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Kv3.3b: A Novel *Shaw* Type Potassium Channel Expressed in Terminally Differentiated Cerebellar Purkinje Cells and Deep Cerebellar Nuclei

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A two-step hybridization/subtraction procedure was employed to isolate markers for the later stages of Purkinje cell differentiation. From this screen, a novel *Shaw* potassium channel cDNA (Kv3.3b) was identified that is developmentally regulated. Expression of this channel is highly enriched in the brain, particularly in the cerebellum, where its expression is confined to Purkinje cells and deep cerebellar nuclei. Sequence analysis revealed that it is an alternatively spliced form of the mouse Kv3.3 gene, and that the previously reported Kv3.3 mRNA (Ghanshani et al., 1992) is not expressed in cerebellum. Expression of the Kv3.3b mRNA begins in cerebellar Purkinje cells between postnatal day 8 (P8) and P10 and continues through adulthood, coinciding with elaboration of the mature Purkinje cell dendritic arbor. The timing of expression of Kv3.3b mRNA is maintained in mixed, dissociated primary cerebellar cell culture. These results suggest that the Kv3.3b K^+ channel function is restricted to terminally differentiated Purkinje cells, and that analysis of the mechanisms governing its expression *in vivo* and *in vitro* can reveal molecular mechanisms governing Purkinje cell differentiation.

[Key words: Purkinje cells, deep nuclei, Shaw, potassium channel, terminal differentiation, development]

The development of the mammalian CNS is programmed by a complex series of genetic and epigenetic events that regulate the differentiation of neuronal and glial cell types. Although one can expect that certain molecular mechanisms that regulate development in the CNS will be employed broadly to program specific stages of differentiation in many different neuronal cell types, these mechanisms must be precisely regulated by the microenvironment in which a given neuron is developing. For example, one might expect that proper temporal regulation of gene products involved in synaptogenesis is required for establishment of proper neuronal circuitry, and that this regulation may be achieved through mechanisms that impinge directly on individual neurons. Our interest is both to identify general subprograms for neuronal differentiation and to discover how these programs are epigenetically regulated in specific neuronal cell types. To investigate these processes, we have chosen to focus on mechanisms regulating development of the mammalian cerebellum.

Cerebellar Purkinje cells provide the sole cerebellar efferent pathway. Since the initial description of cerebellar Purkinje cell development by Ramon y Cajal (1911), analysis of Purkinje cell development under a wide variety of experimental conditions has established that differentiation of these neurons involves complex interactions between intrinsic and extrinsic programs. Purkinje cells originate from the primitive ependymal layer, and in the mouse they become postmitotic by embryonic day 13 (E13) (Uzman, 1960; Miale and Sidman, 1961). Three general phases of Purkinje cell differentiation have been recognized. Until approximately birth, Purkinje cells are small and fusiform, and constitute a multilayered band several cells thick in the cerebellar anlage (Ramon y Cajal, 1911; Uzman, 1960). They then organize themselves into a discrete monolayer forming the boundary between the internal granule layer and the molecular layer in the developing cerebellar cortex (Miale and Sidman, 1961). In this second stage of differentiation, Purkinje cells are multipolar, possessing numerous somatic processes (Ramon y Cajal, 1911). In the final phase of their differentiation, coincident with migration of cerebellar granule cells from the external germinal layer into the internal granular layer during the second and third postnatal weeks, the elaborate dendritic arbor that is the anatomical hallmark of mature cerebellar Purkinje cells is formed. It is during this time that Purkinje cells undergo extensive synaptogenesis with their presynaptic excitatory partners, the cerebellar granule neurons (Herndon et al., 1981).

Examination of Purkinje cell differentiation in the absence of cerebellar granule neurons has demonstrated that Purkinje cells are formed in the absence of granule cells, but that the final stage of Purkinje cell development is critically dependent upon granule cell interaction. Neurologic mutant mouse strains in which granule cells fail to differentiate and descend into the internal granule layer exhibit gross abnormalities of the Purkinje cell dendritic arbor, and fail to develop the characteristic spiny branchlets that characterize normal Purkinje cells (Sidman, 1968; Hirano and Dembitzer, 1973; Rakic and Sidman, 1973a,b; Sotelo, 1975; Bradley and Berry, 1978). Destruction of the devel-

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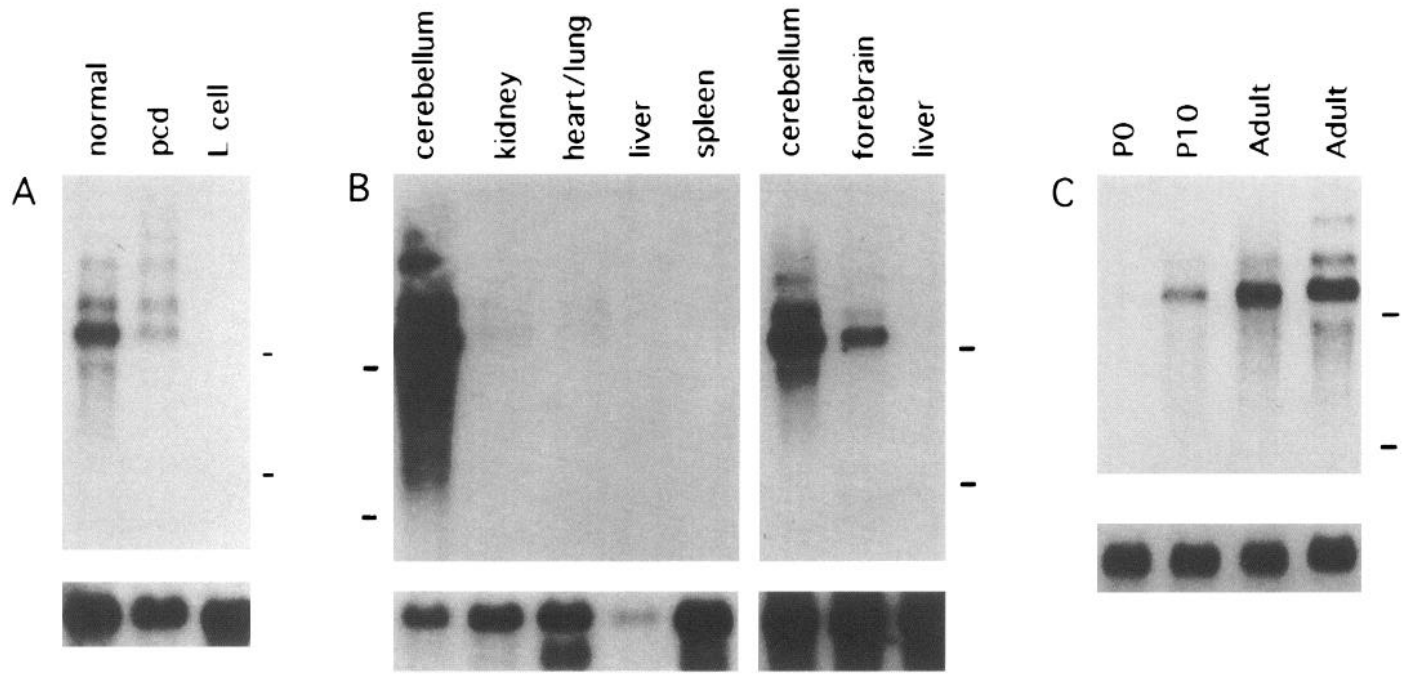


