# *Png-1,* a Nervous System-Specific Zinc Finger Gene, Identifies Regions Containing Postmitotic Neurons During Mammalian Embryonic Development

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## ABSTRACT

To identify genes associated with early postmitotic cortical neurons, gene fragments were examined for expression in postmitotic, but not proliferative, zones of the embryonic murine cortex. Through this approach, a novel member of the zinc finger gene family, containing 6 $C_2HC$ fingers, was isolated and named *postmitotic neural gene-1,* or *png-1.* Embryonic *png-1* expression was: 1) nervous system-specific; 2) restricted to zones containing postmitotic neurons; and 3) detected in all developing neural structures examined. In the cortex, *png-1* expression was first observed on embryonic day 11, correlating temporally and spatially with the known generation of the first cortical neurons. Gradients of *png-1* expression throughout the embryonic central nervous system further correlated temporally and spatially with known gradients of neuron production. With development, expression remained restricted to postmitotic zones, including those containing newly-postmitotic neurons. *Png-1* was also detected within two days of neural retinoic acid induction in P19 cells, and expression increased with further neuronal differentiation. These data implicate *png-1* as one of the earliest molecular markers for postmitotic neuronal regions and suggest a function as a panneural transcription factor associated with neuronal differentiation. J. Comp. Neurol. 381:130–142, 1997.   © 1997 Wiley-Liss, Inc.

Indexing terms: neurogenesis; neuronal differentiation; cerebral cortex; mouse; zinc finger

A general feature of embryonic neurogenesis in the mammalian central nervous system (CNS) is the production of neuroblasts in proliferative zones overlying the lumen of the neural tube, followed by migration of young postmitotic neurons to more superficial positions. The dynamics of these processes have been particularly well defined in the cerebral cortex, in which neuroblasts proliferate in the ventricular zone, adjacent to the lateral ventricles. A cortical neuron is "born" after its last round of mitosis, following which it may: 1) migrate superficially through the intermediate zone to reach the cortical plate (Boulder Committee, 1970; see Fig. 1); or 2) undergo programmed cell death (Blaschke et al., 1996). Ventricular zone neurogenesis occurs within a defined embryonic period and is completed before birth. Estimates on precisely when cortical neurons are born have relied on "birthdate" labeling at the embryonic day in question (with [³H]thymidine or bromodeoxyuridine [BrdU]; Angevine and Sidman, 1961; Bayer and Altman, 1991; Takahashi et al., 1995) followed by subsequent examination of cortical tissue sections. On the basis of these studies, murine cortical neurogenesis begins around embryonic day 11–12 (E11–E12) and continues through E17 (Caviness, 1982; Bayer and Altman, 1991; Wood et al., 1992).

Although the molecular mechanisms underlying early neuronal differentiation are incompletely understood, they likely involve the expression of distinct families of transcription factors, members of which have been identified in the embryonic nervous system. These include the POU-homeodomain (He et al., 1989; Turner et al., 1994; Alvarez-Bolado et al., 1995), homeobox (Simeone et al., 1992; Bulfone et al., 1993), winged-helix (Xuan et al., 1995) and basic helix-loop-helix (bHLH) families (Murre et al., 1989; Lo et al., 1991; Begley et al., 1992; Bartholomä and Nave,
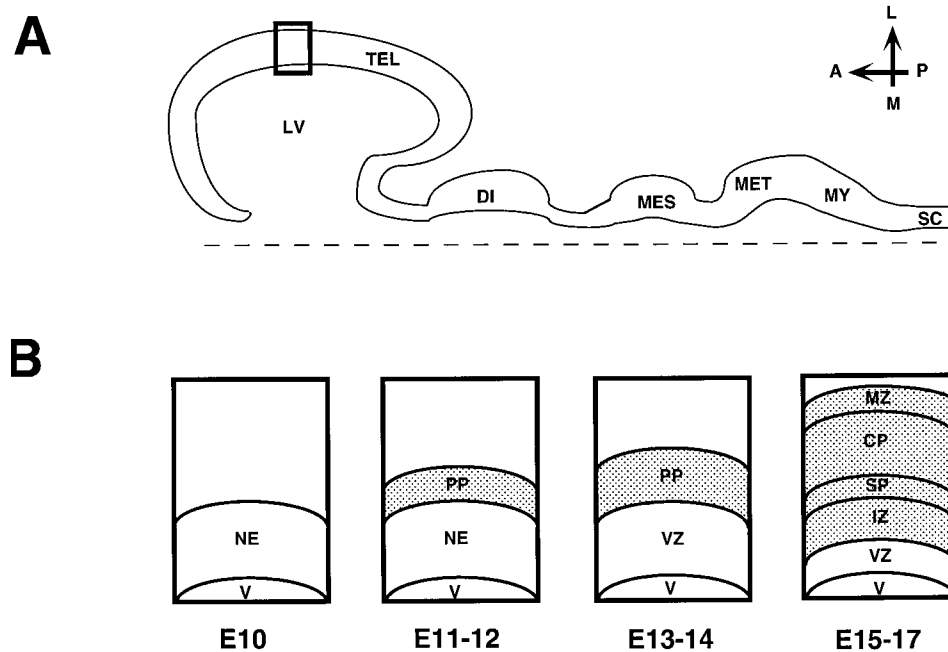
Fig. 1. The embryonic cerebral cortex contains distinct zones of proliferation and differentiation. **A:** A schematic horizontal cross section through the neural tube. Neuroblasts proliferate in zones overlying the tube's lumen (nearest the dashed midline). Postmitotic neurons migrate away from these ventricular zones (VZs) to differentiate in superficial regions. The cortex arises from the telencephalon (TEL). **B:** An expanded view of the box in (A). Embryonic days (E10–E17) refer to murine development (gestation is ~19–20 days). At E10, the telencephalic wall consists of a proliferative neuroepithelium (NE); the first postmitotic neurons are generated between E11 and E12. By E12, neurons have migrated to form the preplate (PP), beneath the pial surface. The PP thickens on E13–E14, as more neurons are generated and migrate from the VZ. By E15–E17, the PP has been split into the marginal zone (MZ) and subplate (SP) by the migration through the intermediate zone (IZ) of neurons forming the cortical plate (CP), which gives rise to the adult cortex. Stippled zones contain postmitotic neurons. LV, lateral ventricle; DI, diencephalon; MES, mesencephalon; MET, metencephalon; MY, myelencephalon; SC, spinal cord; A, anterior; P, posterior; L, lateral; M, medial; V, ventricle.

1994; Lee et al., 1995). Of the many members of these families thus far identified, few are expressed exclusively in young postmitotic neurons during embryonic development. Of those that appear to be (e.g., *NEX-1*, Bartholomä and Nave, 1994; *T-Brain-1*, Bulfone et al., 1995; *Brn-3.2*, Turner et al., 1994; and *NeuroD*, Lee et al., 1995), all have expression in limited regions of the developing nervous system. Transcription factors that are expressed in postmitotic neurons *throughout* the developing nervous system and that thus are candidates for the control of early differentiative events shared by all neurons have yet to be described.

A class of transcription factors that has been associated with early cell fate determination and differentiation in a variety of tissues is the zinc finger family (Wilkinson et al., 1989; Swiatek and Gridley, 1993; Turner et al., 1994; Georgopoulos et al., 1994; Winandy et al., 1995; Yang et al., 1996). In the immune system, for example, members of this family may precede bHLH genes in the transcriptional hierarchy that leads to fully functional B or T cells (Georgopoulos et al., 1994; Winandy et al., 1995). The zinc finger gene *Ikaros* is required for the early determination of lymphocytes (Georgopoulos et al., 1994) and is further important for the later control of proliferation and differentiation of T cells (Winandy et al., 1995). Similarly, *Blimp-1* can drive the differentiation of B cell lines into immunoglobulin-secreting cells (Turner et al., 1994). In the nervous system, the zinc finger gene *Krox-20* is necessary for the specification and development of rhombomeres in the hindbrain (Swiatek and Gridley, 1993), and, similarly,

*RU49* may play a role in the differentiation of granule neurons (Yang et al., 1996).

During a screen to identify genes associated with the differentiation of newly postmitotic cortical neurons, we identified a novel zinc finger gene expressed in postmitotic but not in proliferative regions throughout the embryonic nervous system. Here, we report the initial characterization of this gene, named *postmitotic neural gene-1*, or *png-1.* We demonstrate that *png-1* expression is associated temporally and spatially with the known generation of the first cortical neurons, with postmitotic neuronal regions throughout the developing nervous system, and with P19 cells following neural induction. These results implicate zinc finger transcription factors as potential mediators of early neuronal differentiation and provide support for the use of *png-1* expression as an early marker for postmitotic neurons.

