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Molecular cloning of a neuron-specific transcript and its regulation during normal and aberrant cerebellar development

(neuropeptide/PEP-19/Purkinje cell/mutants/gene expression)

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ABSTRACT **PEP-19 is a brain-specific polypeptide whose levels increase dramatically during the late maturation of the rodent nervous system. By using immunocytochemistry, PEP-19 is shown to be localized to several regions of the central nervous system, notably cerebellum, thalamus caudate putamen, and olfactory bulb. We have isolated a 0.5-kilobase cDNA clone that encodes the entire PEP-19 protein sequence, making this one of the smallest primary transcripts and translation products ever identified in eukaryotes. The cDNA was used to investigate the developmental expression of PEP-19 in rodent brain. PEP-19 mRNA rises from low levels at embryonic day 17 of gestation in the rat to a plateau value by day 18 postpartum. This mirrored the levels of the protein determined by radioimmunoassay. Since the rise coincided with the formation of synaptic contacts onto Purkinje cells (a major site of PEP-19 expression), the hypothesis was tested that the activity and/or presence of afferent input modulated PEP-19 expression. Parallel fiber innervation was disrupted either by killing granule cells with the cytostatic agent methylazoxymethanol or by examining PEP-19 levels in cerebellar granuloprival mutant mice (reeler and weaver). The influence of climbing fiber input was assessed by either eliminating them with 3-acetylpyridine or stimulating them with harmaline in both neonatal and mature rats. None of the above altered PEP-19 gene expression in cerebellum, leading us to propose that the signals triggering the PEP-19 gene do not emanate from granule cells or neurons in the olivary nucleus. However, preliminary evidence suggests that PEP-19 is under posttranscriptional regulation.**

A general quest in neurobiology is to elucidate the cellular and molecular mechanisms that concert the orderly assembly and maturation of the nervous system. One approach to this problem is to investigate the transcriptional regulation of defined, neuron-specific, genes in situations where the normal developmental program of the cells can be perturbed. To identify such specific gene products, we have used HPLC technology to construct a polypeptide database from rat cerebellum at various stages of maturation (1, 2). The cerebellum was selected for a number of technical reasons (1), predominant among these being that rodent cerebellar development can be impaired by both chemical and genetic means (3–5). This provides the opportunity to investigate the expression of identified gene products in circumstances where specific input pathways or neuronal populations have been eliminated.

In an initial study a polypeptide, termed PEP-19 in the database, was identified as being developmentally regulated in rat cerebellum (2). The molecule was isolated and sequenced and found to be a 7.6-kDa polypeptide with limited structural homology to several calcium binding proteins (2). Furthermore, biochemical evidence suggested that PEP-19 was unique to the nervous system (2). This study establishes the nature of the developmental regulation of PEP-19 by analyzing the levels of the polypeptide and its mRNA in the cerebella of normal and neurodevelopmentally mutant rodents. To perform such analyses we have isolated a recombinant cDNA clone encoding PEP-19.†

MATERIALS AND METHODS

cDNA Cloning and Sequencing of PEP-19. An adult rat brain λgt11 library (Clontech) consisting of 1.2×10^6 independent clones with 0.15- to 1.5-kilobase (kb) insert size was screened with the polyclonal antibody according to the procedure of Young and Davis (6). The second antibody used in the detection system was a goat anti-rabbit IgG horseradish peroxidase conjugate followed by a color reaction with 4-chloro-1-napththol and H_2O_2. Eighteen positive clones were picked from ≈300,000 plaques. The one with the longest insert, clone 20 b.2, was subcloned into pBR322 and then rescued by cutting with *Eco*RI. By restricting the insert with appropriate enzymes, fragments were obtained and sequenced by the method of Maxam and Gilbert (7).

Isolation of DNA and RNA. Phage minilysates were prepared by the method of Helms *et al.* (8). Large-scale phage and plasmid DNA was prepared by using standard procedures (9). Total RNA was prepared from various tissues and cell lines with 3 M LiCl/8 M urea as described by Auffray and Rougeon (10).

