglion from an E20 rat embryo (Fig. 4 *Lower*) shows that nclk mRNA expression is high in the neurons of the dorsal root ganglion (Fig. 4*B*). These neurons also contain high levels of both NF-H and NF-M mRNA (M. E. Goldstein, personal communication). *In situ* hybridization plus immunocytochemistry will be necessary to determine whether nclk mRNA is localized in one or more subpopulations of neurons in the dorsal root ganglion.

The identification of nclk expands the family of cdc2-related protein kinases. The functional consequence of the structural differences between nclk and the other members of the cdc2 kinase family are presently unclear. The predominant expression of nclk in postmitotic neurons makes it a good candidate for a neurofilament KSP kinase and suggests the possibility that the cdc2 family of kinases may play an important role in cell functions other than cell cycle regulation.

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