Figure 1. *A*, Northern blot analysis of 5 μg each of total adult cerebellar RNA, adult *pcd/pcd* RNA, and L-cell RNA with the pcd8 probe (*top*) or an actin probe (*bottom*). *Tick marks* indicate the positions of the 28S and 18S ribosomal RNAs. *B*, Northern blot analysis of adult mouse tissues. Two micrograms of polyA$^+$ RNA from adult cerebellum, kidney, heart/lung, liver, spleen, cerebellum, forebrain, and liver were analyzed by Northern blot with the 940 bp (8118) probe. The blot was then stripped and reprobed with an actin probe (*bottom*). *Tick marks* indicate the position of the 28S and 18S ribosomal RNAs.*C*, Northern blot analysis of mouse cerebellum at different postnatal ages. Two micrograms of polyA$^+$ RNA from mouse cerebellum at P0, P10, and adult and 3 μg of polyA$^+$ RNA from adult mouse cerebellum (*far right lane*) were analyzed by Northern blot using the 940 bp clone (8118) as a probe (*top*). The blot was then stripped and reprobed with GAPDH (*bottom*). The *tick marks* indicate the positions of the 28S and 18S ribosomal RNAs.

oping external germinal layer by x-irradiation (Berry and Bradley, 1976), viral infection (Herndon et al., 1971a,b), or cytotoxic drug treatment (Shimada and Langman, 1970; Woodward et al., 1975) results in stunted and malformed Purkinje cell dendritic arbors and the absence of spiny branchlets. Recent experiments in which purified embryonic Purkinje cell precursors were cultured in the presence or absence of cerebellar granule cells also demonstrate that development of mature Purkinje cell morphology, in particular the presence of dendritic spines that are indistinguishable from those present *in vivo*, occurs *in vitro* only in the presence of differentiating cerebellar granule cells (C. Baptista, M. E. Hatten, and C. Mason, unpublished observations). Since dendritic spines are the sites of synapse formation and specialization between the Purkinje cell dendritic arbor and the granule cell parallel fibers, one might surmise that epigenetic mechanisms that control the final stages of Purkinje cell differentiation are critical for proper construction of mature cerebellar circuitry.

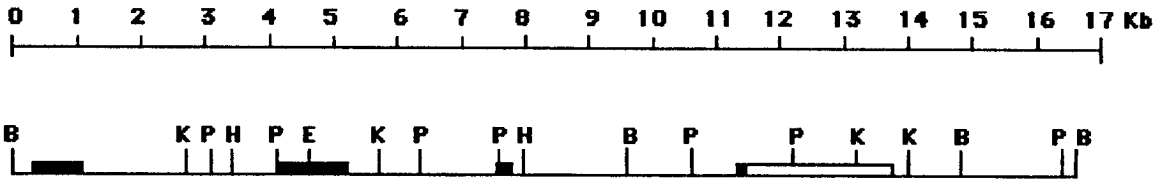
One approach toward definition of the precise molecular mechanisms underlying the epigenetic regulation of this stage of Purkinje cell differentiation is the biochemical and molecular genetic analysis of gene expression. Thus, dissection of the signal transduction pathways that control the cell type and temporal specificity of expression of genes activated in the terminal stages of Purkinje cell differentiation can reveal mechanisms critical for these events in Purkinje cells that may also play important roles in other neuronal cell types. Furthermore, the identification of genes whose expression is limited to terminally differentiated Purkinje cells implicates their cognate gene products in mature Purkinje cell function. In this study, we report the identification of a novel potassium channel cDNA whose mRNA is expressed specifically in Purkinje cells and in deep cerebellar nuclei. The temporal pattern of expression of this gene in developing cerebellar cortex, and the precise distribution of the mRNA across the Purkinje cell population suggest that its expression may be responsive to the epigenetic mechanisms that regulate the timing of Purkinje cell terminal differentiation. Cell-specific and temporally correct expression of this K$^+$ channel cDNA is observed in mixed dissociated cerebellar cell cultures. These results strongly support a role for this K$^+$ channel in mature Purkinje cell function, and demonstrate that expression of this gene in primary cell culture may be useful for definition of mechanisms regulating Purkinje cell terminal differentiation.

Materials and Methods

Mice. pcd mice B6C3Fe-a/a-*pcd* and their littermates were purchased from The Jackson Laboratory, Bar Harbor, Maine. All other mice were bred and housed at the Laboratory Animal Research Center at the Rockefeller University.

mRNA preparation. Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). PolyA$^+$ RNA was prepared using an oligo-dT cellulose (Bethesda Research Laboratories, Gaithersburg, MD) column (Aviv and Leder, 1972). When total RNA instead of mRNA was used for Northern analysis, total RNA was isolated by LiCl precipitation (Cathala, 1983).

Northern analysis. RNAs were run in 1% agarose, 1 × MOPS buffer, 1.1% formaldehyde gels and visualized by ethidium bromide staining. The gels were transferred overnight onto Gene Screen Plus membranes (Dupont, Inc.). Prehybridization for a minimum of half an hour and hybridization overnight were at 42°C in 50% formamide, 1 M sodium chloride, 1% SDS, and 100 μg/ml boiled sheared salmon sperm DNA. Probe synthesis was done by random hexanucleotide labeling (Feinberg and Vogelstein, 1983); 1 × 10^6 cpm/ml of boiled probe was added in the hybridization buffer. Blots were washed to a final stringency of either

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Gln Gln Gln Gln Cys Ser Gln Pro Gly Thr Gly Pro Ser Pro Gly Val Pro Ala Phe Leu Arg Pro Gly Gly Arg Arg Ala Glu Pro Cys Pro Gly Leu Pro Ala Val Ala Met Gly Arg