Northern Blots. Northern blot analyses of RNA were performed on 0.8% agarose/formaldehyde gels. DNA probes were either nick-translated or random prime labeled with [^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham). After the electrophoretic run the gels were blotted onto nitrocellulose filters, which were then baked in a vacuum oven at 80°C for 1 hr. They were prehybridized for 4 hr at 42°C in 5× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate), 5× Denhardt's reagent (1× Denhardt's reagent = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 50% formamide, and 250 μg of salmon sperm DNA per ml and hybridized with 5× SSC, 5× Denhardt's reagent, 50% formamide, 250 μg of salmon sperm DNA per ml, 10% dextran sulfate, and 1×10^6 cpm of ^{32}P-labeled probe per ml at 42°C for 18 hr as described (11). At the end of hybridization the filter was washed three times with 2× SSC/0.1% SDS at room temperature and 0.1× SSC/0.1% SDS at 50°C for 15–30 min. The filter was then exposed to Kodak XAR-5 film at −70°C with an intensifying screen. For all experiments 10 μg of total RNA per lane was used. In the case of mutant mice, which exhibit cerebellar atrophy, this may cause an inadvertant enrichment of certain mRNAs. In the context of the

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Abbreviations: 3-AP, 3-acetylpyridine; P, postpartum day; E, embryonic day; MAM, methylazoxymethanol acetate.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M24852).

experiments reported here this would lead to an overestimation of the abundance of PEP-19 in weaver and reeler. Unlike the granule cells, the Purkinje cells do not represent a significant proportion of the cerebellar mass and their selective loss does not result in overt skewing of the RNA ratios.

Animals and Tissues. Sprague–Dawley-derived CD rats were purchased from Charles River Breeding Laboratories. Mutant mice and their sex-matched littermate controls (25–35 days old) were obtained from The Jackson Laboratory. The specific strains were as follows: weaver (*wv*), B6CBA-$A^{w\text{-}J}$/A-wv; reeler (*rl*), B6C3-a/a-rl; staggerer (*sg*), B6C3-A/A-sg; Purkinje cell degeneration (*pcd*), C57BL/6J-pcd; nervous (*nr*), C3HeB/FeJ-nr; Lurcher (*Lc*), B6CBA-$A^{w\text{-}J/A}$-LcT7Ca Mi^{wh}. Cerebella and cerebral cortices were removed from mice, immediately frozen in liquid nitrogen, and stored at −70°C until used.

3-Acetylpyridine (3-AP) and Harmaline Treatment. Male rats weighing 200–250 g were given a single intraperitoneal injection of 3-AP (Sigma) at a dose of 65 mg/kg. The rats, both control and test, were fed with a special diet because of the motor impairment that follows the 3-AP injection. The diet comprised powdered Rat Chow mixed with sweetened condensed milk. The tremors accompanied by lack of coordination and impaired locomotion continued until the rats were sacrificed 3 days and 3 weeks after treatment (4, 12). Tissues were processed as described above. In a second series of experiments 2-day-old rat pups were given a single dose of 3-AP (65 mg/kg). Animals were sacrificed at postpartum days (P) 4, 9, and 16 for assessment of cerebellar PEP-19 mRNA.

A single intraperitoneal injection of harmaline (Sigma) at a dose of 15 mg/kg was given to male rats weighing about 150 g (13). Symptoms of fine tremors were apparent. The animals were sacrificed 3 days after the injection.

Methylazoxymethanol Acetate (MAM) Treatment. Newborn rats at P1 and P2 were injected with MAM at 20 mg/kg and 15 mg/kg, respectively, in phosphate-buffered saline (5). Symptoms of motor impairment were apparent in the surviving rats. Depending on the severity of the symptoms the rats were classified as either severely affected or moderately affected.

Immunocytochemistry. Rats were anesthetized and perfused via the ascending aorta with 4% paraformaldehyde as described by Friedrich and Mugnaini (14). After fixing and sectioning, the floating sections were allowed to react with the PEP-19 antiserum by the peroxidase-antiperoxidase procedure of Sternberger (15). Control sections were also incubated with preimmune serum or antiserum absorbed with a 10-fold molar excess of the synthetic peptide used as immunogen (16).

Radioimmunoassay (RIA). Rats of the indicated ages were sacrificed, their cerebella were removed and homogenized in a RIA buffer, and their PEP-19 content was determined by a specific RIA (16). The antiserum used for RIA and immunocytochemistry has been previously characterized with regard to its specificity (16).

