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Expression of the mitotic motor protein CHO1/MKLP1 in postmitotic neurons

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Abstract

The kinesin-related motor protein CHO1/MKLP1 was initially thought to be expressed only in mitotic cells, where it presumably transports oppositely oriented microtubules relative to one another in the spindle mid-zone. We have recently shown that CHO1/MKLP1 is also expressed in cultured neuronal cells, where it is enriched in developing dendrites [Sharp *et al.* (1997a) *J. Cell Biol.*, **138**, 833–843]. The putative function of CHO1/MKLP1 in these postmitotic cells is to intercalate minus-end-distal microtubules among oppositely oriented microtubules within developing dendrites, thereby establishing their non-uniform microtubule polarity pattern. Here we used *in situ* hybridization to determine whether CHO1/MKLP1 is expressed in a variety of rodent neurons both *in vivo* and *in vitro*. These analyses revealed that CHO1/MKLP1 is expressed within various neuronal populations of the brain including those in the cerebral cortex, hippocampus, olfactory bulb and cerebellum. The messenger ribonucleic acid (mRNA) levels are high within these neurons well after the completion of their terminal mitotic division and throughout the development of their dendrites. After this, the levels decrease and are relatively low within the adult brain. Parallel analyses on developing hippocampal neurons in culture indicate that the levels of expression increase dramatically just prior to dendritic development, and then decrease somewhat after the dendrites have differentiated. Dorsal root ganglion neurons, which generate axons but not dendrites, express significantly lower levels of mRNA for CHO1/MKLP1 than hippocampal or sympathetic neurons. These results are consistent with the proposed role of CHO1/MKLP1 in establishing the dendritic microtubule array.

Introduction

Neurons are terminally postmitotic cells that use their microtubule arrays for the formation of axons and dendrites rather than for cell division. As such, neurons do not form mitotic spindles, but instead generate highly organized microtubule arrays within these processes. Microtubules in the axon are uniformly oriented with their plus-ends distal to the cell body (Heidemann *et al*., 1981), whereas microtubules in the dendrite have a mixed or non-uniform polarity orientation (Baas *et al*., 1988). These distinct microtubule polarity patterns are essential for directing different complements of cytoplasmic constituents into each type of process (Baas *et al*., 1988), and are also important for establishing morphological features that distinguish axons and dendrites from one another (Sharp *et al*., 1997b). How are the microtubule arrays of axons and dendrites established?

Although progress on this issue has been slow, significant advancements have been made in recent years on the means by which dividing cells organize their microtubules into a bipolar mitotic spindle. Specifically, it appears that microtubule-based motor proteins move microtubules together to form the spindle and then apart to separate the half-spindles (see for example Heald *et al*., 1996; Barton & Goldstein, 1996; Moore & Endow, 1996). These mitotic motors include certain kinesin-related proteins that are specialized not to transport organelles along microtubules, but to transport and organize microtubules themselves. There are essentially two schools of thought regarding how neurons organize their microtubules after their terminal mitotic division. One theory maintains that neurons abandon the principals underlying microtubule organization in mitotic cells. In this view, neurons cease all microtubule movements, and develop entirely novel mechanisms based on stationary microtubules (see Miller & Joshi, 1996; Hirokawa *et al*., 1997). The other theory, which we have proposed, is that neurons organize their microtubules by modifications of the same mechanisms used in mitotic cells. In this view, motor proteins transport microtubules from the cell body into axons and dendrites, thereby determining both their distribution and their patterns of polarity orientation within these processes (see Baas & Yu, 1996). If this latter theory is correct, we would expect neurons to express motor proteins, such as those utilized in the mitotic spindle, that are capable of transporting and organizing microtubules.

In recent studies, we have focused on a motor termed CHO1/MKLP1, which localizes to the mid-zone region of the mitotic spindle, where it is thought to drive oppositely oriented microtubules apart during late anaphase (Sellito & Kuriyama, 1988; Nislow *et al*., 1992). In support of this view, *in vitro* studies have established that CHO1/MKLP1 transports microtubules with their minus-ends leading toward the plus-ends of other microtubules (Nislow *et al*., 1992). Interestingly,

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this property would be ideally suited to intercalate minus-end-distal microtubules among plus-end-distal microtubules during the development of the dendrite. In fact, expression of a fragment of CHO1/MKLP1 in insect ovarian cells causes these normally rounded cells to extend dendrite-like processes with non-uniform microtubule polarity orientation (Kuriyama *et al*., 1994; Sharp *et al*., 1996). Most recently, we have shown that CHO1/MKLP1 is expressed in cultured neurons, that it localizes to dendrites, and that inhibition of its expression suppresses dendritic development (Yu *et al*., 1997; Sharp *et al*., 1997a). These results strongly suggest that CHO1/MKLP1 is used both in the mitotic spindle of dividing cells and in the developing dendrites of postmitotic neurons. If this is true, then CHO1/MKLP1 should be expressed within developing neurons *in situ*, and should show patterns of expression consistent with dendritic development. To investigate this, we have now determined the patterns of CHO1/MKLP1 expression in developing neurons of the brain, as well as in developing sympathetic, hippocampal and dorsal root ganglion neurons in culture.

Materials and methods

Animal dissection and tissue preparation

For all of the studies presented here, we used samples obtained from rodents. For the studies on CHO1/MKLP1 expression *in vivo*, we used hamsters because the clone of CHO1/MKLP1 was isolated from Chinese hamster ovary (CHO) cells (Kuriyama *et al*., 1994). We reasoned that using hamsters would optimize the signal-to-noise ratio in the *in situ* hybridization analyses. For studies on prenatal animals, pregnant hamsters were decapitated and embryos were removed by Caesarean section on E15. Postnatal studies were performed on animals at ages P4, P8, P15, P21 and adult. Embryos (E15) removed from the amniotic membrane were frozen whole in isopentane (–50 °C) and stored at –80 °C. The postnatal animals (from P4 pups to adult) were decapitated, and their brains removed and subsequently frozen in isopentane at –50 °C and stored at –80 °C until needed. Sagittal sections (10 μm) of the whole embryos (E15) and brains of P4 pups to adults were cut in a cryostat (–24 °C), mounted onto gelatin-coated slides, and then kept at –80 °C until used.

Cell cultures

For studies on cultured neurons, we obtained the neuronal tissue from rats, because cultures of rat hippocampal, sympathetic and dorsal root ganglion neurons have been well characterized, and because our previous studies showed sufficient cross-reactivity of cultured rat neurons with the hamster probe to provide a reasonable signal-to-noise ratio (Sharp *et al*., 1997a). Cultures of embryonic rat hippocampal neurons were prepared as previously described (Goslin & Banker, 1991; Sharp *et al*., 1995). Briefly, hippocampi were dissected from 18-days-old rat embryos, treated with trypsin for 15 min at 37 °C, and triturated with fire-polished Pasteur pipettes. The cells were plated at a density of 1000 cells/cm^2 onto glass coverslips coated with 1 mg/mL poly D-lysine in Minimum Essential Medium (MEM, Gibco-Bethesda Research Labs, Grand Island, NY, USA) containing 10% horse serum. After 2–4 h attachment, the coverslips plated with neurons were co-cultured into plastic tissue–culture dishes containing a monolayer of astroglial cells. The astroglial cells had been grown in medium containing MEM and 10% foetal bovine serum. One day prior to co-culture, the medium was changed to a fresh medium containing MEM, the N2 supplements described by Bottenstein (see Goslin & Banker, 1991), 0.1% ovalbumin and 0.01 mg/mL sodium pyruvate.