## MATERIALS AND METHODS
### Isolation and characterization of *png-1* cDNA clones

A probe comprising bases 1,013–1,964 of the mouse recombination activating gene-2 (RAG-2) cDNA (Oettinger et al., 1990) was used to screen at low stringency for neural RAG-2 homologues, or putative transcription factors with activation domains similar to an acidic region in the probe. RAG-2 activates somatic gene recombination in B and T cells (Schatz et al., 1992) through interaction with RAG-1,

a gene that is also expressed in the developing nervous system (Chun et al., 1991). A cDNA library containing mRNAs from postmitotic cortical neurons was used; because cortical neurogenesis ceases before birth, a postnatal day 20 (P20) Balb/c mouse brain λ-ZAP cDNA library (Stratagene, La Jolla, CA) was chosen. One million recombinants were screened by using 1–2 × $10^6$ cpm/ml of $^{32}$P-labeled random-primed probe in hybridization solution containing 5× SSC (standard saline citrate: 1× = 150 mM NaCl, 15 mM $Na_3$citrate · $2H_2O$, pH 7.0), 0.6% sodium dodecyl sulfate (SDS), 4× Denhardt's solution, 5 mM EDTA, 40 mM sodium phosphate (pH 7.0), and 0.1 mg/ml salmon sperm DNA, at 50°C. Lifts were washed successively in 2× SSC/0.1% SDS, 1× SSC/0.1% SDS, and 0.5× SSC/0.1% SDS at room temperature, followed by a repeat of the final wash at 50°C. Twenty-one positive pBluescript cDNA clones were rescued by in vivo excision following manufacturer's protocols (Stratagene).

Initial northern blot analyses were carried out to identify cDNA clones expressed in the postnatal brain but not in the neocortical neuroblast cell lines TR and TSM (Chun and Jaenisch, 1996) representing immature neuronal phenotypes. Clone 19 detected a transcript present in brain but absent from TR and TSM. This 3.6-kb cDNA encoded an open reading frame incomplete at the 5′ end; thus, the library was rescreened at high stringency to isolate a full-length cDNA of ~4.6 kb. The clone was sequenced (USB, Cleveland, OH), and northern blots of 20 μg of cytoplasmic or total RNA were made by using standard protocols (Ausubel et al., 1994). Blots were probed with a $^{32}$P-labeled *png-1* Eco RI 4.2-kb fragment at 5 × $10^6$ cpm probe/ml of hybridization solution (25% formamide, 0.5 M $Na_2HPO_4$, 1% bovine serum albumin [BSA], 1 mM EDTA, 5% SDS) at 55°C, followed by SSC/SDS washes of increasing stringency (final wash of 0.2× SSC/0.1% SDS at 65°C). Blot autoradiographs were scanned by using a Macintosh OneScanner; contrast was adjusted, and labels were added in Adobe Photoshop 3.0 and Canvas 3.5.

## In situ hybridization

All animal protocols have been approved by the Animal Subjects Committee at the University of California, San Diego, and conform to NIH guidelines and public law. Pregnancy-timed Balb/c mice were killed by cervical dislocation, and embryos of various ages (day of vaginal plug = E0) were rapidly frozen by using Tissue-Tek OCT (Optimal Cutting Temperature, Miles, Elkhart, IN) and Histofreeze (Fisher, Pittsburgh, PA). Parasagittal and coronal cryostat sections (20 μm) were cut, thaw-mounted onto charged microscope slides (Superfrost Plus, Fisher), and fixed and processed as previously described (Chun et al., 1991). Digoxigenin-labeled riboprobes were transcribed in the sense and antisense orientations from linearized *png-1* plasmid by using standard protocols (Turka et al., 1991). Hybridization was carried out by using 2 ng/μl of labeled riboprobe in hybridization solution (50% formamide, 2× SSPE [standard sodium phosphate-EDTA; 2× = 300 mM NaCl, 20 mM $NaH_2PO_4$ · $H_2O$, 25 mM EDTA, pH 7.4], 10 mM dithiothreitol, 2 mg/ml yeast tRNA, 0.5 mg/ml polyadenylic acid, 2 mg/ml bovine serum albumin [fraction V], and 0.5 mg/ml salmon sperm DNA) for 12–16 hours at 65°C. Slides were washed twice for 45 minutes at room temperature in 2× SSPE/0.6% Triton X-100, followed by three 30-minute washes at 65°C in high-stringency buffer (2 mM $Na_4P2O_7$, 1 mM $Na_2HPO_4$, 1

mM sodium-free EDTA, pH 7.2). Following washes, slides were incubated in a humidified chamber in blocking solution (1% blocking reagent [Boehringer Mannheim] 0.3% Triton X-100 in Tris-buffered saline [TBS]) for at least 1 hour, followed by overnight incubation with alkaline phosphatase-conjugated antidigoxigenin Fab fragments (Boehringer Mannheim) at 1:500 in blocking solution. Slides were washed in TBS and then processed for colorimetric detection with nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolyl-phosphate). Before coverslipping, sections were fluorescently counterstained with 0.35 μg/ml DAPI (4′,6-diamidino-2-phenylindole, Molecular Probes, Eugene, OR).

## BrdU/in situ hybridization double labeling

Pregnant mice carrying E14 embryos were injected intraperitoneally with 20 μl/g body weight of 10 mM bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) in normal saline. Mice were killed by cervical dislocation, and embryos were removed at 1 hour postinjection; coronal cryostat sections were cut and processed as above. After *png-1* in situ hybridization was carried out, the color solution was rinsed off, and sections were incubated at 60°C in 1 N HCl for 15 minutes, followed by washes in phosphate-buffered saline (PBS). Slides were then blocked for 1 hour in a humidified chamber with 2.5% BSA, 0.3% Triton X-100 in PBS, followed by a 1-hour incubation with mouse monoclonal anti-BrdU (Boehringer Mannheim) at 6 μg/ml in the same solution. Bound antibody was detected with biotinylated anti-mouse IgG, avidin biotin complex (ABC) reagent (Vector Labs, Burlingame, CA), and diaminobenzidine (DAB; Chun and Shatz, 1989).

## P19 cell culture and neuronal induction

The P19 embryonal carcinoma cell line was grown in Dulbecco's modified Eagle medium (DMEM; GIBCO, Gaithersburg, MD) with 10% fetal calf serum. Neuronal induction of aggregated cells with retinoic acid (RA; all-trans; Sigma) was conducted as previously described (Rudnicki and McBurney, 1987; Chun et al., 1991). Cells were aggregated in the presence of 0.5 μM RA for up to 4 days, followed by plating on tissue culture plastic in the absence of RA for 2 days. Cytoplasmic RNA was isolated by using standard protocols (Ausubel et al., 1994) at 1, 2, and 4 days of RA aggregation, and at 6 days after the first RA exposure (2 days after plating on tissue culture plastic). Northern blot analysis of 30 μg cytoplasmic RNA was carried out by using probes for *png-1,* and for γ-actin, as above. For in situ hybridization, both uninduced cells and cells at day 4 of RA treatment were dispersed and plated (without RA) onto polylysine-coated microscope slides. After 2 days, slides were fixed, processed, and hybridized with *png-1* riboprobes as above.

## RESULTS
### cDNA cloning of *png-1,* a novel zinc finger gene

To identify novel genes expressed in postmitotic neurons but not neocortical neuroblast cell lines (Chun and Jaenisch, 1996), a cDNA library representing postmitotic neurons was screened at low stringency with a probe encoding an acidic domain of the mouse RAG-2 gene (see Materials and Methods). The resulting positive clones

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  1 CGGTGCTATGCGTGGTCCTCCTCGGCCTCCCACTTGTGGGAAAGGGTTTTCTCTTTCTTTCTGTGTGTTTTGAGCCAGCACAGTTACCAAAATTGAACTTGCCGTCACTTGTGAGCGGTG
121 TGGTCATGGTGTGAGGGGTCCCACAGAGGCTGCAGCTGAGGTCTGGGTGTGTGCAATTCTCAGCTGGGCTTTGCTACCCAGGTTGACGACTGAAGAATCACAGAGTGTGGGAAAAAACAC
241 TAGGAGGAATTGGTGAGAACATCTAGCTAGCATCTCCAAGTCCTGTGGAGGGAGCCAGCAGTGCTGTCCAAAGAGGACCCAGAGAGTGAACTCAGAGTGACCACATCTGATAGAAGAGGG
361 GAAGATGTAGTTTCTGAGTCCAGTCCAGTGTTTGTGTCTCTCACATTGTCAACAAAAGAAAGGCTCCAGCTGTCCCCACAGACATATGGATATTCCAGGAGCCACGTAAAGATGGAGAAA
481 TGGAGGCACAGAGAAATTAAGTGACTTGGCCACAGTCACAAGCTGGGGAGGACCAGGGAAAGCCTAGAGAGAGCTGGCTCTGGGCCTGCATCCTGCCCACGGAGTCACCCTGCCTCCGTC
601 CTCAGGAGAGAAGGCTTCCTACAAG
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   1 M  D  V  D  S  E  E  K  R  H  R  T  R  S  K  G  V  R  V  P  V  E  P  A  I  Q  E  L  F  S  [C  P  T  P  G  C  D  G  S  G
 626 ATGGACGTGGACTCTGAGGAGAAGCGCCATCGCACACGGTCCAAAGGGGTTCGAGTTCCTGTGGAGCCAGCCATACAAGAGCTGTTCAGCTGTCCCACTCCAGGCTGCGACGGCAGTGGT
                   ZF1
  41 H  V  S  G  K  Y  A  R  H  R  S  V  Y  G  C]  P  L  A  K  K  R  K  T  Q  D  K  Q  P  Q  E  P  A  P  K  R  K  P  F  A  V
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  81 K  A  D  S  S  S  V  D  E  C  Y  E  S  D  G  T  E  D  M  D  D  K  E  E  D  D  D  E  E  F  S  E  D  N  D  E  Q  G  D  D
 866 AAAGCAGATAGTTCCTCAGTAGACGAATGTTATGAGACGTGATGGTACTGAAGACATGGATGATGATAAGGAGGAAGATGATGATGAGGAGTTCTCTGAAGACAATGATGAGCAAGGGGATGAT