RESULTS

By using a sequence-directed rabbit polyclonal antiserum to PEP-19 (2, 16), immunoreactivity for PEP-19 was observed in cells throughout the rat brain (Fig. 1). In particular, forebrain regions including the granular layer of the olfactory bulb (Fig. 1*a*) and pyriform cortex (Fig. 1*b*) had intense staining for PEP-19. There was also substantial immunoreactivity in the thalamus as well as the hippocampus and caudate putamen (Fig. 1 *c* and *d*). The cerebellum, however, contained the greatest amount of PEP-19-like immunoreactivity, which was associated predominantly with Purkinje cells (Fig. 1) (16).

The sequence-directed antiserum to PEP-19 was used to screen a rat brain λgt11 library (for details see *Materials and Methods*). The positive clone with the largest insert (20 b.2) was sequenced and found to contain the entire coding region of PEP-19 (Fig. 2). Furthermore, the deduced amino acid sequence of 20 b.2 was in perfect agreement with that previously determined for the PEP-19 protein by Edman degradation (2). The 20 b.2 cDNA contained, in addition to the coding region, 43 nucleotides 5′ to the start codon and 273

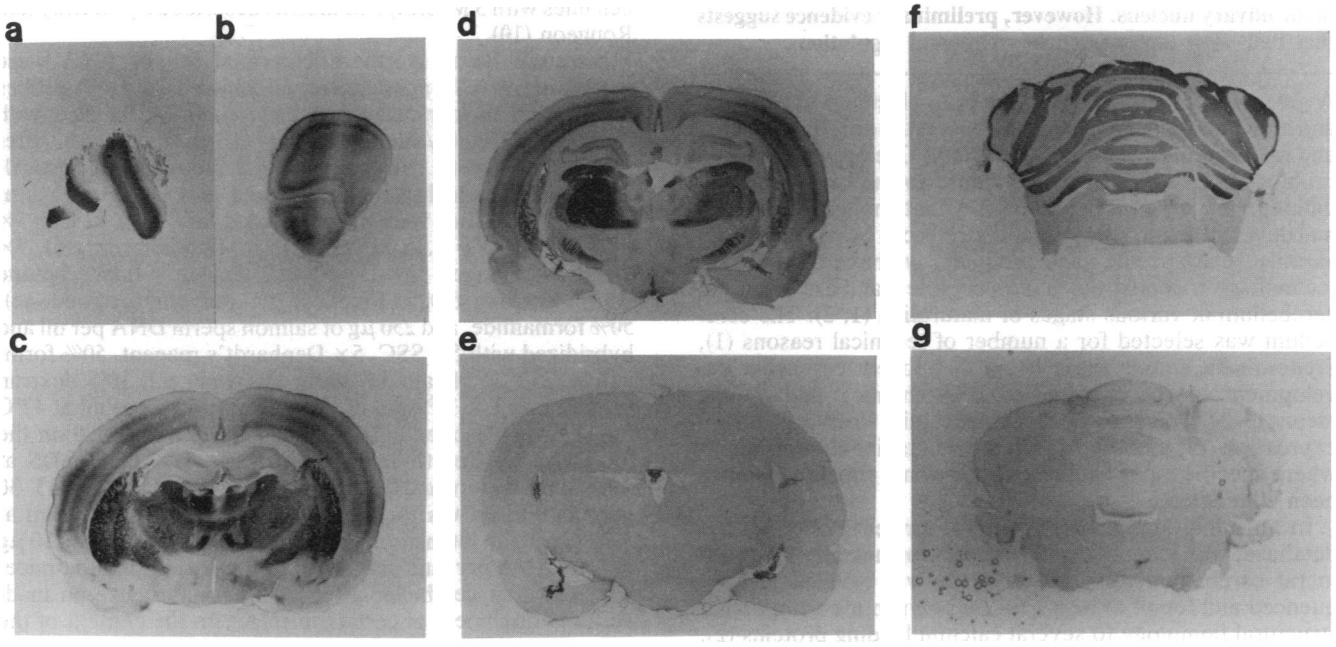

FIG. 1. Distribution of PEP-19-like immunoreactivity in adult rat brain. The sections in *a–d* and *f* represent progressively more caudal coronal sections of rat brain immunostained for PEP-19. Immunoreactivity can be observed in the granular layer of the olfactory bulb (*a*), the piriform cortex (*b*), numerous thalamic nuclei and caudate putamen (*c*), cortex and hippocampus (*c* and *d*), and cerebellum (*f*). Preimmune serum (*e*) does not stain an adjacent section to *d*. Furthermore, absorption of the antiserum with an excess of PEP-19 ablates staining of the cerebellum (*g*). (*a* and *b*, ×7.2; *c–g*, ×3.6.)