Cultures of sympathetic neurons from the superior cervical ganglia and cultures of sensory neurons from the dorsal root ganglia were prepared from newborn and E18 rat pups, respectively. After dissection, the ganglia were treated with 0.25% collagenase for 1 h followed by 0.25% trypsin for 45 min, and then triturated with fire-polished Pasteur pipettes into a single cell dispersion as previously described (Baas & Ahmad, 1993). Before plating the cells, the glass coverslips were coated for 3 h with 1 mg/mL poly-D-lysine, rinsed extensively, and then treated with 10 μg/mL laminin for 4 h as described by Higgins *et al*. (1991). Cells were then plated in Leibovitz's L15 medium (Sigma, St. Louis, MO) supplemented with 0.6% glucose, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% FBS (Hyclone, Logan, UT) and 100 μg/mL nerve growth factor (NGF) for 24 h. For long-term culture, the medium was replaced the next morning by N2 medium (see Baas & Ahmad, 1993) supplemented with 5% FBS and 100 ng/mL NGF. Cytosine arabinoside was added at 10 μM to reduce the proliferation of non-neuronal cells.

Probes

In vitro transcription of ^{35}S-UTP- or digoxigenin-UTP-labelled CHO1/MKLP1 riboprobes was performed from linearized pBluescript plasmids with an Ambion (Austin, TX) or Boehringer-Mannheim (Indianapolis, IN) *in vitro* transcription kit, respectively, according to each manufacturer's protocol. The sense and antisense riboprobes were prepared, respectively, from 1 kb and 815 bp hamster cDNA fragment cloned into pBluescript vector flanked by T3 and T7 promoters. The 1-kb fragment cDNA corresponds to the N-terminal region of the CHO1/MKLP1 protein. The sense riboprobes (radioactive and non-radioactive) were transcribed *in vitro* from an *Nsi*I linearized plasmid using T3 RNA polymerase (Ambion). The *Pst*I 815-bp fragment spans bases 2424–3239 of the hamster cDNA and encodes a part of the carboxy terminal region of the CHO1/MKLP1 protein (809–953) and the entire 3′ UTR (Kuriyama *et al*., 1994). This 815-bp fragment shows no homology with any known proteins including other kinesin-related proteins. The antisense riboprobes (radioactive and non-radioactive) were transcribed from a *Pst*I linearized plasmid using T3 RNA polymerase purchased from Ambion and Boehringer-Mannheim, respectively.

Northern blot analyses

Hamsters ranging from P7 to adults were killed and brains rapidly removed for RNA extraction. Total RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform extraction method as previously described by Chomczynski & Sacchi (1987). Briefly, brains were homogenized at room temperature in GuSCN solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). After the isopropanol RNA precipitation, pellets were washed with 75% ethanol and resuspended in diethylpyrocarbonate-treated water (DEPC-H_2O). Aliquots of the RNA were used for quantification by optical density scanning (210–320 nm) and the integrity of the extracted RNA was confirmed by running 2 μg total RNA in a denaturing (formaldehyde 2.2 M) agarose (1%) gel in 1 × MAE buffer (20 mM 4-morpho-linepropanesulphonic acid, pH 7.0, 8 mM sodium acetate, 1 mM EDTA, pH 8.0). For Northern blot analysis, RNA (20 μg/lane) was separated on 1% agarose formaldehyde gel, transferred onto positively charged nylon membrane (Boehringer-Mannheim), probed with the digoxigenin CHO1/MKLP1 antisense riboprobe and washed at high stringency at 68 °C with 0.1 × SSC, 0.1% SDS (w/v) as recommended in the manufacturer's protocol. Finally, the membranes were directly used for detection of the hybridized RNA by the chemiluminescent

detection of the digoxigenin-labelled probe with CDP-star, as described in the Boehringer-Mannheim procedure.

In situ *hybridization on brain sections*

Frozen sections were brought to room temperature and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, 1×) for 15 min and then rinsed in PBS, 1×. After acetylation in triethanolamine for 10 min, dehydration in 30, 50, 70, 85, 95 and 100% ethanol, and delipidation in chloroform for 5 min, the sections were prehybridized for 2 h in 4× SSC buffer containing 50% formamide, 1× Denhardt's solution, 300 μg/mL yeast RNA, 300 μg/mL salmon sperm DNA, and 100 mM dithiothreitol (DTT). The sections were hybridized with 5×10^5 c.p.m./100 μL of the antisense or sense riboprobe overnight at 50 °C. The tissue was then rinsed three times in 2 × SSC for 15 min at room temperature, treated with 20 μg/mL RNase A (Boehringer-Mannheim), and finally washed in increasingly stringent conditions up to 0.1 × SSC at 60 °C for 30 min. All rinse and wash buffers contained 0.25 g/mL sodium thiosulphate. The sections were processed for both film (hyperfilm-βmax, Amersham) and emulsion autoradiography (NTB2, Kodak), with exposure times of 10–13 days and 3–4 weeks, respectively. After development of emulsion autoradiograms, the sections were counterstained with cresyl violet and mounted with Permount.

In situ *hybridization on primary neuron cultures*

Cells that had been grown on glass coverslips were fixed for 15 min at room temperature in 4% paraformaldehyde in PBS, 1 ×, and dehydrated in graded alcohols (30, 50, 70, 85, 95 and 100%). Hybridization was carried out overnight at 50 °C with the same hybridization mixture described above using 7.5 ng/100 μL of the sense or antisense riboprobe. Following hybridization, cells were rinsed, treated with RNase and then subjected to high stringency washes as described above. The cells were then washed twice for 10 min each in Tris–HCl buffer (100 mM Tris–HCl, pH 7.4, 150 mM NaCl). After exposure for 30 min to a blocking solution containing 0.1% Triton X-100 and 2% normal sheep serum (Sigma) in Tris–HCl buffer, the cells were incubated overnight at 4 °C with sheep antidigoxigenin alkaline phosphatase antibody (Boehringer-Mannheim) diluted 1/1000 in blocking buffer. The coverslips were rinsed twice for 10 min in Tris–HCl buffer and then exposed for 10 min to colour development buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$), after which they were incubated with Tris–HCl buffer substrate solution (100 mM Tris–HCl, pH 9.5, 50 mM $MgCl_2$) containing nitro-blue tetrazolium (NBT, 340 μg/mL) and bromochloroindolylyl phosphate (BCIP, 170 μg/mL). To reduce the endogenous phosphatase activity, 5 mM levamisole was added to the colour development buffer. The colour signal was monitored by microscopy and in our initial experiments on sympathetic neurons, the reaction was stopped when a strong cellular signal was developed against a low background. For all subsequent experiments on sympathetic, hippocampal and dorsal root ganglion neurons, this same development time was used so that comparison of signal levels among these different types of neurons would be meaningful. After transferring them to buffer containing 10 mM Tris–HCl, pH 8.0 and 1 mM EDTA, the coverslips were washed twice for 10 min in distilled water, air dried and mounted in mounting aqueous solution. Cells were visualized and photographs were taken using bright-field microscopy to reveal the reddish alkaline-phosphate reaction product.