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CAT TTA CAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA A  5089
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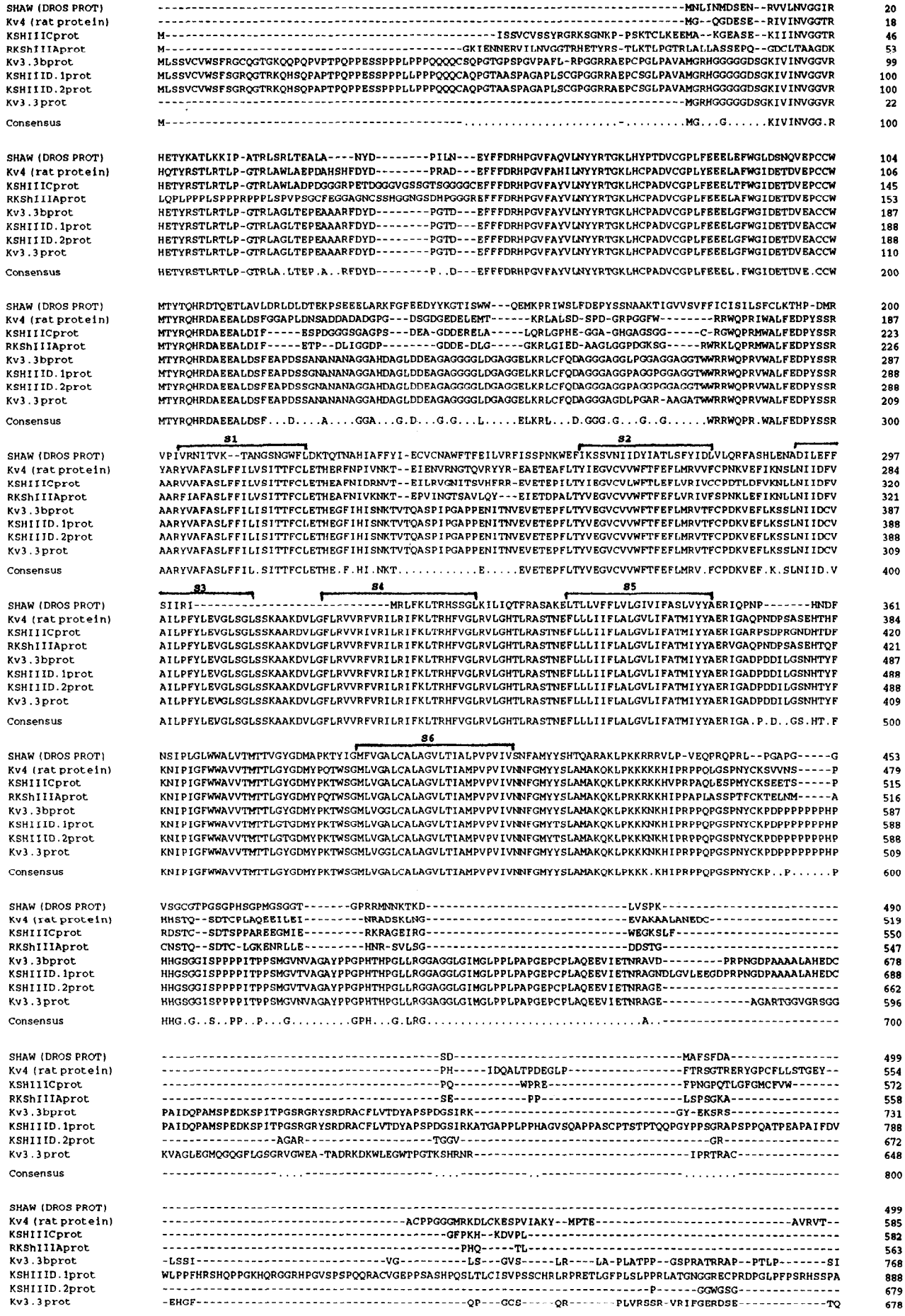
Figure 2. Genomic map of Kv3.3 gene. The restriction map was derived from the part of a cosmid that contained the Kv3.3 gene. The *boxes* indicate the location of the exons from our cDNAs. The 5′ end of exon one was derived from the published sequence (Ghanshani et al., 1992) and from genomic sequencing. The deduced Kv3.3b cDNA sequence is shown. The *arrows* indicate splice sites. The *solid boxed regions* indicate the coding portions of the cDNA.


S1
S2
S3
S4
S5
S6

2× saline–sodium citrate (SSC), 1% SDS or 0.2× SSC, 0.1% SDS at 65°C and exposed onto Kodak XAR-5 film at −70°C with an intensifying screen.

cDNA subtraction. Three micrograms of polyA+ RNA isolated from normal mouse cerebellum were transcribed into ^{32}P-dCTP–labeled cDNA (Riboclone cDNA synthesis kit from Promega, Madison, WI). This cDNA was subjected to two rounds of subtractive hybridization. It was hybridized for 40 hr in a small volume at 65°C in a 1:10 ratio with photobiotinylated L-cell mRNA (Sive and St. John, 1988). Photobiotinylation was performed with photoprobe biotin according to manufacturer's instructions (Vector Laboratories, Burlingame, CA). The cDNA/mRNA hybrids were removed by the addition of streptavidin and organic extraction, and the remaining RNA was hydrolyzed. Approximately 40% of the cDNA remained after the first round of subtraction. The subtraction was repeated using biotinylated mRNA from adult *pcd/pcd* mice. Less than 10% of the starting cDNA was recovered. Second-strand cDNA synthesis was performed. EcoRI adaptors were ligated to double-stranded cDNA, phosphorylated, and size selected on a CL4B (Sigma) column (Ausubel et al., 1987). The cDNAs had an average size of less than 500 base pairs (bp). cDNAs were ligated to λGT10 (Huynh et al., 1985) and packaged *in vitro* following manufacturer's instructions (Gigapack II Gold packaging extract from Stratagene, La Jolla, CA). A total of 3.4×10^4 plaques were obtained. cDNA insert sizes were determined by polymerase chain reaction (PCR) analysis. Approximately 20% of the resulting plaques had insert sizes >100 bp.

cDNA insert size determination. Phage were eluted from isolated plaques in 100 μl of suspension medium (SM) for several hours at room temperature. Two microliters of the eluant were used in a 100 μl PCR reaction with oligo primers that flanked the λGT10 cloning site. The sizes of the PCR products were determined by gel electrophoresis on agarose gels.

Normal cDNA libraries. Total RNA was subjected to two rounds of oligo-dT (Bethesda Research Laboratories, Gaithersburg, MD) selection. Double-stranded cDNA was prepared using an oligo-dT NotI primer-adaptor (Promega) and the Riboclone cDNA synthesis kit (Promega) according to manufacturer's instructions. Hemiphosphorylated EcoRI adaptors (Promega) were ligated onto the cDNA. The cDNA was digested with NotI, kinased, and size selected over a CL4B (Sigma) column (Ausubel et al., 1987). The cDNA was ligated to the dephosphorylated EcoRI/NotI-digested arms of the λgt11 SfiI-NotI vector and packaged *in vitro* (Gigapack II Gold packaging extract from Stratagene, La Jolla, CA); 1.5×10^6 individual clones were obtained. The average insert size was 1.5 kilobases (kb), and 9% contained no inserts. The library was amplified and stored at 4°C.

A second cDNA library was made as above but without NotI digestion. It was ligated into the EcoRI site of λGT10 and packaged *in vitro* (Gigapack II Gold packaging extract from Stratagene, La Jolla, CA).

A 5′ cDNA library was made by priming mRNA with the oligo CGCCAGCTGGCTCCTCCAAGCCC to make double-stranded cDNA using the Riboclone cDNA synthesis kit (Promega). EcoRI adaptors (Promega) were attached to the ends, and the cDNA was size selected on a CL4B (Sigma) column (Ausubel et al., 1987). A fraction of the cDNA was cloned into dephosphorylated EcoRI-digested Bluescript KS(+) vector (Stratagene, La Jolla, CA). The DNA was transformed into frozen competent XL1-Blue cells (Stratagene, La Jolla, CA).

Cosmid library. The genomic library was a C57B/6 genomic library (Stratagene, La Jolla, CA) in the pWE 15 vector. Screening was done according to manufacturer's instructions.

In situ *hybridization of tissue slices.* Dissected brains from normal adult mice were embedded in OCT (Miles, Inc., Elkhart, IN) and frozen at −70°C. Ten-micron serial sagittal sections were cut on a cryostat and mounted on subbed slides. They were fixed in 4% paraformaldehyde, 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature, washed in PBS twice for 10 min each, dehydrated through an ethanol gradient, and stored at −20°C.

For the P6, P8, P10, and P12 slides, sections were prepared as previously described (Kuhar et al., 1993).

Riboprobe synthesis using digoxigenin-11-UTP (Boehringer Mannheim) and *in situ* hybridization were as previously described (Kuhar et al., 1993).