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                                                                    50
TTCGGAACTGTTCTGTGGGACCAAGCTGCGGAGTCAGGCCAACATG AGT GAG AGA CAA AGT GCT GGA GCA
                                                   S   E   R   Q   S   A   G   A

                                                100
ACC AAT GGA AAA GAC AAG ACA TCA GGA GAT AAT GAT GGG CAG AAG AAG GTC CAA GAA GAA TTT GAT
 T   N   G   K   D   K   T   S   G   D   N   D   G   Q   K   K   V   Q   E   E   F   D

                150                                                             200
ATC GAC ATG GAT GCA CCA GAG ACA GAG CGT GCA GCT GTG GCC ATT CAG TCT CAG TTC AGA AAA TTC
 I   D   M   D   A   P   E   T   E   R   A   A   V   A   I   Q   S   Q   F   R   K   F

                                                            250
CAG AAG AAA AAG GCA GGA TCA CAG TCC TAGTGGGGAAGCTGCCTTCCTTGTTCACCCGAAGACACCAAGT
 Q   K   K   K   A   G   S   Q   S

                                        300
TCAACCACCGTCCATCCAGAAATGAGAAGAACAATACCCTAGAGCAAAGTCATCCACACCCAGTACACACTCCGC

 350                                                            400
TGCTAACCTGAAATGCATGAACAGAAACCCATAGTATTTATGCCCCTCTAGGCAGGTGTCCACAATAAAATTGTGA

                                    450                                         500
GCAGCTTAATCTGTCTGTCTCCATTACAATTCCTCTGCAACGATTTTCCTCGATGTTGTAATAAAAAGGAGGTAAGA

GCCGA
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FIG. 2. Nucleotide sequence of the cDNA clone, 20 b.2, encoding PEP-19. The predicted amino acid sequence is written below the nucleotides. Start and stop codons are indicated by boldface. There are consensus AATAAA, poly(A) sequences in the 3′ untranslated region at positions 411–416 and 483–488.

nucleotides 3′ to the stop codon in the single open reading frame.

Having confirmed that 20 b.2 encoded the cDNA for PEP-19, this insert was used to assess PEP-19 mRNA levels by Northern transfer and hybridization. The cDNA hybridized to a single mRNA species in total RNA from cerebellum of ≈800 bases (Fig. 3). Since 20 b.2 comprises only 505 bases (Fig. 2), the clone is not full length, and as no poly(A) tail is present, initiation must have occurred at an internal A-rich site. However, there are two consensus poly(A) signals present in the 3′ untranslated region of the mRNA at nucleotides 411–416 and 483–488.

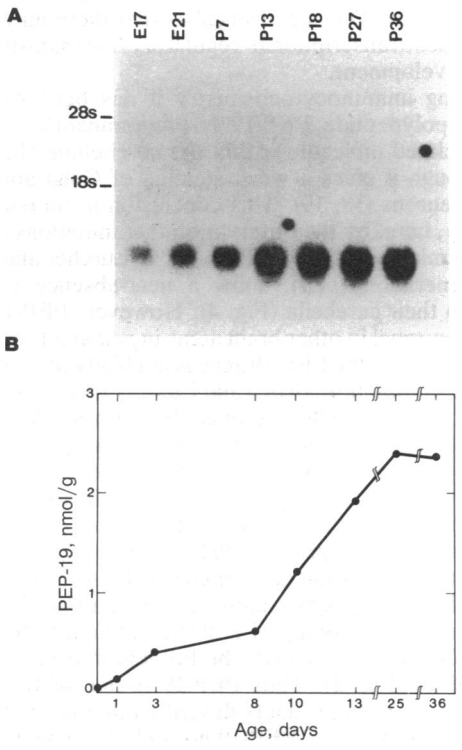

FIG. 3. Developmental expression of PEP-19 mRNA (*A*) and peptide (*B*) in developing rat brain. (*A*) Ten micrograms of total RNA from embryonic days (E) 17 and 21 and P7, P13, P18, P27, and P36 were used for RNA analysis. (*B*) PEP-19 levels determined by a specific RIA (16) in rat cerebellum. Each point in the curve represents the arithmetic mean of three replicate determinations.