Results

The goal of this study was to determine the pattern of expression of CHO1/MKLP1 in various types of central and peripheral neurons, both *in vivo* and *in vitro*. In our previous study on cultured rat hippocampal and sympathetic neurons, we documented CHO1/MKLP1 mRNA expression at one time point, shortly after dendrites had formed (Sharp *et al*., 1997a). The presence of the protein was documented by immunofluorescence microscopy using a monoclonal antibody specific for CHO1/MKLP1. The protein was found to be concentrated in the cell body prior to dendritic development, and then also within dendrites during their development. In the present study, we focused entirely on mRNA expression because we have been unable to successfully adapt the immunodetection procedure to tissue slices.

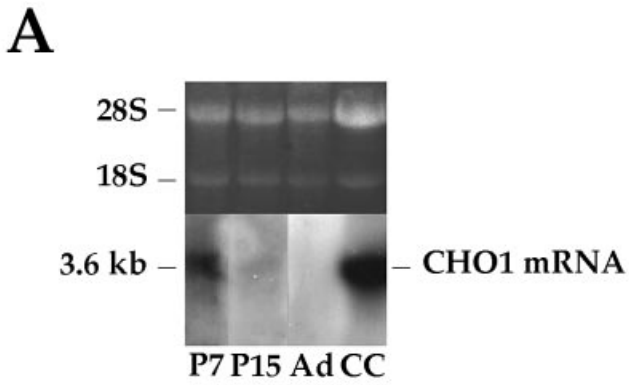
Expression of CHO1/MKLP1 in tissues of the hamster determined by Northern blot and in situ *hybridization*

The specificity of the CHO1/MKLP1 antisense riboprobe was assessed by Northern blot analyses on RNA isolated from the brains of P7, P15 and adult hamsters. As a positive control, we also examined RNA from cultured CHO cells, the cell type from which CHO1/MKLP1 cDNA was originally isolated (Kuriyama *et al*., 1994). Using the antisense CHO1/MKLP1-specific probe, we observed a single band corresponding to a 3.6 kb transcript in the P7 and P15 brains, as well as in the CHO cells (Fig. 1A). These results confirm the specificity of the probe, and demonstrate the presence of CHO1/MKLP1 mRNA in developing brains. Although equal quantities of total RNA were analysed from the P7, P15 and adult brains, the hybridization signal was much weaker in the P15 samples compared with the P7 samples, and no hybridization signal was detected in the adult samples. Thus, CHO1/MKLP1 mRNA is expressed in the hamster brain, but its expression is downregulated during development.

The remaining panels in Figure 1 show *in situ* hybridization analyses using the same riboprobe to detect CHO1/MKLP1 mRNA within an E15 hamster embryo. A slice of the whole embryo is shown in Figure 1(B). The most intense signal appears in the submandibular gland, the kidney and the gut. In the gut, the hybridization signal appears in the smooth muscle and at somewhat higher levels in the mucosal cells (see Fig. 1C,D). The transcript was also found elsewhere, e.g. in the mesenchymal cells surrounding nasal cavities and in the cells surrounding condensing cartilage. Interestingly, CHO1/MKLP1 mRNA was also strongly expressed in nervous tissue, e.g. the cerebral cortex (Fig. 1E), which is consistent with our previous work showing CHO1/MKLP1 expression in cultured neurons (Sharp *et al*., 1997a). The specificity of these results was confirmed by the lack of signal in sections hybridized with a sense riboprobe instead of the antisense probe (not shown; see Materials and methods).

CHO1/MKLP1 expression in developing postnatal hamster brain

The Northern blot analyses indicate that CHO1/MKLP1 mRNA is expressed in the developing brain, but do not reveal the specific cell types in which it is expressed. To investigate this issue, we performed *in situ* hybridization analyses with CHO1/MKLP1 cRNA probes on P8, P15, P21 and adult hamster brain sections (Fig. 2). Consistent with the results of the Northern blot analyses and the *in situ* hybridization analyses on E15 embryos, we observed high levels of hybridization signal in the cerebral cortex that decreased during development. In addition, we detected high levels of expression in other brain structures, e.g. the cerebellum, olfactory bulb and hippocampus. In all of these structures, the levels of expression decreased during development. However, the temporal pattern of expression differed for these different brain regions, with the cortex