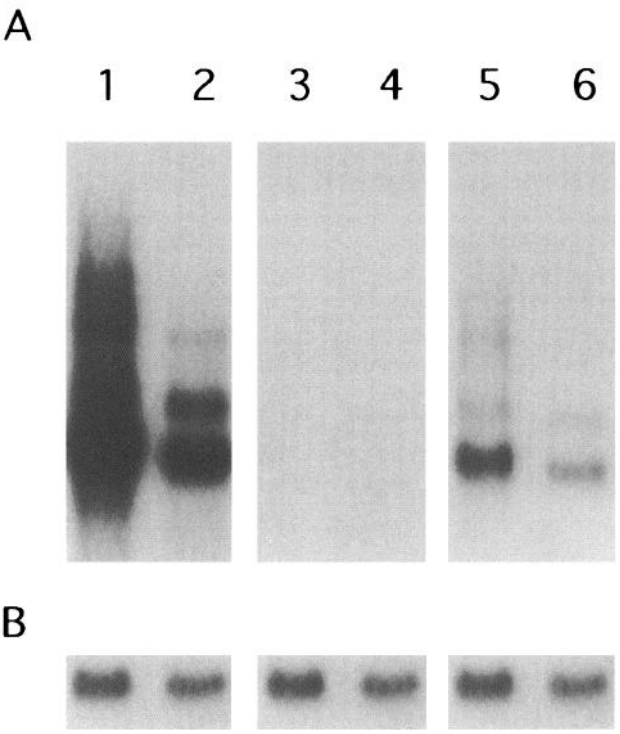


Figure 4. Northern blot analysis of the 3′ ends of the Kv3.3 cDNAs. *Lanes 1, 3,* and *5* contain 2 μg of polyA+ RNA from cerebella of normal adult mice. *Lanes 2, 4,* and *6* contain 2 μg of polyA+ RNA from cerebella of *pcd/pcd* mice. In *A, lanes 1* and *2* were probed with the 940 bp (8118) fragment, *lanes 3* and *4* were probed with the PCR fragment generated from the 3′ end of the Ghanshani message using the primers GAGGCTGGGGCCAGGACT and CACCTGGGTTTCTGAATC, and *lanes 5* and *6* were probed with the PCR fragment generated from the 3′ end of our B3.1 clone using the primers GACCCCCGTCCCA-ATGGA and TGGGCTCTTGTCTTCTG. In *B*, the same Northern strips that were used in *A* were probed with an actin control.

Cerebellar cell culture. Cultures were prepared from late embryonic (E18) mice using methods previously used to dissociate cerebellar cells (Hatten and Sidman, 1978; Trenkner and Sidman, 1981; Hatten, 1985; Baird et al., 1992). Timed-pregnant female mice were deeply anesthetized with ketamine/xylazine, and embryos were removed. Brains were dissected from the embryos. Cerebella were obtained by dissection and stripped of meninges under chilled calcium/magnesium-free phosphate-buffered saline (Tyrode's solution, PBS). Cerebella were immersed in calcium-free PBS containing trypsin (Worthington; 1%) and DNase (Worthington; 1%) for 3 min at 20°C. Trypsin/DNA solution was replaced with Eagle's basal medium (BME) supplemented with 0.33% glucose containing DNase (0.05%), and cerebella were dissociated by trituration in three Pasteur pipettes fire polished to progressively finer bores. The resulting cell suspension was pelleted with brief centrifugation and resuspended in serum-containing medium. Clumps of cells were removed by passing the suspension through a 33 μm nylon mesh (Tetco, Inc., Elmsford, NY) in a syringe-mounted filter holder. The cell concentration of the suspension was determined using a hemocytometer and adjusted to 8×10^5 cells/ml; 0.25 ml of the suspension was plated into each well of 16-well Lab-Tek chamber slides (Nunc) coated with poly-D-lysine hydrobromide (Sigma; MW > 300,000; 0.5 mg/ml), for a plating density of 2×10^5 cells/well (4000 cells/mm^2). Cultures were incubated at 35.5°C in 5% CO_2. Two hours after plating, cells had adhered sufficiently to replace the serum-containing medium with serum-free medium. Neuron survival is increased and glial proliferation is decreased in serum-free medium (Baptista, Blazeski, Hatten, and

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Figure 3. Comparison of the amino and carboxyl termini of our cDNA with other published *Shaw* channel carboxyl termini (Butler et al., 1990; McCormack et al., 1990; Wei et al., 1990; Luneau et al., 1991).

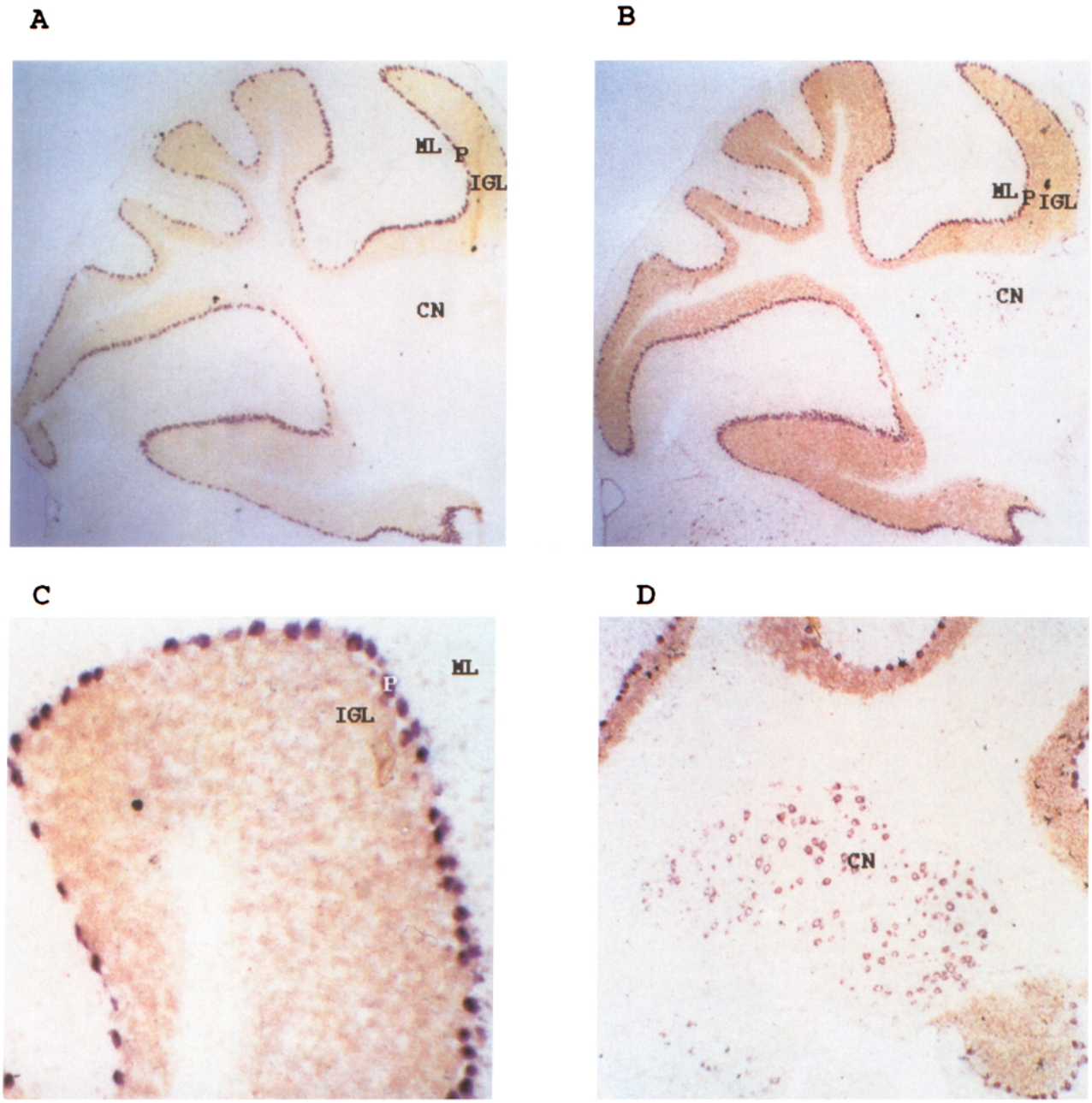

Figure 5. *In situ* hybridization analysis of adult mouse brain sections. *A* shows hybridization with the antisense calbindin probe. *B–D* show hybridization with the antisense 8118 probe. *IGL*, internal granule layer; *P*, Purkinje cell layer; *ML*, molecular layer; *CN*, deep cerebellar nuclei.