20 b.2 hybridized to a single species of mRNA in total RNA isolated from rat cerebellum, cerebral cortex, thalamus/hypothalamus, and brainstem (data not shown). The relative hybridization signals in the various rat brain regions were cerebellum = 1.0; cerebral cortex = 0.79; thalamus/hypothalamus = 0.67; brainstem = 0.30; retina and spinal cord = 0.04. No signal was detected in liver, kidney, lung, spleen, thymus, testis, or adrenal (data not shown). PEP-19 mRNA was also undetectable in the PC12 and B104 cell lines (data not shown).

A previous study established that PEP-19 was developmentally regulated in rat cerebellum, the polypeptide appearing at around birth and rising to adult levels by the third postnatal week (2). This has been confirmed and extended here by using a specific RIA for PEP-19 (Fig. 3). The added sensitivity of this assay enabled PEP-19 to be detected at birth and in addition revealed two phases of increase of the polypeptide postnatally. As also shown in Fig. 3, a similar pattern was determined for PEP-19 mRNA levels in rat brain. In fact the message for PEP-19 was detectable as early as E17 in rat, well before the peptide can be measured by HPLC or RIA. The levels of mRNA subsequently rise to maximum values by P18, mirroring the results obtained by HPLC (Fig. 3) (2).

Having established that the alterations in PEP-19 levels in rat brain during development are attributable, at least in part, to transcriptional activation of the PEP-19 gene, the question next arose as to the mechanism of this induction. Our initial studies have attempted to address the possibility that synaptic contacts regulate the expression of PEP-19.

The Purkinje cell receives a number of inputs (3). Two major sets of inputs, one from climbing fibers emanating from the inferior olivary nucleus and the other from parallel fibers of granule cells (17), may be experimentally manipulated. To eliminate parallel fiber input two strategies were employed. First, granule cell division (and thereby number) was attenuated by two consecutive injections of the cytostatic agent MAM. However, even in cerebella of severely affected rats the levels of PEP-19 mRNA remained comparable to normal values at all stages of investigation (data not shown). Since granule cell depletion is incomplete following MAM (5), a second strategy was employed. The expression of PEP-19 in rat and mouse cerebellum is essentially identical (16, 18, 19), although no extensive developmental analysis has been performed. Thus, PEP-19 levels were determined in mice having genetically determined cerebellar anomalies. Mutations that

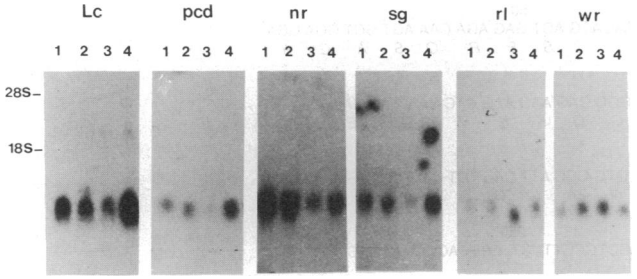

FIG. 4. PEP-19 mRNA in neurologic mutant mice. Brains were dissected from Lurcher (*Lc*), Purkinje cell degeneration (*pcd*), nervous (*nr*), staggerer (*sg*), reeler (*rl*), weaver (*wv*), and their heterozygotes as controls. Ten micrograms of total RNA from a pool of five cerebella or cortices was loaded in each lane. The experiment was repeated three times with the same results and a representative example is given here. Lanes 1, cortex from mutant mouse; lanes 2, cortex from control; lanes 3, cerebellum from mutant mouse; lanes 4, cerebellum from control.

result in a loss of granule cells [reeler and weaver (20, 21)] have essentially normal levels of PEP-19 in both their cerebella and cerebral cortices (Fig. 4). This is despite the marked diminution in granule cell numbers in both strains of mice and a maximal loss of 50% of Purkinje cells. It should be borne in mind that we may be slightly overestimating PEP-19 levels in weaver and reeler, which may account for the near-normal levels despite the loss of some Purkinje cells in these mutants. In contrast, mutations that cause a postnatal loss of Purkinje cells (Lurcher, Purkinje cell degeneration, and nervous) showed substantial deficits in PEP-19 mRNA levels (Fig. 4). In addition, the staggerer mouse, which has effects upon both