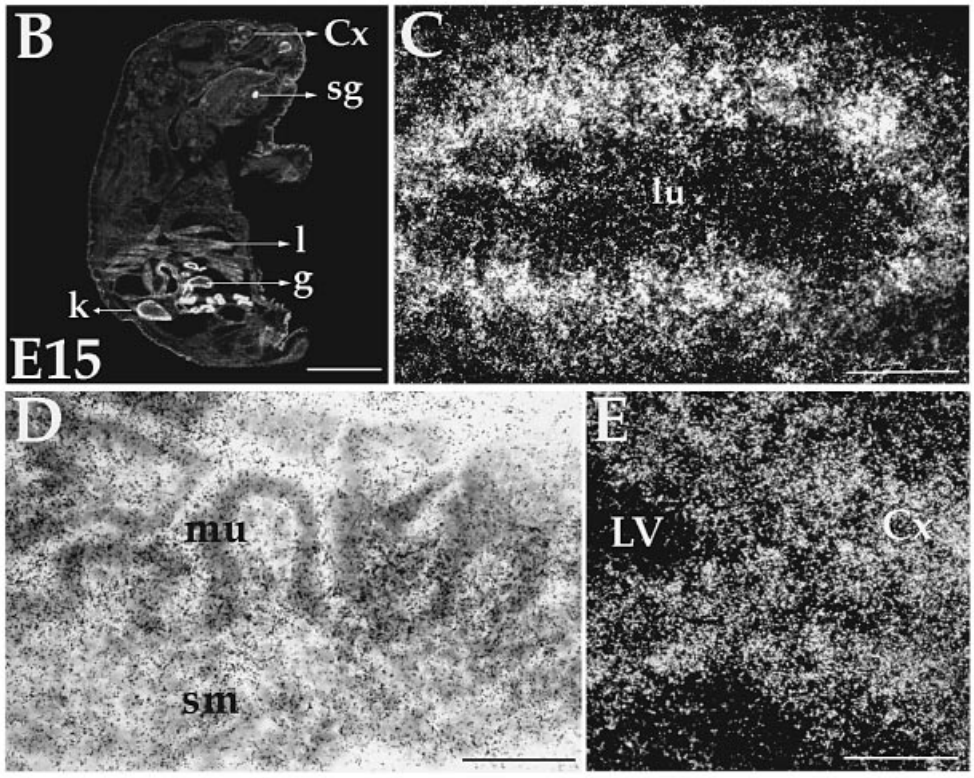


FIG. 1. CHO1/MKLP1 messenger ribonucleic acid (mRNA) expression in hamster tissues. (A) Northern blot analyses of CHO1/MKLP1 during postnatal development of hamster brain. Total RNA (20 μg/lane) isolated from postnatal P7, P15 and adult (Ad) hamster brains, and from Chinese hamster ovary (CHO) cells (CC) was electrophoresed in a formaldehyde 1% agarose gel containing ethidium bromide (upper panel), transferred to a nylon membrane, and then probed with the digoxigenin-labelled probe for CHO1/MKLP1 (lower panel). Shown are 28S and 18S ribosomal RNA demonstrating integrity of the RNA samples. The 3.6-kb transcript detected at P7 and P15 was identical in size to that found in CHO cells (used as a positive control). (B) Autoradiograph representative of the hybridization pattern obtained with CHO1/MKLP1 antisense riboprobe at embryonic day 15 (E15). A strong hybridization signal was detected within the cortex (Cx), submandibular salivary gland (sg), liver (l), gut (g) and kidney (k). Dark-field (C) and corresponding bright-field (D) illumination of a sagittal section of the gut hybridized for CHO1/MKLP1 mRNA. (E) Dark-field illumination of the cerebral cortex. Lu indicates lumen of the gut, mu indicates mucusal cells of the gut, sm indicates smooth muscle cells of gut, LV indicates lateral ventricle. Bar, 0.3 cm (panel B), 10 μm (panel C) and 20 μm (panels D and E).

showing the earliest developmental decrease, followed by the hippocampus, cerebellum and finally the olfactory bulb (see Fig. 2; olfactory bulb not shown). These observations are consistent with the differing timetables of neuronal differentiation in these regions of the brain (see Discussion).

Analyses of emulsioned slices allowed the identification of some of the cell types expressing the CHO1/MKLP1 mRNA (Figs 3–5). In the hippocampus of P15 hamsters, high expression was seen in pyramidal neurons and granule neurons of the dentate gyrus (Fig. 3A). These levels were decreased at P21 (Fig. 3B) and were further diminished to barely detected levels in adult animals (Fig. 3C). In the cerebellum of P8 hamster pups, the expression of CHO1/MKLP1 mRNA was observed in both external and internal granular layers (Fig. 4A). Expression was higher in the external granular layer than in the internal granular layer. Figure 4(B) shows that at P15, both external and internal granular layers express high levels of CHO1/

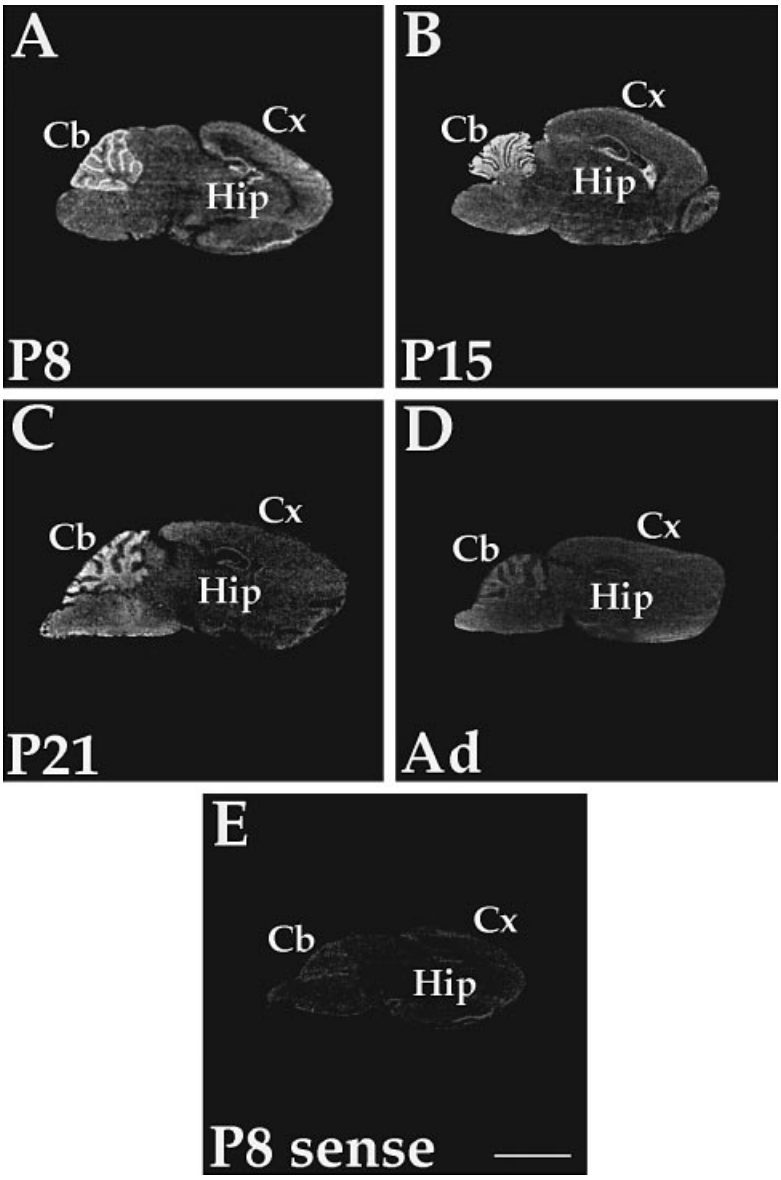


FIG. 2. Regional localization of CHO1/MKLP1 mRNA during postnatal hamster brain development determined by *in situ* hybridization. Autoradiographs (A)–(D) are representative of the hybridization patterns obtained with CHO1/MKLP1 antisense riboprobe at the postnatal days P8, P15, P21 and adult hamster brain (Ad), respectively. The autoradiograph (E) corresponds to the hybridization obtained with control CHO1/MKLP1 sense riboprobe in the P8 brain. Abbreviations: Cb: cerebellum; Hip: hippocampus; Cx: cortex. Bar, 0.3 cm.

MKLP1 mRNA, thus indicating an increase in expression in the internal granular layer compared with P8. At P21 after all the neurons had migrated from the external to the internal layer, the neurons continued to express CHO1/MKLP1 mRNA, but at slightly reduced levels compared with the earlier time points (Fig. 4C). In the adult cerebellum, the hybridization signal was barely detectable compared with the levels in younger animals (Fig. 4D). The same diminution of signal was observed in the olfactory bulb, where CHO1/MKLP1 mRNA expression was higher in younger animals (Fig. 5A,B) than in the adult (Fig. 5C). Prominent expression of CHO1/MKLP1 mRNA was observed in the glomerular, mitral and granular neurons, as shown in higher magnification micrographs (Fig. 5B). In the adult, all of these neurons showed only very low levels of expression (not shown at high magnification).