Mason, unpublished observations). Cultures were incubated 7, 14, and 21 d before fixation.

Serum-containing medium for cerebellar cell culture (Baird et al., 1992) consisted of Eagle's basal medium with Earle's salts (GIBCO) supplemented with 10% horse serum (GIBCO), 2 mM L-glutamine, glucose (final concentration 32 mM), and penicillin/streptomycin (GIBCO; 20 U/ml each).

Serum-free medium suitable for long-term culture of Purkinje cells (Baptista, Blazeski, Hatten, and Mason, unpublished observations) was used, consisting of Eagle's basal medium with Earle's salts (GIBCO) supplemented with insulin (10 μg/ml), transferrin (Sigma; 100μg/ml), sodium selenite (Sigma; 30 nM) putrescine (Sigma; 100 μM), progesterone (Sigma; 20 nM), 2 mM L-glutamine, glucose (final concentration of 32 mM), and penicillin/streptomycin (GIBCO; 20 U/ml each).

In situ *hybridization of cultured cells.* Cultures to be hybridized were fixed in 4% paraformaldehyde in Sorensen's phosphate buffer for 30 min at 20°C. Cultures were washed three times in sterile PBS treated with 0.1% diethyl pyrocarbonate (Sigma) and stored overnight at 4°C. PBS was then replaced with 70% ethanol (Groslin and Banker, 1991) to preserve RNA and reduce microbial growth until the day of hybridization, when they were returned to PBS. Cultures were washed three times in PBS immediately before prehybridization treatment. The digoxigenin method for nonisotopic *in situ* hybridization to sections (Kuhar et al., 1993; present results) was used for cultured cells, with the following minor modifications. Tops and sides of the Lab-Tek chamber slides were removed, leaving the gasket, which forms a well to contain solutions for hybridization. Proteinase K treatment was reduced to 5 min to minimize the removal of cells from the slides. Cultures were not air dried preceding application of probe to prevent shrinkage and associated damage to cell cultures. After dehydrating in an ethanol series to 100% ethanol, cultures were returned to 2× SSC via the same ethanol series; 2× SSC was removed, and 15 μl of probe was applied per well

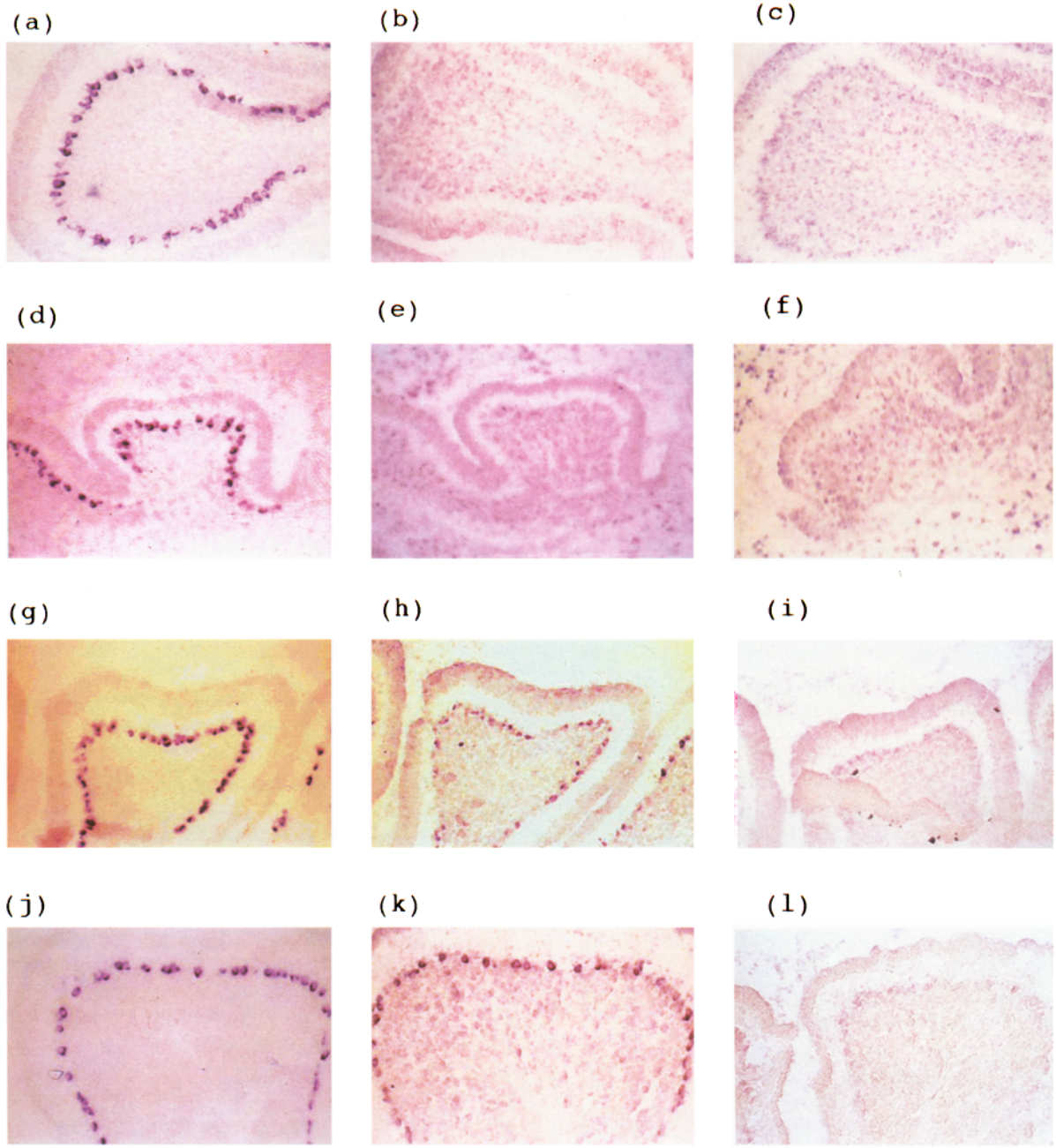

Figure 6. *In vivo* time course of Kv3.3b expression in P6, P8, P10, and P12 mouse cerebellar sections. *a–c* are P6 cerebellar sections, *d–f* are P8 cerebellar sections, *g–i* are P10 cerebellar sections, and *j–l* are P12 cerebellar sections. *a, d, g, and j* are stained with a calbindin antisense probe to identify the Purkinje cells. *b, e, h, and k* are stained with the 8118 antisense probe to identify Kv3.3b-positive cells. *c, f, i, and l* are control slides stained with the 8118 sense probe. Expression of the Kv3.3b gene is visible by P10 in a subset of the Purkinje cells (*h*). By P12, virtually all of the Purkinje cells are expressing the potassium channel (*k*).

at a final dilution of 1:400 in hybridization buffer. No coverslips were applied. Cultures were hybridized 12–16 hr at 55°C in a humid, sealed container. The remainder of the procedure was similar to that described for sections, except that cultures were immunolabeled before the alkaline phosphatase reaction to label hybridization.