 121 D  D  E  D  E  V  A  S  R  E  D  E  E  I  E  E  E  D  D  E  D  D  D  D  D  E  D  G  D  D  V  E  E  E  E  D  D  D
 986 GACGACGAAGATGAGGTGGCATCGAGGGAAGACGAGGAGGAGATCGAGGAGGAAGATGATGAAGATGATGATGATGATGAAGATGGTGACGATGTAGAAGAGGAAGAAGAGGATGATGAT

 161 E  E  E  E  E  E  E  E  E  E  N  E  D  H  Q  M  S  C  T  R  I  M  Q  D  T  D  K  D  D  N  N  N  D  E  Y  D  N  Y  D
1106 GAAGAGGAGGAAGAAGAGGAAGAGGAAGAAGAAATGAAGACCATCAAATGAGTTGTACTCGAATAATGCAGGACACAGACAAGGATGATAACAACAATGATGAGTATGATAACTATGAT

 201 E  L  V  A  K  S  L  L  N  L  G  K  I  A  E  D  A  A  Y  R  A  R  T  E  S  E  M  N  S  N  T  S  N  S  L  E  D  D  S  D
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 241 K  N  E  N  L  G  R  K  S  E  L  S  L  D  L  D  S  D  V  V  R  E  T  V  D  S  L  K  L  L  A  Q  G  H  G  V  V  L  S  E
1346 AAAAACGAAAACCTCGGTCGGAAAAGCGAACTGAGTCTAGACTTAGCACAGTGATGATGTTGTTAGAGAAACAGTGGACTCCCTTAAGCTGTTAGCACAAGGACATGGTGTTGTGCTATCAGAG

 281 N  I  S  D  R  S  Y  A  E  G  M  S  Q  Q  D  S  R  N  M  N  Y  V  M  L  G  K  P  M  N  N  G  L  M  E  K  M  V  E  E  S
1466 AATATCAGTGACAGAAGTTATGCTGAGGGGATGTCACAGCAGGACAGTAGAAATATGAACTATGTCATGCTAGGGAAGCCCATGAACAATGGACTCATGGAGAAGATGGTGGAGGAGAGT

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 361 Q  H  V  R  Q  E  D  D  F  P  G  R  T  P  D  R  S  Y  S  D  M  M  N  L  M  R  L  E  E  Q  L  S  P  R  S  R  T  F  S  S
1706 CAACATGTCCGGCAAGAGGACGACTTCCCTGGGAGGACGCCAGACAGGAGCTACTCGGATATGATGAATCTTATGCGGCTGGAGGAGCAGCTCAGCCCCAGGTCTAGAACGTTCTCCAGC

 401 C  A  K  E  D  G  C  H  E  R  D  D  D  T  T  S  V  N  S  D  R  S  E  E  V  F  D  M  T  K  G  N  L  T  L  L  E  K  A  I
1826 TGTGCCAAGGAGGATGGGTGTCATGAGAGGGATGATGACACCACTTCAGTGAACTCAGACAGGTCTGAGGAAGTGTTTGACATGACCAAGGGCAACCTGACTCTGCTAGAGAAAGCCATT

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                                                                                                              ZF2
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 521 G  L  Y  P  H  H  R  S  L  S  G  C]  P  H  K  D  R  V  P  P  E  I  L  A  M  H  E  N  V  L  K  [C  P  T  P  G  C  T  G  R
2186 GGGCTTTACCCGCATCACCGCAGTCTGTCTGGATGCCCGCACAAAGATAGGGTCCCTCCAGAAATTCTTGCCATGCATGAAAATGTTCTCAAGTGTCCCACTCCAGACCAGGGCCA
           ZF3
 561 G  H  V  N  S  N  R  N  S  H  R  S  L  S  G  C]  P  I  A  A  A  E  K  L  A  K  A  Q  E  K  H  Q  S  C  D  V  S  K  S  N
2306 GGGCATGTGAATAGCAACAGGAACTCGCACAGAAGCCTCTCTGGATGCCCCATTGCTGCTGCAGAAAAACTGGCAAAGGCCCAAGAGAAACACCAGAGCTGTGATGTGTCCAAATCCAAC

 601 Q  A  S  D  R  V  L  R  P  M  C  F  V  K  Q  L  E  I  P  Q  Y  G  Y  R  N  N  V  P  T  T  T  P  R  S  N  L  A  K  E  L
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 641 E  K  Y  S  K  T  S  F  E  Y  N  S  Y  D  N  H  T  Y  G  K  R  A  I  A  P  K  V  Q  T  R  D  I  S  P  K  G  Y  D  D  A
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 681 K  R  Y  C  K  N  A  S  P  S  S  S  T  T  S  S  Y  A  P  S  S  S  S  N  L  S  C  G  G  G  S  S  A  S  S  T  C  S  K  S
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 721 S  F  D  Y  T  H  D  M  E  A  A  H  M  A  A  T  A  I  L  N  L  S  T  R  C  R  E  M  P  Q  N  L  S  T  K  P  Q  D  L  C
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 761 T  A  R  N  P  D  M  E  V  D  E  N  G  T  L  D  L  S  M  N  K  Q  R  P  R  D  S  C  C  P  V  L  T  P  L  E  P  M  S  P
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                                                                                                        ZF4
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 921 N  Y  A  S  H  R  S  L  S  G  C]  P  R  A  K  K  S  G  I  R  I  A  Q  S  K  E  D  K  E  D  Q  E  P  I  R  [C  P  V  P  G
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3626 TCGGTCAAGACGGAGGGCATGTCCTGCCCTACCCCTGGGTGTGATGGGTCAGGACACGTCAGTGGCAGCTTCCTCACACACCGCAGCTTGTCAGGATGTCCAAGAGCCACATCAGCAATG

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1081 N  S  Q  M  E  A  D  M  I  K  L  R  T  Q  I  T  T  M  E  S  N  L  K  T  I  E  E  E  N  K  V  I  E  E  Q  Q  N  E  S  L  L
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1121 H  E  L  A  N  L  S  Q  S  L  I  H  S  L  A  N  I  Q  L  P  H  M  D  P  I  N  E  Q  N  F  D  A  Y  V  T  T  L  T  E  M
3986 CACGAGTTGGCCAACCTGAGCCAGTCCCTGATCCACAGCCTCGCCAACATCCAGCTGCCTCACATGGATCCAATCAATGAACAAAATTTTGATGCTTACGTGACTACTTTGACGGAAATG

1161 Y  T  N  Q  D  R  Y  Q  S  P  E  N  K  A  L  L  E  N  I  K  Q  A  V  R  G  I  Q  V  *
4106 TATACAAATCAAGATCGTTATCAGAGTCCAGAAAATAAAGCCCTACTGGAAAATATAAAGCAGGCTGTGAGAGGAATTCAGGTCTGA
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4193 ACAGCTGCTCTAGTGGTGACTCATGCTTAAAAAGGATGCCTCTTGTTTCTTGCTGCTGTAACTTACCAGAAAGTGTTATATATTTATTTCTGTCGGAACAGTGTTATGCTACAAGACTTC
4313 ATAATGGTTTTGTGTGCTCTTGAGGAGTACCTGCAGACTAGTTTTGGATACATTCACATTTTGTACGTTTTCATATAAGCTGACATAGTGTGATTTGCCATGTAATGTTTATAGCTGCT
4433 GCTGTCTGCACATTTGGGGGTCTCTATATTTCTGAAGAGGTAAGCTGATGAAAATAAATAGAGTGTAAATTCTTTTTAATGCTTTAGTGATTAAATGTTTTAGTATTTTGAACTGAAATG
4553 GACAAAAAAAA
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Fig. 2. *Png-1* encodes a member of the zinc finger family. The approximately 3.6-kb *png-1* open reading frame encodes a predicted protein of 1,188 amino acids, with a calculated molecular weight of 133 kDa. The six zinc finger domains are boxed and numbered. A highly acidic domain (77% aspartate or glutamate residues) common to many eukaryotic transcription factors is indicated by a dashed overline. The *png-1* cDNA sequence has been deposited in GenBank, accession number U86338.

were screened by Northern blot analyses to identify differentially expressed clones, and one partial cDNA detected transcripts in postnatal brain but not in neocortical neuroblast cell lines. Isolation and sequencing of a 4.6-kb *png-1* cDNA revealed an open reading frame of ~3.6 kb. The *png-1* open reading frame encoded a protein of 1,188 amino acids with a predicted molecular weight of 133 kDa, containing six similar zinc finger domains of the $C_2HC$ type (Fig. 2). Database searches revealed that *png-1* was >95% homologous to rat *neural zinc finger factor-1* (*nzf-1*), whose product binds to β-retinoic acid response elements and activates transcription (Jiang et al., 1996). The predicted *png-1* zinc finger domains were also highly homologous with zinc fingers found in the human MyT1 protein, which binds to the promoter of the PLP gene (Kim and Hudson, 1992). An acidic domain (77% aspartate or glutamate residues) spanning amino acids 88–174, similar to those found in many eukaryotic transcription factors (Ptashne, 1988), probably represented the region detected by the probe.