Expression of CHO1/MKLP1 in neuronal cultures revealed by in situ *hybridization*

If CHO1/MKLP1 is essential for dendritic differentiation, then we would expect its pattern of expression to be consistent with such a role. To explore this issue, we followed the expression of CHO1/MKLP1 in two different well-characterized neuronal culture systems, one from the central nervous system and one from the peripheral nervous system. In the first system, hippocampal neurons are obtained from E18 rat foetuses. At this time, most of the neurons had completed their final mitotic division, but had not yet developed dendrites. In the second system, sympathetic neurons are obtained from the superior cervical ganglia of newborn rat pups. At this time, the neurons had also completed their terminal mitotic division, but unlike the hippocampal neurons at E18, these neurons had already developed

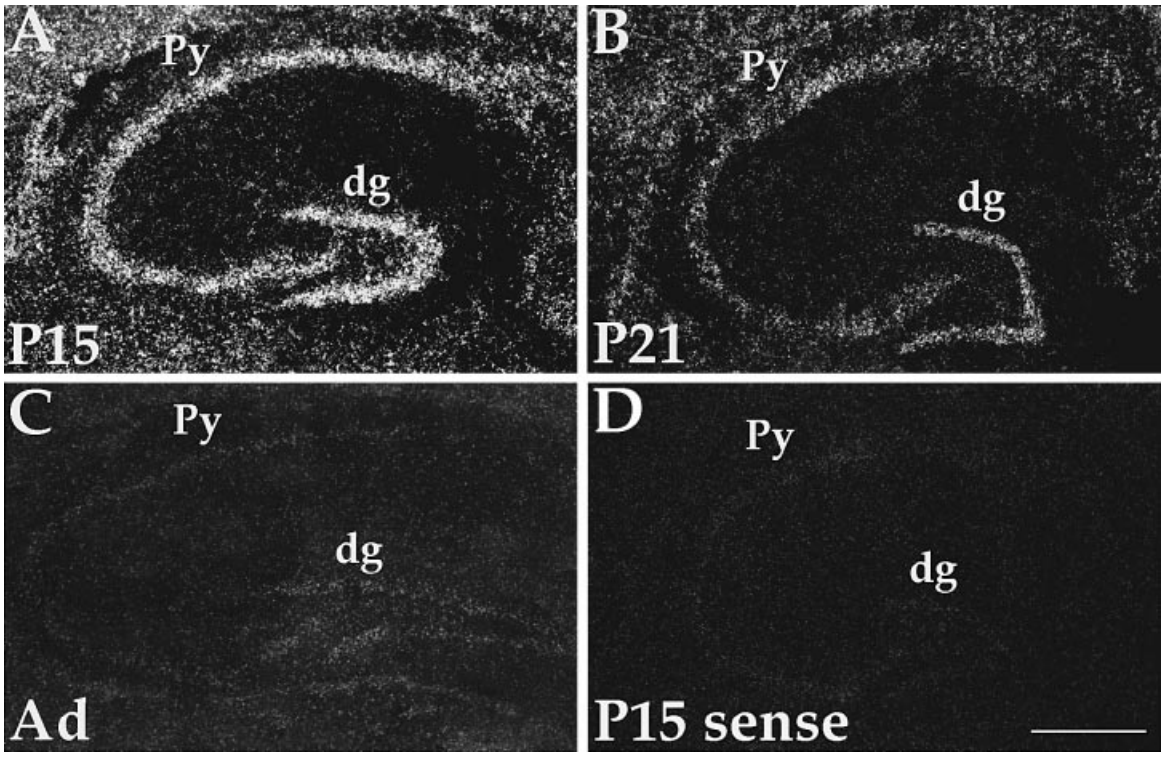


FIG. 3. Cellular localization of CHO1/MKLP1 mRNAs during development of the hippocampus. (A) Sagittal section showing the hippocampus of P15. Note the intense hybridization signals in the granular cells of the dentate gyrus (dg) and in the pyramidal cells (Py). (B), (C) Sagittal sections showing the hippocampus of P21 and adult, respectively. At P21, the granular cells and pyramidal cells still express CHO1 mRNA, but at lower levels than at P15. In the adult, the granular cells and pyramidal cells are only slightly labelled. (D) Sagittal section showing the absence of hybridization signal with labelled CHO1/MKLP1 sense riboprobe. Bar, 40 μm.

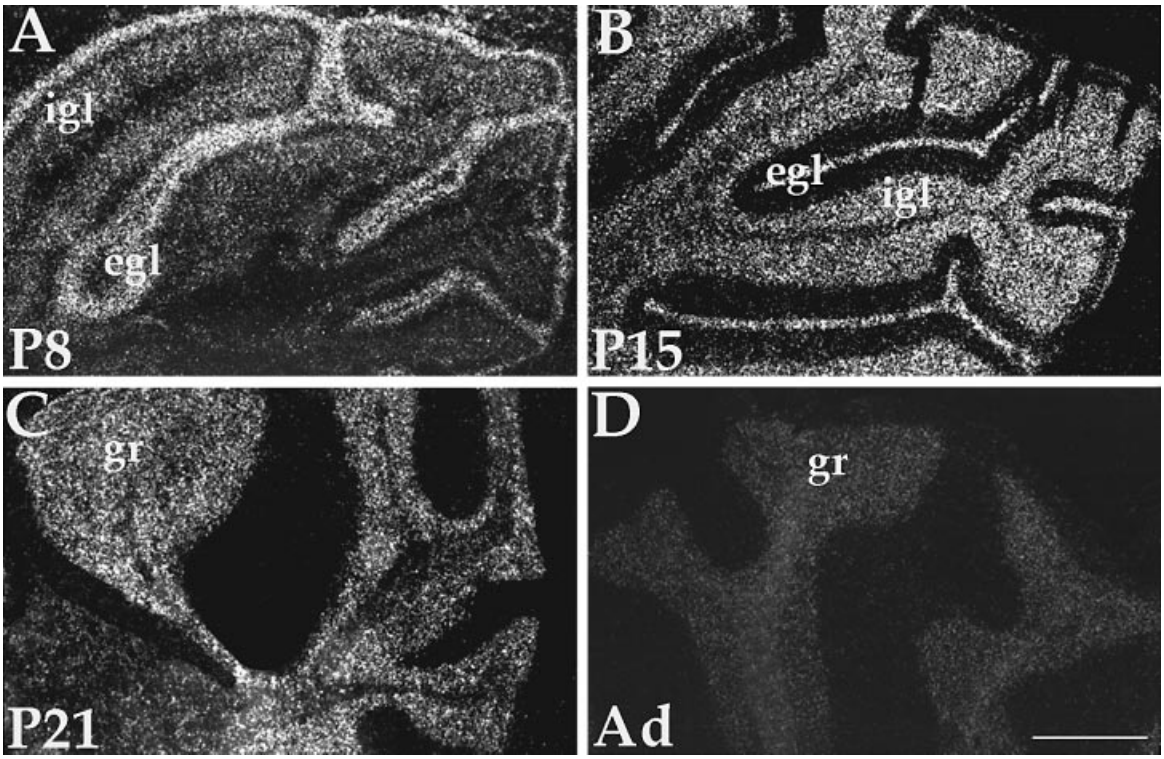


FIG. 4. Cellular localization of CHO1/MKLP1 mRNAs during development of the cerebellum. (A) Sagittal sections showing the cerebellum of P8 (A) and P15 (B). At P8 and P15, the external granular cell layer (egl) and internal granular cell layer (igl) were labelled. (C), (D) Sagittal sections of P21 and adult cerebellum, respectively. Note that at P21, the granular neurons (gr) still express CHO1/MKLP1 mRNA, but at slightly lower levels than at P8 or P15. In the adult, the granular cells are only slightly labelled. Bar, 40 μm.