Combining in situ *hybridization with immunolabeling in a single culture.* Antiserum raised in rabbit against calbindin-D_{28K} (1:1000; gift of Dr. Sylvia Christakos) was applied together with alkaline phosphatase–conjugated anti-digoxigenin antibody raised in sheep (Boehringer Mannheim; 1:500); cultures were incubated in a humid chamber at 4°C for 16–22 hr. Cultures were then washed three times, 10 min each wash, in Tris-buffered saline (TBS) with 0.1 M Tris, 150 mM NaCl, pH 7.5. Rhodamine-conjugated anti-rabbit secondary antibody was applied (1:100) for 30 min at 20°C in a humid chamber. Cultures were washed three times, 10 min per wash, followed by a 10 min wash in alkaline phosphatase reaction buffer (TBS with 100 mM NaCl, 50 mM MgCl, pH 9.5). Alkaline phosphate substrate solution (0.33 mg/ml 4-nitro blue tetrazolium chloride (NBT); 0.165 mg/ml 5-bromo-4-chloro-2-indoyl-phosphate (BCIP); 0.24 mg/ml levamisole) was applied, and incubated in a humid chamber for 60 min. The reaction was stopped by immersion in 10 mM Tris; 1 mM EDTA, pH 8.0. Gaskets forming wells were removed from chamber slides, and slides were dipped in distilled water. Slides were then coverslipped with Gelmount (Biomeda), an aqueous mountant. Cultures were photographed using a microscope equipped

with a 25× phase objective. Fluorescence photomicrographs using rhodamine optics were taken to record calbindin-D_{28K} immunolabeling, and hybridization photographed using bright-field optics.

Results

Preparation of Purkinje cell–enriched cDNA library

To prepare a cDNA library enriched in genes expressed in Purkinje cells or whose expression was dependent upon the presence of functioning and viable Purkinje cells, we took advantage of the existence of Purkinje cell degeneration (*pcd*) mice. Adult *pcd* mice have lost greater than 99% of their Purkinje cells without a corresponding loss of other cerebellar cell types (Mullen et al., 1976). Thus, we employed a two-step subtractive hybridization procedure to generate a cDNA library enriched for Purkinje cell–specific cDNA clones. The first step involved hybridization against a fivefold excess of mouse L-cell mRNA to eliminate abundant housekeeping genes. The second round of subtraction was against a fivefold excess of *pcd/pcd* mouse cerebellar mRNA. This second round of hybridization/subtraction eliminated brain cDNAs that were not Purkinje cell specific or whose expression was Purkinje cell independent. Through this double-step hybridization/subtraction, we hoped to enrich for Purkinje cell cDNAs without requiring that they encode very abundant mRNAs or that their expression be absolutely restricted to Purkinje cells.

Northern blot analysis of 10 randomly picked cDNAs from the resulting subtracted cDNA library revealed five clones that were expressed equally in normal cerebellum, *pcd* cerebellum, and L-cells: one cDNA that was expressed at equal levels in normal and *pcd* cerebella but not in L-cells, three cDNAs that were expressed at undetectable levels by Northern analysis, and one clone (pcd8) that was expressed at elevated levels in normal versus *pcd* cerebella and was undetectable in L-cells (data not shown). Although we do not yet have definitive data on the three clones expressed at undetectable levels, we believe that they encode rare Purkinje cell–specific messages whose expression can best be analyzed by *in situ* hybridization. From this limited sample of the cDNA library, it appears that the subtraction procedure effectively enriched for Purkinje cell cDNAs (they represent 10–40% of the population).

Tissue-specific expression

The original 258 bp cDNA fragment (pcd8) isolated in this screen was sequenced and no significant homology was found to any sequences in the GenBank or EMBL databases. This fragment was then used to isolate a 940 bp clone from an oligo-dT–primed normal adult cerebellar cDNA library. This new fragment (8118) was also not represented in the databases. This larger cDNA fragment was used to examine the expression of its cognate mRNA, both to confirm and extend the initial results obtained with the original 258 bp fragment (data not shown). As shown in Figure 1*A*, Northern blot analysis of mRNA isolated from normal adult cerebellum reveals a major transcript of 5.5 kb, and less abundant transcripts of 6.5, 9.0, and 12 kb. Comparison with the transcripts evident in mRNA prepared from adult *pcd* cerebellum demonstrates that the 5.5 kb transcript is significantly depressed relative to the minor bands in the mutant mRNA. None of these transcripts are present in mRNA prepared from mouse L-cells. These results indicated that the 8118 cDNA encoded a transcript whose expression may be restricted to Purkinje cells, and that the 8118 gene is either alternatively spliced or a member of a gene family.

To determine whether the 8118 transcript is specifically expressed in brain, Northern blot analysis was used to assay its abundance in several other tissues in adult mouse. As shown in Figure 1*B*, mRNAs hybridizing to the 8118 probe are expressed at very high levels in adult cerebellum and at lower levels in forebrain, but are not present at significant levels in mRNA isolated from several other tissues.

Developmental regulation

Since our particular interest is to isolate cDNA clones that mark the final stages of Purkinje cell differentiation, expression of the 8118 mRNA during development was analyzed by Northern blot (Fig. 1*C*). No expression of the 8118 mRNA was detected in cerebellar RNA isolated in the first postnatal day (P0). Low levels are evident in RNA prepared at P10, and these levels increase and become maximal in adult cerebellum. Thus, the expression of this clone is positively developmentally regulated with a time course that parallels the time course of the terminal differentiation of Purkinje cells.

Identification of a novel potassium channel cDNA

Since the 940 bp fragment did not contain an open reading frame, and was not homologous to sequences in the GenBank or EMBL databases (Pearson and Lipman, 1988), additional cDNAs were isolated to identify the proteins expressed from this gene. A 1.8 kb cDNA fragment (5-2) was isolated that upon sequencing was found to contain more 3′ sequence but no additional 5′ sequence. This new sequence was still not represented in the databases and, as expected, it also did not contain an open reading frame. To obtain 5′ sequence that would contain coding sequences, a cDNA library primed with a 24-mer approximately 400 bp away from the 5′ end of the 940 bp fragment was prepared. Using the 940 bp fragment as a probe, we identified a clone (B3.1) containing a 1.6 kb insert. This 1.6 kb insert contained an open reading frame that upon sequencing was found to be a novel cDNA from the previously reported Kv3.3 *Shaw* type potassium channel gene (Pearson and Lipman, 1988; Ghanshani et al., 1992). Figure 2 shows the full cDNA sequence of this novel channel. Comparison of this sequence with the published Kv3.3 sequence reveals that the first 1744 nucleotides of the KV3.3 DNA sequence are present in our clone. However, the sequence we have determined contains an additional 231 nucleotides at the 5′ end because we noticed an in-frame ATG initiation codon upstream from that reported for Kv3.3. In addition, after nucleotide 1744 of Kv3.3 the two sequences diverge completely. The remaining 3111 nucleotides of the transcript we have identified are not present in the predicted Kv3.3 mRNA and result in a deduced K^+ channel protein that is different from the reported Kv3.3 protein for the C-terminal 110 amino acids. The amino acids coded for after the splice site and the additional amino acids at the 5′ end are highly homologous to the analogous region of KShIIID.1, a previously cloned rat *Shaw* channel (Vega-Saenz de Miera et al., 1992).