## Nervous system-specific expression of *png-1*

Northern blot analysis of embryonic and adult brain using a *png-1* probe detected a major transcript of ~7.0 kb and a minor transcript of ~3.5 kb. These transcripts were absent both from neuroblast cell lines TR and TSM, derived from the ventricular zone and representing immature neuronal phenotypes (Chun and Jaenisch, 1996), and from nonneural adult tissues examined, including liver, kidney, and spleen (Fig. 3). The spatial expression of *png-1* was examined by in situ hybridization to embryonic tissue sections. At E15.5, *png-1* mRNA expression was detected throughout the CNS and in developing peripheral neural structures, including the dorsal root ganglia and trigeminal ganglia (expression in autonomic and enteric ganglia was not determined) (Fig. 4). *Png-1* expression appeared panneural, with heavy labeling seen in all developing neural structures examined, including the cerebral cortex, thalamus, midbrain, cerebellar primordium, hindbrain, and spinal cord. Hybridization signal was absent from all nonneural embryonic tissues examined.

## Restriction of *png-1* expression to postmitotic neuronal regions

In the CNS, *png-1* expression was further restricted to postmitotic neuronal regions. *Png-1* hybridization signal appeared to be absent from proliferative zones abutting the ventricles, including those of the cortex, midbrain, and ganglionic eminence (Fig. 4, arrows). This pattern of *png-1* expression was further examined in the developing cerebral cortex, in which zones of proliferating and postmitotic cells can be identified clearly (Angevine and Sidman, 1961; Caviness, 1982; Bayer and Altman, 1991) (Fig. 5). At E10.5, before the generation of postmitotic neurons has begun (Caviness, 1982; Takahashi et al., 1995), *png-1* expression was absent from the telencephalic wall (Fig. 5A), which at this stage consists of a thin proliferative neuroepithelium. On E11 in the mouse, the first postmitotic neurons of the cortex are generated (Caviness, 1982; Wood et al., 1992) and migrate superficially to begin forming the preplate. At E11.5, *png-1* expression was confined to a thin band, one to three cells thick, at the outermost portion of the cerebral wall where the preplate forms (Fig. 5C). Expression was absent from the rest of the telencephalon that contained proliferative neuroblasts.
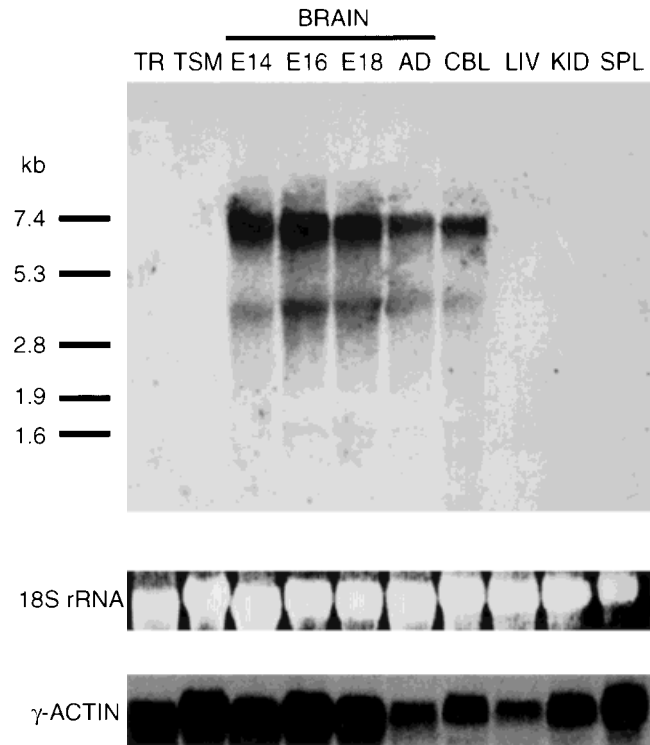


Fig. 3. *Png-1* is expressed in embryonic and adult brain. Northern blot analyses of 20 μg cytoplasmic RNA from cortical neuroblast cell lines TR and TSM (Chun and Jaenisch, 1996), E14, E16, E18, and adult (AD) brain, adult cerebellum (CBL), liver (LIV), kidney (KID), and spleen (SPL) are shown. A major transcript of 7.0 kb and a minor transcript of 3.5 kb are detected in embryonic and adult brain and cerebellum. Neither transcript is detectable in TR and TSM nor in adult liver, kidney, and spleen. A γ-actin loading control is shown; because of differential expression of this isoform in some adult tissues (Erba et al., 1988), ethidium bromide staining of 18S rRNA is also shown.

At E13.5, as the preplate thickens because of the continuing generation and migration of postmitotic neurons, so did the band of *png-1*-positive cells (Fig. 5E). On E15.5, as the stratification of the cortex continues with the emergence of the cortical plate, the restriction of *png-1*-positive cells to postmitotic zones was maintained (Fig. 5G): labeled cells were found throughout the marginal zone, cortical plate, subplate, and intermediate zone, all of which contain postmitotic neurons (Bayer and Altman, 1991). Again, *png-1* expression was virtually absent from the proliferative ventricular zone. Similarly, near the end of neurogenesis on E17.5, most cells of the cortical plate, subplate, and intermediate zone continued to be positive for *png-1* expression (Fig. 5I). Thus, in the embryonic cerebral cortex, *png-1* expression correlated closely with zones containing postmitotic neurons, beginning with those first generated. *Png-1* was also detected in the adult cerebral cortex (Fig. 5K), where its expression was localized within large cells of layers II–VI.

To ascertain that *png-1*-positive regions in the embryonic cortex were distinct from the proliferative ventricular zone, *png-1* in situ hybridization was combined on the same tissue section with BrdU immunohistochemistry. These experiments took advantage of the fact that exposure to a 1-hour pulse of BrdU labels S-phase nuclei at the
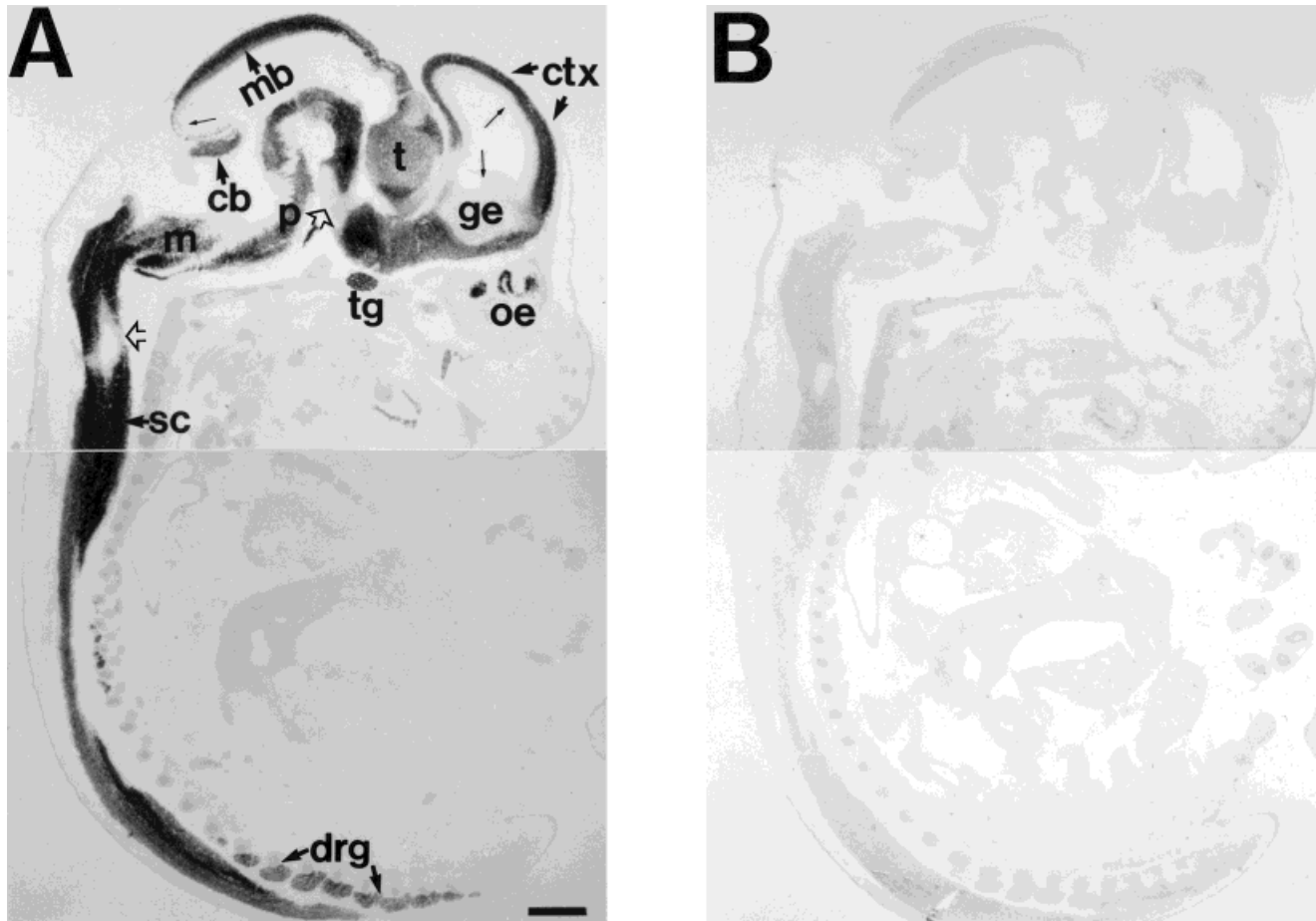
Fig. 4. *Png-1* expression is restricted to, and present throughout, the embryonic nervous system. In situ hybridization to adjacent sagittal sections through an E15.5 mouse embryo with digoxigenin-labeled antisense (**A**) and sense (**B**) *png-1* riboprobes is shown. *Png-1* antisense probe labels cells throughout all regions of the developing central nervous system (CNS), including the cerebral cortex (ctx), thalamus (t), midbrain (mb), cerebellar primordium (cb), hindbrain (pons, p, and medulla, m), and spinal cord (sc). *Png-1* expression is also detected in developing peripheral neural structures, including the olfactory epithelium (oe), trigeminal ganglion (tg), and dorsal root ganglia (drg). In the CNS, *png-1* expression is further restricted to postmitotic regions; hybridization signal is absent from proliferative zones abutting the ventricles (thin arrows). No signal is detected in nonneural embryonic tissues nor in an adjacent section hybridized with *png-1* sense riboprobe (B). Note that the lack of hybridization signal in some regions (open arrows) is a result of the plane of section deviating into the proliferative ventricular zone; consecutive adjacent sections exhibited normal *png-1* hybridization in these regions. ge, ganglionic eminence. Scale bar = 800 μm.