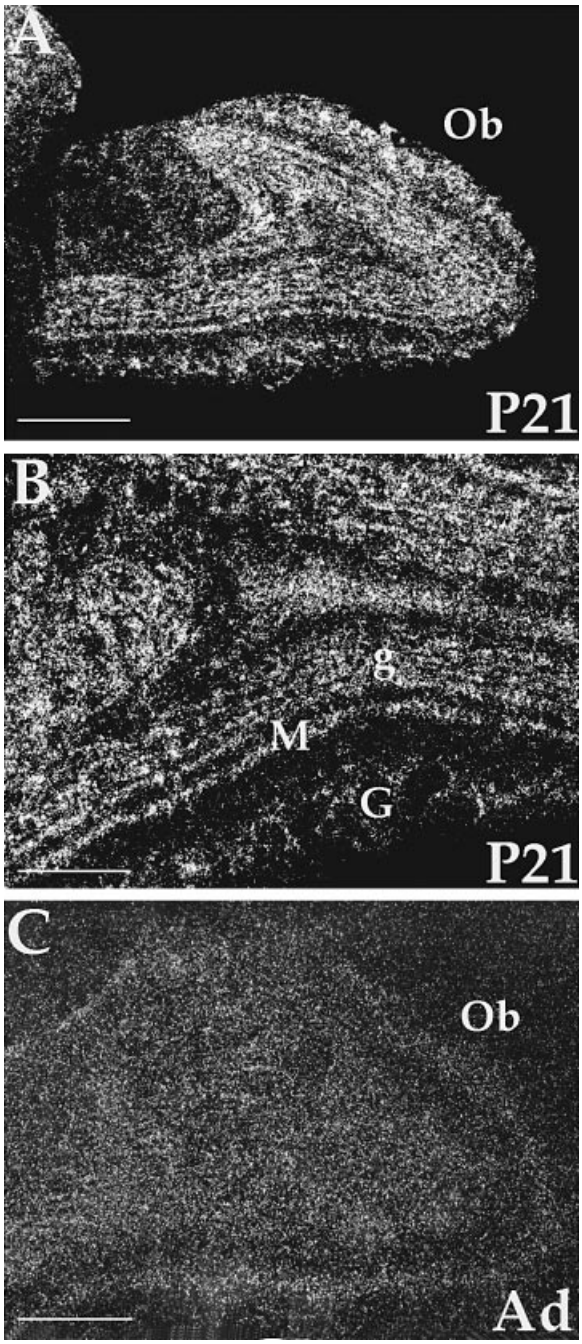


FIG. 5. Cellular localization of CHO1/MKLP1 mRNA during development of the olfactory bulb. Shown are sagittal sections of the olfactory bulb of at P21 (A and B) and adult (C). Higher levels of CHO1/MKLP1 mRNAs are detected at P21 in all cell layers: the glomerular cells (G), mitral cells (M) and granule cells (g). Bar, 80 μm (panels A and C), 160 μm (panel B).

axons and dendrites *in vivo*. In both cases, cultures were grown for 1, 3, 8 and 14 days. The cultured hippocampal neurons undergo a stereotyped sequence of developmental events that presumably reflect their *in vivo* development (Dotti *et al*., 1988). Initially the cells extend immature processes, one of which becomes the axon, by 1–1.5 days in culture. Then after a few additional days, the remaining immature processes differentiate into dendrites.

In situ hybridization analysis showed that mRNAs encoding CHO1/MKLP1 were expressed in hippocampal neurons at all of the time periods examined. Expression was relatively low at 1 day in culture (Fig. 6A), higher at 3 days (Fig. 6B) and higher yet at 8 days (not shown). At each of these time points, the individual neurons within the cultures were generally similar to one another with regard to their levels of expression. At 14 days, most cells displayed levels of expression that were even higher than at 8 days (Fig. 6C), but some cells showed substantially diminished expression (Fig. 6D). In the cells showing high levels of expression, the hybridization signal was observed in proximal regions of dendrites, as well as in cell bodies (Fig. 6C).

It is unclear whether sympathetic neurons develop in stages similar to hippocampal neurons, but it is known that they regenerate their processes in the culture dish, forming axons within the first few hours and dendrites within the first few days. As with the hippocampal neurons, CHO1/MKLP1 expression was detected at all time points. At 1 day, some neurons displayed high levels while others displayed relatively low levels of the hybridization signal (Fig. 7A). At 3 days, all of the neurons showed higher levels of expression (Fig. 7B). At 8 days, expression levels were substantially decreased compared with 1 or 3 days (Fig. 7C). At 14 days, the sympathetic neurons uniformly expressed high levels of CHO1/MKLP1 mRNA, levels that were similar to those detected at 1 day (Fig. 7D). Unlike the case with the 14-day hippocampal cultures, we did not observe any neurons with markedly diminished levels of signal. Also, despite the fact that the hybridization signal was strong in the cell body at 14 days, the signal was not detected in the proximal dendrites of the sympathetic neurons, as was the case with the hippocampal neurons.

If CHO1/MKLP1 principally functions in dendritic differentiation, we would expect its expression to be significantly lower in neurons that do not form dendrites. To test this, we used dorsal root ganglion neurons, which are known to generate only axonal processes both *in vivo* and *in vitro*. Cultures of rat dorsal root ganglion neurons were analysed at 1, 3, 8 and 14 days. CHO1/MKLP1 expression was detected at all of these time points (Fig. 8), but at markedly lower levels compared with those detected in the cultured hippocampal and sympathetic neurons. CHO1/MKLP1 mRNA was barely detectable at 1 day in culture (data not shown). At 3 days, there was a slight increase in expression compared with 1 day (Fig. 8A). At 8 days, expression levels were somewhat lower compared with those at 3 days (Fig. 7B). At 14 days, some neurons showed levels that were higher than at 1, 3 and 8 days culture (Fig. 8C), but the levels were still markedly lower than those detected in cultured sympathetic or hippocampal neurons.