Since the KShIIID1 (Vega-Saenz de Miera et al., 1992) and Kv3.1 (Luneau et al., 1991) genes are alternatively spliced at precisely the position at which the cDNA we have isolated, now referred to as Kv3.3b, and the predicted Kv3.3 mRNA diverge, it had been suggested that alternatively spliced Kv3.3 transcripts might also exist (Ghanshani et al., 1992). Therefore, to determine whether our cDNA encoded a new member of this family or an alternatively spliced version of the Kv3.3, we isolated and

characterized genomic clones using the unique Kv3.3b 3′ sequences. A genomic map of the portion of a cosmid containing the Kv3.3 gene is shown in Figure 2. This genomic clone overlaps that previously isolated and identified as the Kv3.3 gene, proving that the Kv3.3b transcript is encoded by that locus. The structure of the Kv3.3b mRNA was mapped onto the genomic clones revealing two additional 3′ exons in the cDNA that together encode the carboxyl-terminal 110 amino acids and ~3.0 kb of 3′ untranslated mRNA (Fig. 2). As shown in Figure 3, the deduced protein from the Kv3.3b mRNA is very closely related to other *Shaw* type potassium channels. The Kv3.3b specific C-terminal region is unique, although significant homology between Kv3.3b and the rat KShIIID.1 and Kv4 proteins is evident.

Taken together, our results and those reported by Ghanshani et al. (1992) suggest that the Kv3.3 gene is alternatively spliced, leading to K^+ channels with different C-terminal domains. Since this has important implications for both the function and the regulation of expression of this gene, and because the 3′ terminus of the Kv3.3 mRNA was deduced from the genomic clone rather than an expressed cDNA, we sought to determine whether the predicted Kv3.3 mRNA was expressed. Thus, unique 3′ probes were prepared from the Kv3.3 and Kv3.3b sequences and hybridized to Northern blots containing adult cerebellar mRNA from adult normal and *pcd* mice. As shown in Figure 4, the Kv3.3b-specific probe hybridized to the same transcripts seen using the larger Kv3.3b-specific 8118 cDNA. No transcripts hybridizing to the Kv3.3-specific 3′ probe are evident even though the specific activity and size of this probe are equivalent to the Kv3.3b-specific probe. In fact, both probes detect single bands on genomic Southern blots, proving that the Kv3.3-specific probe should detect an mRNA if it were expressed (data not shown). At extremely long exposures, the Kv3.3-specific probe does detect very faintly the upper two bands on Northerns. We believe these faint signals come from RNAs that are not yet fully processed. From these data, we conclude that the previously reported Kv3.3 deduced mRNA is not expressed in cerebellum, although it remains possible that the Kv3.3 gene is alternatively spliced in other ways to generate transcripts containing as yet undetected 3′ sequences.

Cell-specific expression

The pattern of expression of the Kv3.3b mRNA as assayed by Northern blot analysis demonstrated very high levels of expression in cerebellar cortex, and suggested that this gene might be specifically expressed in Purkinje cells. To determine whether this is the case, the 8118 cDNA was used to localize the expression of the Kv3.3b transcripts by *in situ* hybridization to adult mouse cerebellar sections. As shown in Figure 5, the Kv3.3b transcript is expressed at high levels in Purkinje cells (Fig. 5*B–D*). Direct comparison with the expression of calbindin mRNA (Fig. 5*A*), whose expression is known to be restricted to Purkinje cells, suggests that this mRNA is also restricted to Purkinje cells in the cerebellar cortex, but that it is also present in the deep cerebellar nuclei (Fig. 5*D*).

Time course of expression in vivo *and in primary cell culture*

To define more precisely the time of expression of the Kv3.3b transcript in cerebellar Purkinje cells, we examined its expression by *in situ* hybridization to a series of slides prepared at different ages. As demonstrated in Figure 6, expression of the Kv3.3b mRNA begins between P8 and P10, and it is present in virtually all of the Purkinje cells by P12. Inspection of the data obtained from P10 (Fig. 6*g–i*) clearly indicates that the expression of this mRNA is not temporally synchronized in all Purkinje cells in a given folium. Thus, adjacent sagittal sections hybridized to calbindin versus Kv3.3b probes demonstrates that at P10 only some of the Purkinje cells are positive for expression of this K^+ channel. This suggests that the epigenetic mechanisms controlling the final stages of Purkinje cell differentiation may be triggered by interactions that impinge upon single Purkinje cells. Since previous studies have demonstrated the regulation of K^+ channel gene expression in hippocampus as a result of seizure (Tsaur et al., 1992), it is possible that this heterogeneity in the onset of Kv3.3b mRNA in Purkinje cells is related to developmental variations in the acquisition of mature Purkinje cell synapses with cerebellar granule cells during the second postnatal week. Alternatively, the biochemical heterogeneity of Purkinje cells (Hawkes et al., 1985; Ingram et al., 1985; Koh et al., 1989; Stainier and Gilbert, 1989; Brochu et al., 1990; Pioro and Cuello, 1990; Sotelo and Wassef, 1991) may in some way influence the precise temporal pattern of expression of this ion channel.

To provide an avenue for delineation of interactions regulating Kv3.3b expression and its subsequent contribution to mature Purkinje cell function, we were interested in determining whether the cell type specificity and developmental regulation of this gene are maintained in dissociated primary cultures of mouse cerebellum. To examine this question, cells were isolated from late embryonic mice (E18) as previously described (Hatten and Sidman, 1978; Trenkner and Sidman, 1981; Hatten, 1985; Baird et al., 1992). Mixed populations of cerebellar cells were then cultured for various periods, and analyzed using double staining by immunocytochemistry for calbindin to identify Purkinje cells and *in situ* hybridization for Kv3.3b mRNA to determine the time course of its expression. Since Purkinje cells become postmitotic at approximately E12 *in vivo* (Uzman, 1960; Miale and Sidman, 1961) and can be identified by calbindin staining shortly thereafter (Shamley et al., 1992), all cultures contained readily identifiable Purkinje neurons (Fig. 7*b,e,h,k*). Expression of the Kv3.3b mRNA in culture follows its *in vivo* time course: it is not present in E18 Purkinje cells cultured for 1 week, but it is expressed in virtually all of the calbindin-positive cells after 2 and 3 weeks in culture (Fig. 7*c,f,i,l*). These results indicate that the timing of expression of this gene is quite faithfully maintained in primary cerebellar cell culture. Inspection of the phase-contrast and calbindin-stained cultures further reveals clearly identifiable Purkinje cell neurites after 1 week in culture in cells that do not express this K^+ channel, suggesting that its expression is not simply dependent on neurite extension, but correlates with subsequent morphologic maturation of Purkinje cells that has been previously documented in these cultures (Cohen-Cory et al., 1991; Baptista, Blazeski, Hatten, and Mason, unpublished observations).