superficial border of the ventricular zone (Takahashi et al., 1995). The resulting pattern of BrdU-positive cells contrasted with the pattern of *png-1* expression, forming a clear boundary (Fig. 6A). At higher magnification, rare *png-1*-positive cells could be detected within the S-phase zone (Fig. 6B); however, these cells were not BrdU labeled. Conversely, a few BrdU-positive cells were observed in the preplate, within the region of *png-1* expression; however, they did not appear to be positive for *png-1* (Fig. 6C).

This restriction of *png-1* expression was not confined to the cortex. In the developing retina, *png-1* was expressed in the ganglion cell layer, which contains differentiating, postmitotic neurons (Sidman, 1961; Polley et al., 1989), but not in the proliferative neuroblast layer (Fig. 7A). Similarly, in the embryonic olfactory epithelium, *png-1*-positive cells were observed in regions containing differentiating neurons (Fig. 7C; Cuschieri and Bannister, 1975). Thus, *png-1* expression was restricted to postmitotic neuro-

nal regions in every developing neural structure examined.

Patterns of *png-1* expression were also observed, which correlated with previously described gradients of neuronal production. In the developing cerebral cortex, neurons are generated in a ventrolateral-to-dorsomedial gradient, such that ventrolateral neurons are older than dorsomedial neurons in each layer (Smart and Smart, 1982; Bayer and Altman, 1991). Consistent with this gradient, in situ hybridization to a coronal section through the telencephalon at E12.0, early in cortical neurogenesis, labeled a band of *png-1* positive cells that was four to six cells thick ventrolaterally and that gradually tapered to a single layer of positive cells dorsomedially (Fig. 8A). A similar correlation of *png-1* expression with known neurogenetic gradients was observed in the spinal cord at this age (Fig. 8B). Here, postmitotic neurons are first generated ventrally, on E9–E10, followed by intermediate and then
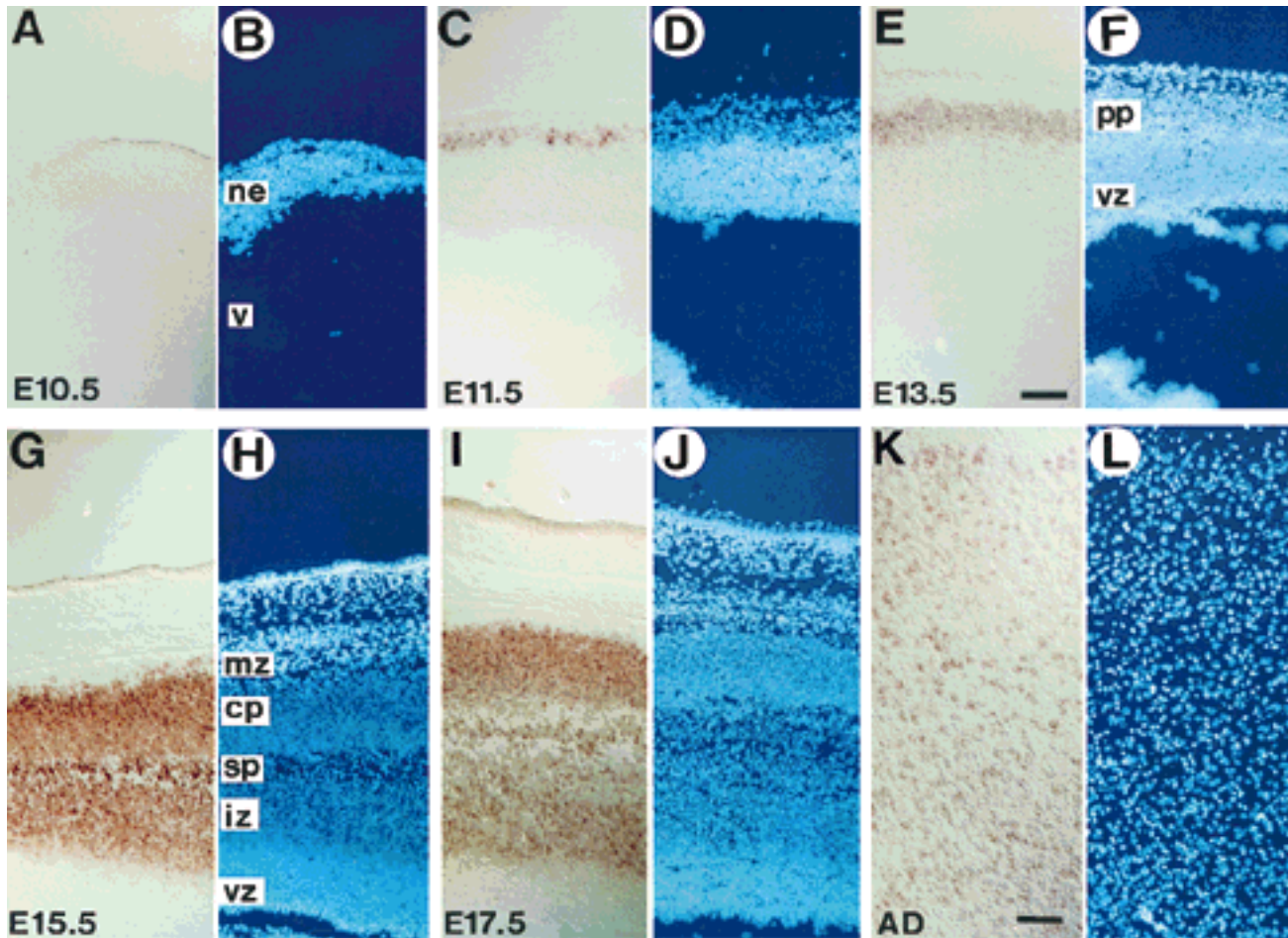
Fig. 5. *Png-1* expression correlates with the generation of the earliest postmitotic cortical neurons. Parasagittal sections through the developing cerebral cortex from E10.5 to E17.5 and the adult cortex are shown. **A,C,E,G,I,K:** Hybridization with *png-1* antisense riboprobe. **B,D,F,H,J,L:** 4,6-diamidino-2-phenylindole (DAPI) fluorescence of the same section, showing location of cell nuclei. The pial surface is at the top. *Png-1* expression is absent on day E10.5, when the telencephalic wall consists of a proliferative neuroepithelium (A). At E11.5, *png-1* expression is detectable in a thin band of cells near the pial surface (C), correlating with postmitotic neurons known to form the preplate (pp), which expands by E13.5 (E). After the stratification of the cortex continues with the emergence of the cortical plate (cp), *png-1* expression remains confined to regions containing postmitotic neurons: the intermediate zone (iz), the subplate (sp), the cp, and the marginal zone (mz) (G,I). Note that *png-1* appears to be expressed in cells as they initially enter the migratory iz. At all ages, hybridization signal is virtually absent from the proliferative ventricular zone (vz). In the adult cortex (K), *png-1* expression is seen in scattered large cells throughout layers II–VI. Expression appears to be absent from the neuron-sparse layer I (K, top). Scale bar = 100 μm.

dorsal neuronal production on E11–E13 (Nornes and Carry, 1978). Consistent with this, the zone of *png-1* expression at E12.0 was thickest ventrally, tapering gradually in more dorsal regions (Fig. 8B), in which neuronal production is just beginning. Thus, gradients of *png-1* expression parallel known gradients of postmitotic neuron generation in the CNS.