Discussion

We previously reported evidence of a potential contribution of CHO1/MKLP1 to dendritic differentiation (Sharp *et al*., 1997a). The main goal of the present studies was to determine whether CHO1/MKLP1 is expressed in various types of postmitotic neurons. CHO1/MKLP1 was originally referred to as the 'CHO1 antigen' because it reacts specifically with a monoclonal antibody termed CHO1. The protein was later named MKLP1 ('Mitosis-specific Kinesin-Like Protein 1) because of its localization within the spindle of mitotic cells, and has subsequently been referred to as CHO1/MKLP1. Consistent with this, we found robust expression in tissues, e.g. epithelium, that exhibit high mitotic activity. Interestingly, we found that the mRNA encoding CHO1/MKLP1 is also expressed in postmitotic cells, e.g. the smooth muscle cells of the gut. In addition, our present results and those of our previous study (Sharp *et al*., 1997a) show that CHO1/MKLP1 is expressed highly in neurons well after their terminal mitotic division. Thus, CHO1/MKLP1 is not mitosis-specific as originally believed. It should be noted that our results

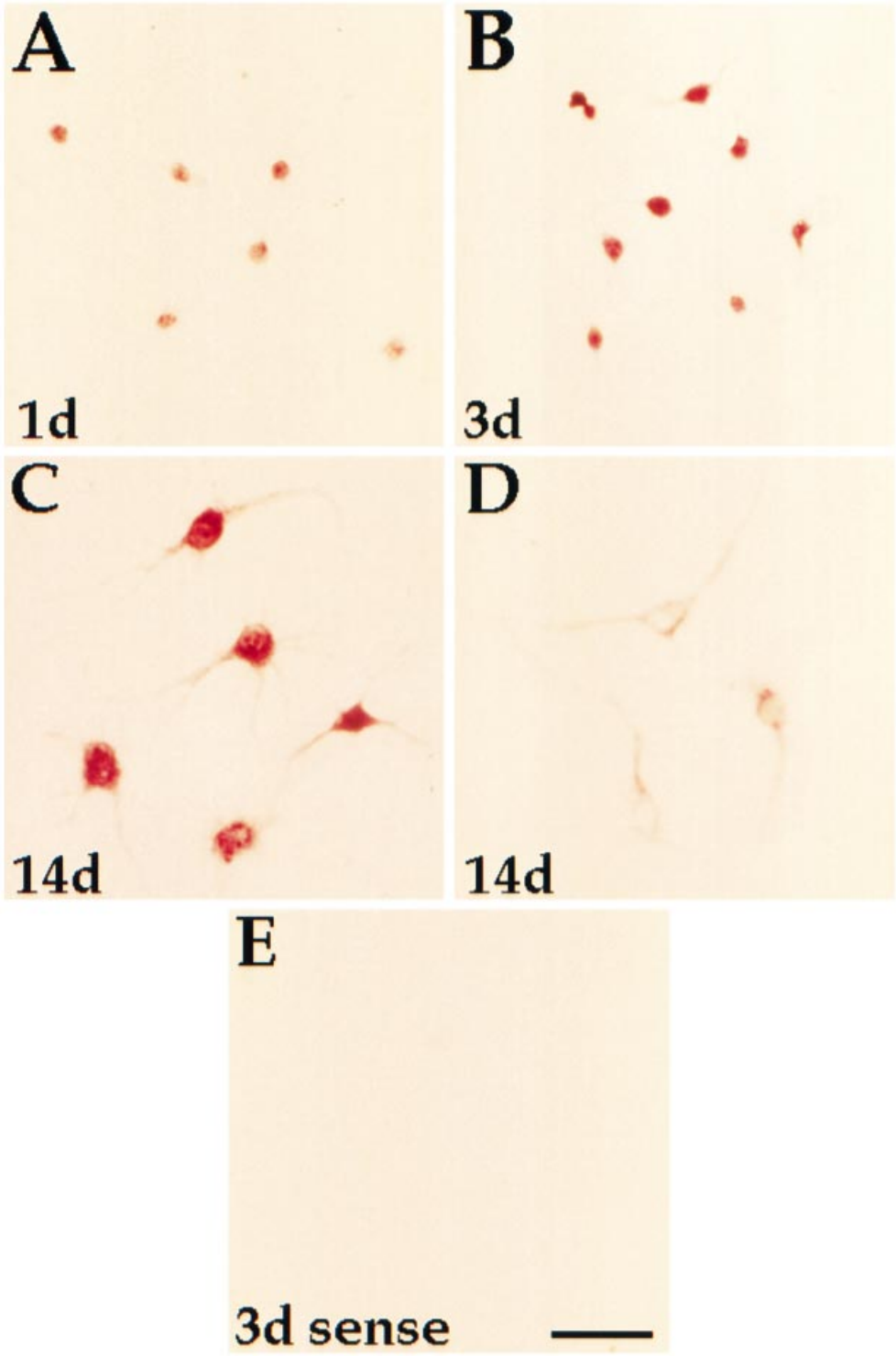


FIG. 6. Expression of CHO1/MKLP1 mRNA in cultured hippocampal neurons determined by *in situ* hybridization. Hippocampal neurons were grown for 1, 3 and 14 days, and fixed with 4% paraformaldehyde. After fixation, cultures were hybridized with the digoxigenin-labelled antisense (A–D) or sense (E) riboprobe for CHO1/MKLP1. Bar, 10 μm.

do not exclude the possibility that the CHO1/MKLP1 present within neurons is a splice variant or a post-translationally modified form of the mitotic protein. However, the strong reactivity of the neurons with a riboprobe and an antibody to the CHO1/MKLP1 of mitotic cells suggests that either the same protein or a very closely related protein is present and presumably used for a similar function in both. On the basis of these findings and the fact that the protein localizes to regions of mixed microtubule polarity orientation both in the mitotic spindle (Sellito & Kuriyama, 1988; Nislow *et al*., 1992) and within the dendrites of postmitotic neurons (Yu *et al*., 1997; Sharp *et al*., 1997a), we would suggest that a better name for the protein might be 'Mixed-polarity Kinesin-Like Protein 1.'