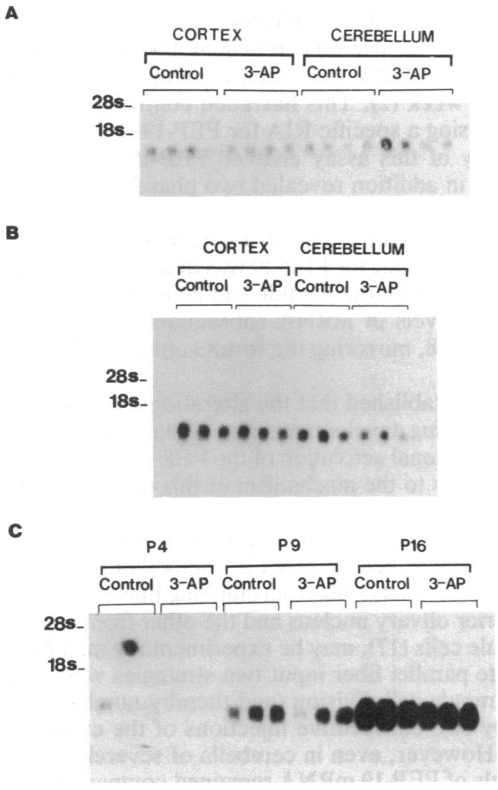

FIG. 5. Effect of climbing fiber ablation on levels of PEP-19 mRNA. Adult rats were given a single dose of 3-AP at 65 mg/kg. Cerebral cortices and cerebella were removed 3 days (*A*) and 3 weeks (*B*) following the injection. In neonatal rats (*C*) brains were removed on P4, P9, and P16 following a single dose of 3-AP on day 2. Each experimental group contained three animals. Only the cerebella were analyzed in *C*. Ten micrograms of total RNA was loaded in each lane.

granule and Purkinje cell survival (22), also shows a loss of PEP-19 (Fig. 4).

Two experiments were performed to determine whether climbing fibers might regulate PEP-19 expression. First, climbing fiber innervation was eliminated at two stages of development (day 2 after birth and in the adult) by treating the rats with 3-AP, a neurotoxin that kills neurons in the inferior olivary nucleus (4, 12). This procedure, though causing marked neurological deficits in the treated rats, had no significant effect upon PEP-19 mRNA levels in either adults or neonates (Fig. 5). Thus, elimination of climbing fibers during their initial period of contact with Purkinje cells as well as after establishment of synaptic contact results neither in an acute nor chronic loss of PEP-19 mRNA. The contrary experiment was to stimulate neurons of the olivary nucleus and thereby Purkinje cells (via climbing fibers) with harmaline (13). The drug produced a pronounced tremor in rats but also had no effect upon PEP-19 mRNA levels (not shown).

DISCUSSION

PEP-19 is a protein specific to certain neuronal populations that include rat and mouse cerebellar Purkinje cells (16, 18, 19). At present no one common phenotype links these various groups of neurons; however, the most intensely PEP-19-positive neurons utilize γ-aminobutyric acid as their neurotransmitter. In most brain regions PEP-19 mRNA levels are in rough agreement with their respective protein levels as measured by histochemistry (Fig. 1) and RIA (16). However, in cerebral cortex, the mRNA is much more abundant than the protein. This may indicate that in the cortex the turnover rate of the peptide is higher than in other brain regions or that the mRNA is under a posttranscriptional regulatory mechanism. The latter might be achieved by altered RNA stability or translation. In the developmental analysis, PEP-19 mRNA was also detected 3–4 days prior to the appearance of the protein (Fig. 3). This again suggests that there may be some form of posttranscriptional regulatory mechanism at play during development.

By using immunocytochemistry it has been established that the polypeptide PEP-19 is predominantly a Purkinje cell-associated molecule within the cerebellum (Fig. 1) (16, 18), although it gives a weak staining of Golgi and basket/stellate neurons (18, 19). This conclusion is supported independently, here, by the observation that mutations that completely eliminate Purkinje cells, such as Lurcher and Purkinje cell degeneration (*pcd*), show a near-absence of PEP-19 mRNA in their cerebella (Fig. 4). However, PEP-19 mRNA levels are normal in other brain areas in *pcd* and Lurcher (Fig. 4), indicating that the PEP-19 gene is not likely to represent the loci of either mutation. In *pcd* and Lurcher there is an essential absence of Purkinje cells, although PEP-19 mRNA and protein are still detectable, albeit at low levels. It is most likely that this material emanates from Golgi, stellate, and basket neurons, which are known to be PEP-19 positive in the *pcd* mouse following the degeneration of Purkinje cells (19).