## Correlation of *png-1* expression with neuronal induction in P19 cells

To determine whether *png-1* expression correlated with neuronal induction, the pluripotent embryonal carcinoma cell line P19 was examined by northern blot analysis at daily time points during RA treatment. When aggregated in the presence of RA, P19 cells give rise to large numbers of postmitotic neurons (Rudnicki and McBurney, 1987; McBurney et al., 1988; Bain et al., 1994). Untreated P19 cells did not express *png-1* (Fig. 9A). However, within 2 days of RA treatment, *png-1* expression was upregulated (Fig. 9A). *Png-1* expression continued to increase in differentiating P19 cells, through at least day 6. The expression of *png-1* in P19 neurons was confirmed by in situ hybridization; although hybridization signal was absent from uninduced P19 cells (Fig. 9B), RA treatment produced *png-1*-positive cells exhibiting characteristic neuronal morphologies (Fig. 9C, D). These data indicate that *png-1* expression correlates with the neuronal phenotype in P19 cells and is induced early in, and increases throughout, the induction of this phenotype.

## DISCUSSION

We have cloned, sequenced, and initially characterized the developmental expression of a new murine member of
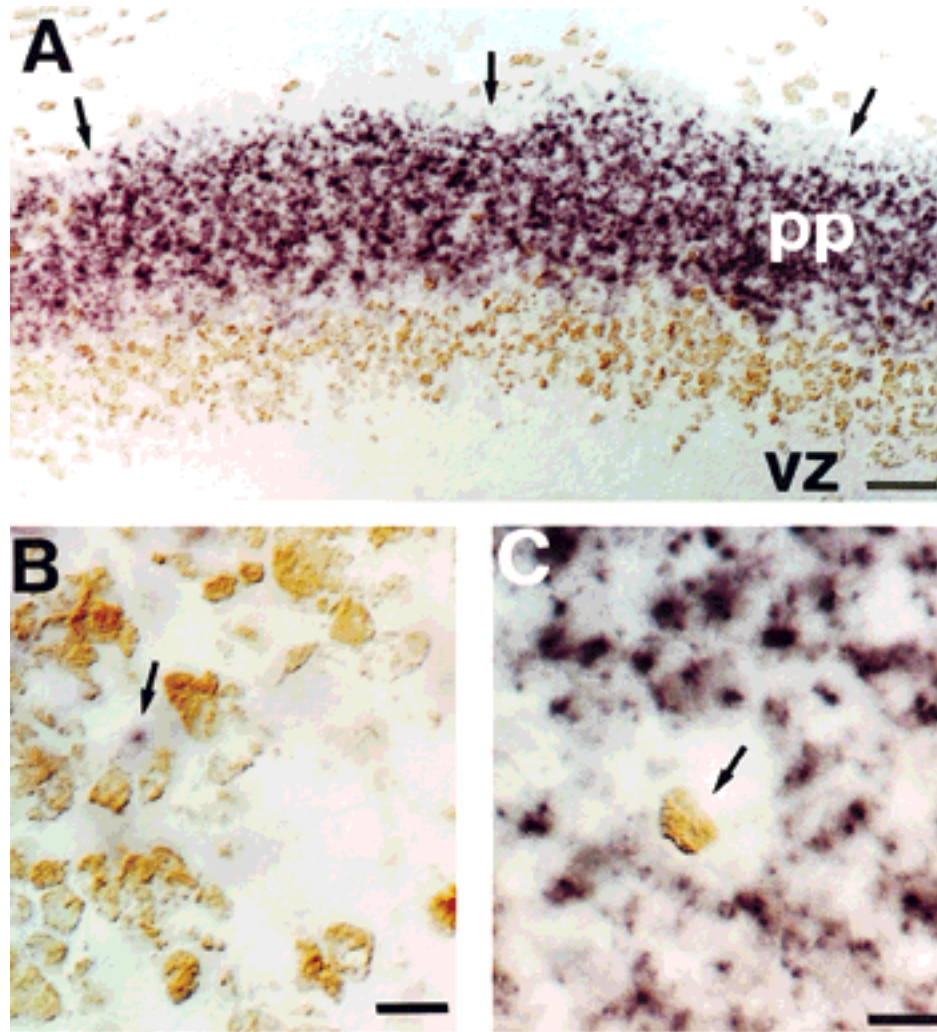
Fig. 6. *Png-1*-positive regions in the embryonic cortex are distinct from the proliferating population. A coronal section through E14.5 cortex, double-labeled for *png-1* in situ hybridization (purple reaction product) and BrdU immunohistochemistry (brown reaction product) is shown. **A:** A 1-hour pulse with BrdU labels S-phase cells at the superficial border of the ventricular zone (vz). *Png-1* expression is confined to the postmitotic preplate (pp), forming a sharp border with the vz, with virtually no overlap. Arrows, pial surface. **B:** A high magnification view of the vz shows that the few *png-1*-positive cells there (arrow) are not BrdU positive. These are likely postmitotic neurons beginning their migration out of the vz. **C:** Conversely, the few BrdU-positive cells in the preplate (arrow) do not appear to be *png-1*-positive. Scale bar = 50 µm in A; 10 µm in B, C.

the zinc finger gene family, *png-1,* using in situ hybridization, BrdU incorporation, and northern blot analyses. *Png-1* expression is restricted to, and present throughout, the embryonic CNS and developing peripheral neural structures. In the embryonic CNS, expression is restricted to postmitotic neuronal regions. In situ hybridization in the cerebral cortex during the neurogenetic period (E11–E17) indicates that *png-1* expression correlates temporally and spatially with the generation of the earliest postmitotic cortical neurons and with known gradients of neuron production; *png-1* expression continues in developing postmitotic cortical zones throughout embryonic development. Further, *png-1* is expressed within 2 days of neuronal induction in P19 cells.