Discussion

To elucidate the role of epigenetic molecular mechanisms in Purkinje cell differentiation, we sought to isolate markers for the later stages of Purkinje cell development by employing a two-step hybridization/subtraction technique that yielded a cDNA library enriched in Purkinje cell clones. Our approach led to the identification of a developmentally regulated novel

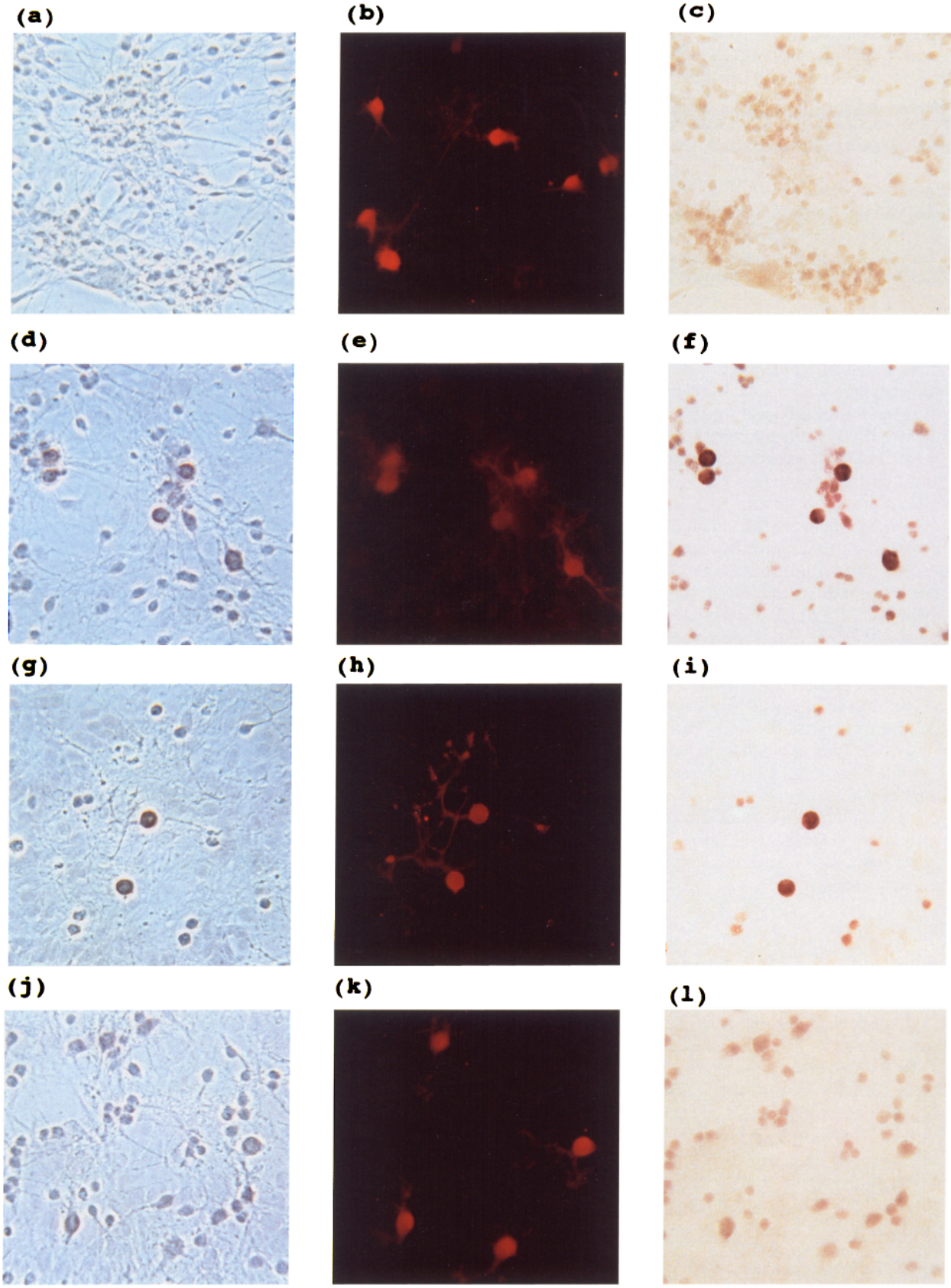

Figure 7. Specific expression of the Kv3.3b gene in Purkinje cells from E18 mouse embryos after 14 d *in vitro*, combining nonisotopic *in situ* hybridization with immunolabeling for calbindin-D_{28K}, a Purkinje cell marker. *a–c* are taken after 7 d *in vitro*. Little or no potassium channel expression is detected in dissociated cerebellar cells using the 8118 antisense probe (*c*) although many cells with neuronal and non-neuronal morphology are visible in phase optics (*a*). Purkinje cells are identified by immunolabeling for calbindin-D_{28K} (*b*) and have not attained a mature

Shaw type potassium channel cDNA that is expressed in Purkinje cells and deep nuclei in the cerebellum.

The *Shaw* type potassium channels are a subfamily of delayed-rectifier potassium channels believed to be important in setting the resting membrane potential (Salkoff et al., 1992) and for repolarization of the membrane potential during an action potential and thus are important modulators of action potential duration (Hille, 1992). Specific expression of the Kv3.3b channel cDNA in mature Purkinje cells may be essential in the development of mature physiologic function in these neurons. Since expression of this K^+ channel is properly regulated in mixed cultures of cerebellar neurons, it will be of interest to correlate the expression of this gene with Purkinje cell physiologic parameters using the *in vitro* culture system. One might expect, for example, that expression of the Kv3.3b gene in mature Purkinje cells could alter their excitability and, perhaps, synaptic function.

Characterization of Kv3.3b cDNAs and mapping onto the genomic clone establish that the deduced mRNA reported in the original study of the Kv3.3 gene (Ghanshani et al., 1992) is probably not correct. Thus, the 3′ sequences thought to comprise part of the Kv3.3 mRNA, based on their colinearity with partial cDNAs in the Kv3.3 genomic clones and on studies of alternative splicing of the Kv3.1 gene (Luneau et al., 1991; Ghanshani et al., 1992), are not expressed as mature mRNA in cerebellum (Fig. 2). However, our experiments do not rule out alternative splicing as a mechanism for generating K^+ channel diversity from the Kv3.3 gene. Rather, we believe from characterization of additional Kv3.3 mRNAs that alternative transcripts arise from differential splicing of the fourth Kv3.3 exon. Further studies will be required to define these alternative transcripts precisely and establish their functional consequences. This seems particularly important given the widespread occurrence of alternative splicing in K^+ channel genes and the potential for very subtle regulation of neuronal physiology resulting from the increased complexity of K^+ channels arising as a result of alternative splicing (Jan and Jan, 1990; Salkoff et al., 1992).

The timing of expression of Kv3.3b mRNA in Purkinje cells coincides with the final stage of their differentiation. Kv3.3b mRNA is first detected between P8 and P10, as granule cells migrate from the external germinal layer to the internal granule layer where they terminally differentiate. It is during this time that Purkinje cells establish synapses with granule cell parallel fibers. Taken together with the aberrant morphology of Purkinje cells that develop in agranular cerebellar cortex (see introductory remarks), it seems probable that expression of this gene is regulated by epigenetic mechanisms involving interactions between Purkinje cells and granule cells. Definition of sequences regulating Kv3.3b mRNA expression in Purkinje cells, and the cognate transcription factors involved in this regulation, will provide an important avenue toward characterization of signal transduction pathways regulated by granule cell–Purkinje cell interactions.

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morphology. *d–f* are taken after 14 d *in vitro*. High levels of expression are detected in a subset of cells with neuronal morphology (compare *d* and *f*). The expressing cells are calbindin positive (compare *e* and *f*) and have morphology characteristic of developing Purkinje cells (*e*). *g–i* are taken after 21 d *in vitro*. High levels of expression are maintained in a subset of cells with neuronal morphology (compare *g* and *i*). The expressing cells are calbindin-positive (compare *h* and *i*) and have features typical of mature Purkinje cells, including a highly branched dendritic arbor (*h*). *j–l* are controls taken after 14 d *in vitro*. Many cells with neuronal and non-neuronal morphology are visible in phase (*j*), including calbindin-positive cells (*k*), but little or no hybridization is detected using the sense 8118 probe (*l*).

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