In the initial description of PEP-19 it was suggested, based upon *in vitro* translation of cerebellar poly(A)$^+$ RNA, that the polypeptide could not be significantly larger than 7.6 kDa (2). As shown by the cloning of a cDNA encoding PEP-19, the 7.6-kDa molecule is, in fact, the full-length primary translation product (Fig. 2). Thus PEP-19 is one of the smallest primary translation products described in eukaryotes.

As noted previously, PEP-19 not only has some sequence homology with a number of calcium binding proteins but also has a putative EF-hand structure (2, 16), suggesting that it might be a calcium binding protein. If PEP-19 is a cytoplasmic calcium binding protein, then it is an example of one that is neuron-specific. Cerebellar Purkinje cells do contain very high levels of several calcium binding proteins (23) and the

relatively high concentration of PEP-19 in these neurons (>10 μM) would be consistent with this property. A further feature of structure apparent from this analysis is the presence of a canonical sequence for a cyclic AMP-dependent protein kinase phosphorylation site in PEP-19 (residues 3–5). Whether PEP-19 exists in a phosphorylated form is unknown at present.

The main goal of this study was to investigate the developmental regulation of PEP-19 in the cerebellum. The developmental expression of the PEP-19 protein begins after birth (Fig. 3), with levels rising sharply in the postpartum period to reach stable adult values at day 18 (2). Since Purkinje cells develop a dense dendritic arbor during the same time course, it is not possible to state unequivocally that the increase in polypeptide concentration is a result of transcriptional activation rather than a reservoir effect. However, the Northern hybridization data presented here show that there is a concomitant accumulation of PEP-19 mRNA during this period, indicating that PEP-19 gene expression is indeed regulated. The period of maximum acquisition of PEP-19 protein (2) and mRNA (Fig. 3) coincides with the main phase of cellular development in the cerebellum—namely, the formation of the internal granular layer, the laying down of parallel fiber synapses onto Purkinje cells, and morphological and electrophysiological maturation of Purkinje cells (24–26). This spurred us to consider the possibility that parallel fiber synapses onto Purkinje cells might regulate PEP-19 expression. Two mutants that have deficits in granule cells (reeler and weaver) have normal levels of PEP-19 mRNA (Fig. 4). In reeler, in particular, this is a telling result inasmuch as most Purkinje cells are ectopic in the cerebellum of this mutant and never receive correct innervation (27). The situation in weaver is less clear since synapses can form onto Purkinje cells although most are subsequently lost (28). These results would indicate that parallel fiber synapses do not play a significant role in regulating PEP-19 expression. Perhaps the most definitive mutant would be staggerer, whose Purkinje cells are unable to form synapses. However, although these mice lose granule cells as a result of the mutation, they also lose 60–90% of Purkinje cells; thus the observed decrease in PEP-19 mRNA levels could simply be a reflection of Purkinje cell loss as they are in Lurcher, nervous, and *pcd*.

Elimination of climbing fibers by 3-AP treatment does not result in a decrement in PEP-19 mRNA. This would lead us to conclude that afferent input from this system is not a major contributor to the regulation of PEP-19 gene expression, leaving us to consider two further alternatives. The first would be that PEP-19 is induced by a signal intrinsic to the Purkinje cell. The second possibility would be that neuronal elements other than those described here, such as the basket cells or noradrenergic inputs, provide the signal. In addition, we cannot exclude the possibility that nonneuronal cells such as Bergman glia are involved in this process.

Macroneurons in the mammalian brain (such as Purkinje cells) are generated relatively early in neurogenesis and undergo terminal division and migration before the birth of microneurons (such as cerebellar granule cells) (29). Thus, it could be argued that the macroneurons, like radial glia, constitute the primary cellular framework of the brain. In this role, they may subserve a guidance or trophic function for later-arriving neurons or glia. As such it may be necessary for the macroneurons to execute their developmental program largely independent of other cellular contacts or inputs and, perhaps explicitly, later developing cellular elements. In this sense one might predict that gene expression in Purkinje cells, such as that of PEP-19, may be largely independent of the presence of granule cells. Indeed, several proteins have been identified in Purkinje cells that do not respond to the elimination of granule cells (30, 31). We would postulate that the genes for PEP-19, as well as those encoding the proteins showing similar developmental characteristics in Purkinje cells, might have common consensus sequences in their promoter/enhancer elements that confer this specificity of expression. The availability of a cDNA to PEP-19 will now permit the isolation of genomic clones to begin the analysis of such promoter regions and ultimately to define the nuclear proteins interacting with them.

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