## *Png-1/nzf-1* is a member of the zinc finger gene family

The *png-1* open reading frame encodes a protein containing six putative zinc finger domains of the $C_2HC$ type and a highly acidic region similar to activation domains found in many eukaryotic transcription factors (Ptashne, 1988). Zinc finger domains, in which the coordination of a $Zn^{2+}$ ion by pairs of cysteine or histidine residues creates a finger-like structure that binds DNA, are present in a large family of transcription factors, estimated to represent 1% of the human genome (Berg and Shi, 1996). The most common type of zinc finger domain contains a pair of cysteines and a pair of histidines ($C_2H_2$ type; Berg, 1990).
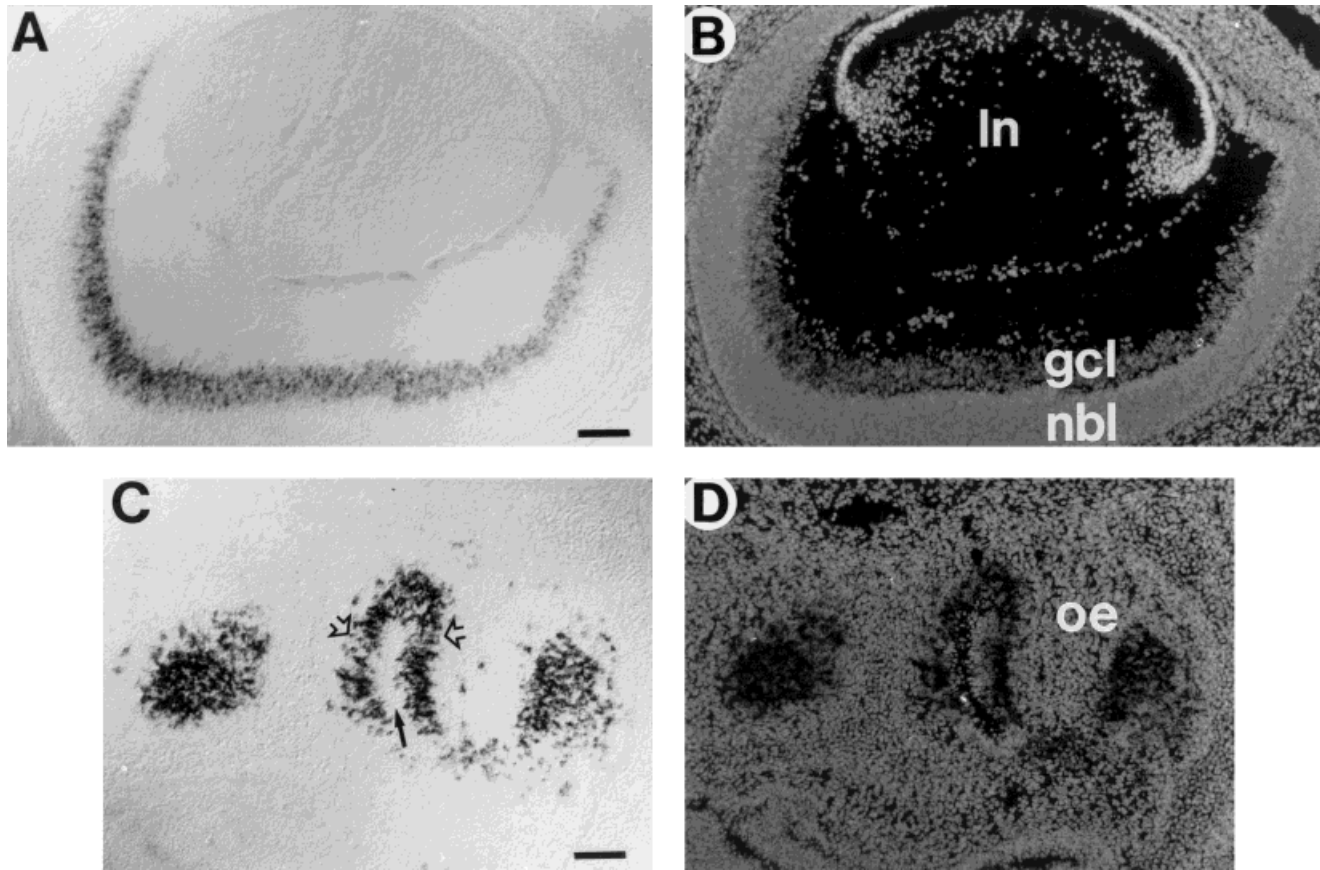
Fig. 7. *Png-1* expression correlates with the presence of postmitotic neurons in other neural structures. *Png-1* antisense in situ hybridization (**A,C**) and same-section DAPI fluorescence (**B,D**) of E15.5 retina and olfactory epithelium are shown. In a sagittal section through the developing retina, (A) *png-1* expression is detected in the postmitotic ganglion cell layer (gcl) but not in the proliferative neuroblast layer (nbl). ln, lens. In (C) *png-1* is expressed in regions of the olfactory epithelium containing differentiating receptor neurons (open arrows) but not in the inner (apical) zones of proliferating stem cells and epithelial supporting cells (filled arrow). Scale bar = 100 µm.

The C$_2$HC type fingers in *png-1* are less common but have been reported in MyT1, a putative transcription factor that binds to promoter elements of the PLP gene in oligodendrocytes (Kim and Hudson, 1992; Armstrong et al., 1995).

Concurrent with our studies, a rat gene was reported, termed *nzf-1,* which is ~95% identical in nucleotide sequence to *png-1* (Jiang et al., 1996). Although *nzf-1* expression was observed in the embryonic nervous system, its restriction to postmitotic zones was not examined in detail. However, it was shown to encode a functional protein that binds to the β-retinoic acid response element and that can activate transcription (Jiang et al., 1996). This binding specificity is particularly interesting in light of our observation of *png-1* upregulation in retinoic acid-induced P19 cells (see below). In view of the high degree of identity with rat *nzf-1,* we will hereafter refer to the murine gene as *png-1/nzf-1.*

### *Png-1/nzf-1* expression is restricted to the nervous system during embryogenesis

*Png-1/nzf-1* expression in the murine embryo is restricted to the nervous system and is detected both throughout the CNS and in peripheral neural structures. Although a number of transcription factors have been reported to have restricted expression in regions of the developing nervous system (e.g., Wilkinson et al., 1989; Lo et al., 1991; Simeone et al., 1992; Bartholomä and Nave, 1994; Frantz et al., 1994; Alvarez-Bolado et al., 1995; Bulfone et al., 1995; Lee et al., 1995), to our knowledge none appear as widely expressed in the nervous system as *png-1/nzf-1.* In all examined regions, *png-1/nzf-1* expression was further restricted to regions containing postmitotic neurons (see below). *Png-1/nzf-1* expression was virtually absent from proliferative zones.

### *Png-1/nzf-1* is expressed in postmitotic neuronal zones

Our data suggest that *png-1/nzf-1* is neuron-specific during embryonic life, based on four observations. First, previous studies have shown that E11–E17 envelops murine cortical neurogenesis, whereas most glia arise around E18 or later in postnatal life (Das, 1979; Woodhams et al., 1981; Caviness, 1982; Bayer and Altman, 1991; Misson et al., 1991; Takahashi et al., 1995). Therefore, *png-1/nzf-1* in situ hybridization labels constituent neuronal regions during the period of cortical neurogenesis. Second, *png-1/nzf-1* expression was never detected in the deepest por-
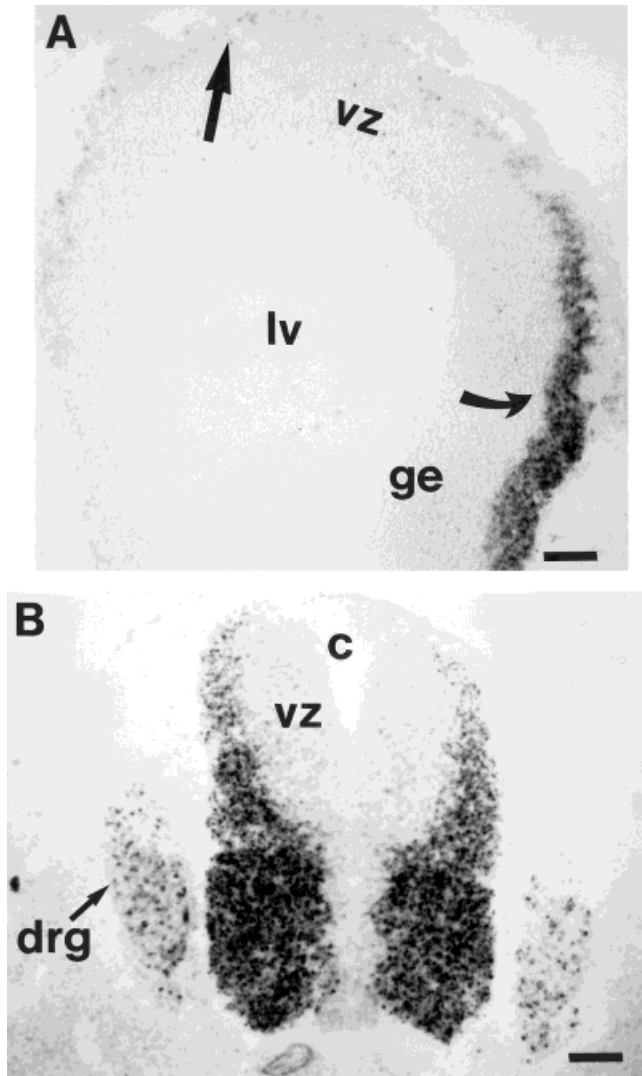
Fig. 8. *Png-1* expression correlates with known gradients of neuronal production. **A:** In a coronal section through the telencephalon at E12.0, early in neurogenesis, a gradient of *png-1* expression is detected; the band of labeled cells is four to six cells thick in the ventrolateral cortex (curved arrow) and tapers gradually in the dorsomedial direction to a single cell thickness (straight arrow). This gradient parallels previously described gradients of postmitotic cortical neuron production. *Png-1*-positive cells are also seen in the postmitotic zone over the ganglionic eminence (ge). lv, lateral ventricle. Scale bar = 100 μm. **B:** A similar correlation of *png-1* expression with known neurogenetic gradients is seen in the E12.0 spinal cord. Here, neurons are first generated ventrally, on E9–E10 and then in more dorsal regions between E11 and E13. At E12.0, the zone of *png-1* expression is most extensive ventrally (toward the bottom of the micrograph) and tapers in more dorsal regions (toward the top), where neuronal production is just beginning. Many cells of the developing dorsal root ganglia (drg) are also *png-1*-positive, consistent with the peak of drg neuronal production on E11.5–E12.5 (Lawson and Biscoe, 1979). vz, ventricular zone; c, central canal. Scale bar = 120 μm.

tions of the ventricular zone that contain the cell bodies of radial glia (Misson et al., 1991), demonstrating that the major glial component of the ventricular zone does not express *png-1/nzf-1.* Third, gradients of *png-1/nzf-1* expression during the neurogenetic period correspond to

previously described gradients of postmitotic neuronal production. Fourth, *png-1/nzf-1* expression increases dramatically in retinoic acid-induced P19 cells; these cultures consist of 85–90% postmitotic neurons when *png-1/nzf-1* expression was observed to be highest (McBurney et al., 1988), and *png-1/nzf-1* in situ hybridization labels cells with clear neuronal morphologies (see Fig. 9). We cannot eliminate the possibility that a small subset of glia or other cells expresses *png-1/nzf-1* embryonically. However, our observations, along with extensive prior data describing neurogenesis in the cortex and other CNS regions, indicate that *png-1/nzf-1* is a strong candidate for a postmitotic neuron-specific gene.

The results of in situ hybridization studies in the adult cerebral cortex further suggest neuronal, rather than glial, expression of *png-1/nzf-1* in the nervous system. Expression was detected in many large cortical cells, likely to be neurons, in layers II–VI (little expression was detected in layer I). Although this observation is consistent with neuronal expression in the adult cortex, formal proof will require double-labeling studies with known neuronal and glial markers. Interestingly, *png-1/nzf-1* expression appears to become more restricted in the adult brain, being maintained in forebrain regions while diminishing in some posterior structures (data not shown). It will be important in the future to establish the function of *png-1/nzf-1* in the adult brain and in the embryonic nervous system.

## *Png-1/nzf-1* expression is one of the earliest hallmarks of postmitotic cortical neurons

In the cerebral cortex, in which the generation and differentiation of neurons occur in distinct laminar zones, *png-1/nzf-1* expression first appeared on E11, in a thin rim of cells at the superficial border of the cerebral wall. This temporospatial pattern of expression correlates closely with prior data describing the generation of the earliest cortical neurons, based on [3H]thymidine or BrdU birthdate labeling (Caviness, 1982; Luskin and Shatz, 1985; Wood et al., 1992). At later ages, *png-1/nzf-1* expression continued to be associated with the location of newly postmitotic neurons, as evidenced by labeled cells at the border of the ventricular zone and the immediately superficial intermediate zone (Figs. 5 and 6). It appears that *png-1/nzf-1* expression begins shortly after a cortical neuron completes its final division and exits the ventricular zone. Previous estimates of the time required for newly postmitotic neurons to exit the ventricular zone varies from 2.8 to 10.7 hours (Hicks and D'Amato, 1968; Takahashi et al., 1993). Therefore, expression of *png-1/nzf-1* likely commences within hours of the final mitosis.

Established protein markers for differentiating neurons, such as MAP2 (Crandall et al., 1986; Chun et al., 1987) and the neurofilaments (Cochard and Paulin, 1984), appear at later stages of neuronal differentiation, after cortical neurons have completed their migration to the preplate or the cortical plate. Class III β-tubulin, recognized by the TuJ1 antibody (Lee et al., 1990; Menezes and Luskin, 1994), has been detected in the embryonic cortex roughly as early as *png-1/nzf-1* expression; however, this protein has also been detected in proliferative zone neuroblasts (Memberg and Hall, 1995). Another protein that is apparently expressed in the earliest postmitotic neurons of the rat cerebral cortex is TOAD-64 (Minturn et al., 1995), which

shares homology with UNC-33, a *C. elegans* protein involved in axon outgrowth. In addition, the gene *T-Brain-1* also appears to be expressed in early postmitotic neurons



**A**

DAY OF NEURONAL INDUCTION

0    0    1    2    4    6

kb

9.5
7.5

4.4

2.4

ACTIN

**B**

**C**

**D**

of the cortex (Bulfone et al., 1995). As an early expressed transcription factor common to most if not all neurons, *png-1/nzf-1* expression could coincide with or precede expression of regional factors, such as *T-Brain-1,* and perhaps regulate the expression of cytoplasmic neuronal proteins. Such a temporal sequence is supported by results of experiments in P19 cells.

*Png-1/nzf-1* expression correlates with early stages of neuronal differentiation in a well-studied model system, the P19 embryonal carcinoma cell line (Rudnicki and McBurney, 1987; McBurney et al., 1988). Temporal changes in expression during P19 neuronal induction have been reported for several genes (McBurney et al., 1988; Johnson et al., 1992; Fujii and Hamada, 1993; Bain et al., 1994; Minturn et al., 1995). The rapid upregulation of *png-1/nzf-1* in the first 2 days of RA treatment places it among the earliest genes induced during P19 neuronal differentiation. Two other transcription factors, *MASH-1,* a bHLH gene, and *Brn-2,* a POU-homeodomain gene, have been detected at day 2 of P19 neuronal induction (Johnson et al., 1992; Fujii and Hamada, 1993), but neither of these genes are restricted to postmitotic neurons in situ, and their expression in the embryo is regional (Lo et al., 1991; Alvarez-Bolado et al., 1995). In contrast, *png-1/nzf-1* is expressed in postmitotic neuronal regions but not in proliferative neuroblasts and is expressed throughout the developing nervous system. *TOAD-64* is also expressed during P19 neuronal induction but not until day 4 of RA treatment (Minturn et al., 1995). Thus, in both the embryonic CNS and the P19 model system of neuronal differentiation, *png-1/nzf-1* is one of the earliest genes expressed by postmitotic neurons.

Determining the functional role of *png-1/nzf-1* will require perturbation of its expression in vivo. However, the data presented here implicate *png-1/nzf-1* as an early molecular hallmark of postmitotic neurons throughout the developing mammalian nervous system and suggest an important role for it in the earliest stages of neuronal differentiation.

## ACKNOWLEDGMENTS
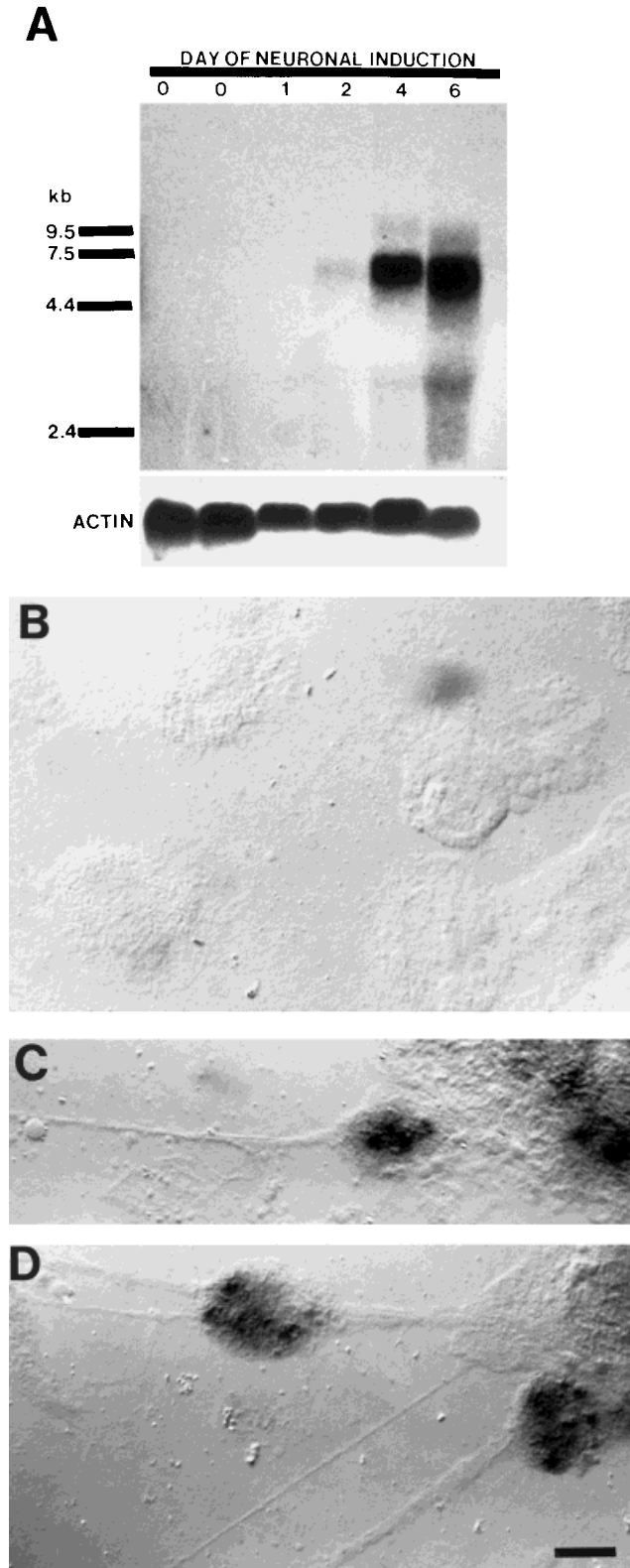
Fig. 9. *Png-1* expression is upregulated during retinoic acid-induced differentiation in P19 cells. **A:** Northern blot analysis of 30-µg cytoplasmic RNA from P19 cells at daily intervals during neuronal induction with retinoic acid (RA, Rudnicki and McBurney, 1987). *Png-1* is not expressed by uninduced P19 cells (day 0; data from two different splits). By day 2 of aggregation with RA, *png-1* transcripts are detectable. *Png-1* expression increases by day 4, the end of RA treatment, and increases further by day 6, two days after plating in the absence of RA. The same blot probed for γ-actin is shown. **B–D:** In situ hybridization with *png-1* antisense riboprobe confirms the Northern blot results. Uninduced P19 cells do not express *png-1* (B). *Png-1* is expressed in cells with clear neuronal morphology by day 6 of RA induction (C, D). Scale bar = 10 µm.

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