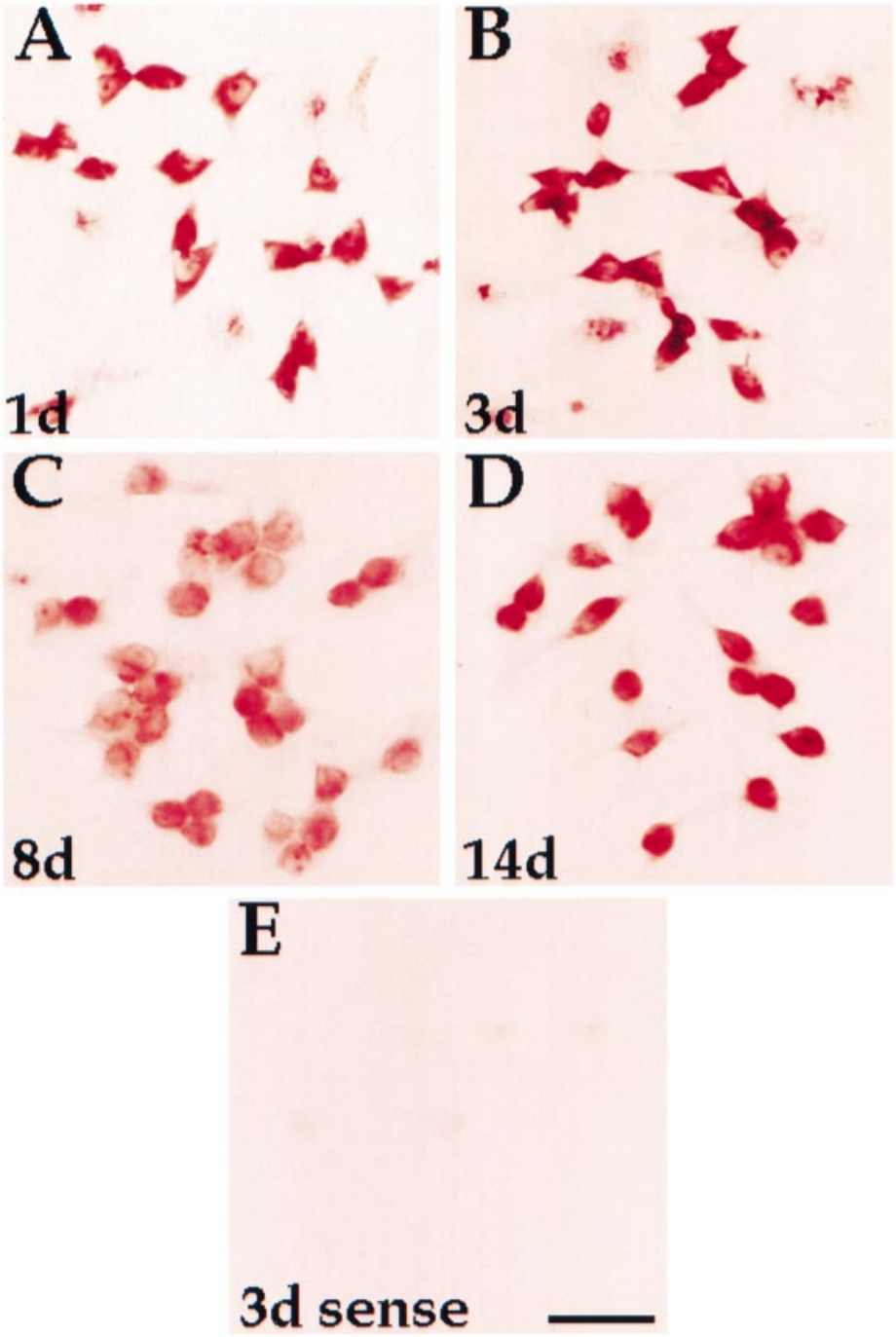


FIG. 7. Expression of CHO1/MKLP1 mRNA in cultured sympathetic neurons determined by *in situ* hybridization. Sympathetic neurons were grown for 1, 3, 8 and 14 days and fixed with 4% paraformaldehyde. After fixation, cultures were hybridized with the digoxigenin-labelled antisense (A–D) or sense (E) riboprobe for CHO1/MKLP1. Bar, 10 μm.

Another goal of this study was to examine the patterns of CHO1/MKLP1 expression in developing neurons. If the principal role of CHO1/MKLP1 in the neuron is to establish non-uniform microtubule polarity orientation in the dendrite, then we would expect its pattern of expression to be consistent with such a role. To explore this issue, we examined the brain at different stages of development, and also examined different regions of the brain in which the neurons differ markedly in their timetable of development. Developing neurons exit the cell cycle, develop an axon and then subsequently develop dendrites. The neurons of the cortex have an earlier timetable of development than those within the hippocampus, which complete their terminal postmitotic division around the time of birth. The neurons of the cerebellum develop later, while the neurons of the olfactory bulb develop still later. In all of these regions of the brain, we found high levels of expression at times well after the neuron's terminal mitotic division, after axon differentiation, and throughout times corresponding to dendritic differentiation. For example, in the cerebellum, CHO1/MKLP1 is highly expressed in the external granular layer prior to the migration of the developing neurons into the internal granular layer. This may correlate with residual mitotic activity of

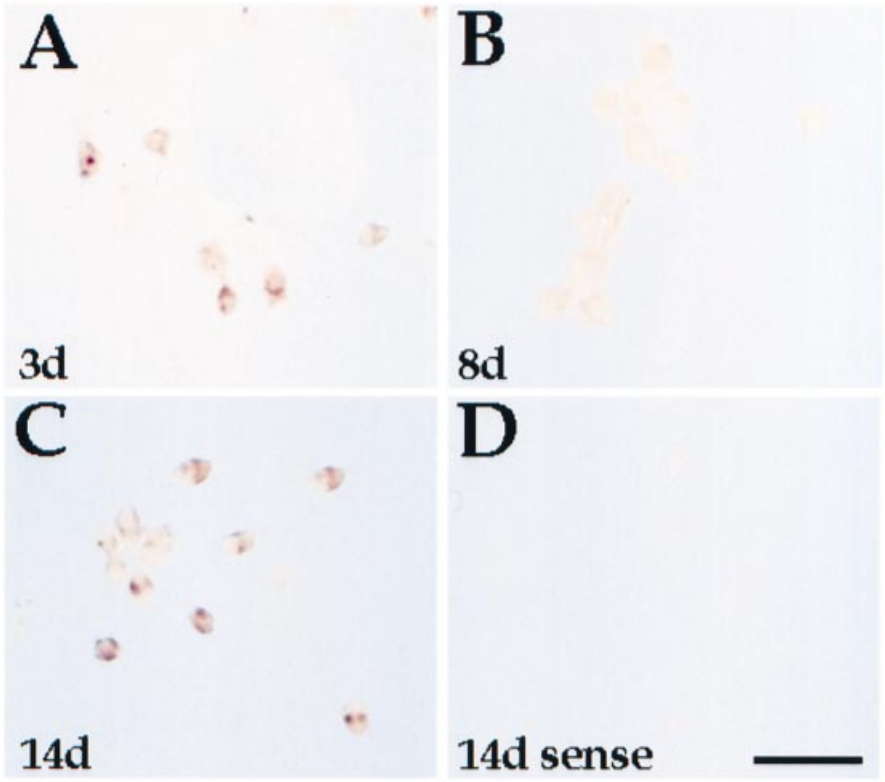


FIG. 8. Expression of CHO1/MKLP1 mRNA in the dorsal root ganglia neurons determined by *in situ* hybridization. Dorsal root ganglia neurons were grown for 3, 8 and 14 days, and fixed with 4% paraformaldehyde. After fixation, cultures were hybridized with the digoxigenin-labelled antisense (A–C) or sense (E) riboprobe for CHO1/MKLP1. Bar, 10 μm.

the neuroblasts in the external layer. Later in development, there is an increase in expression in the internal granular layer during a time well after cell division has ceased and dendritic differentiation is underway. After the dendrites had fully developed, the levels of CHO1/MKLP1 mRNA gradually decreased until they were barely detectable in adult brain.

The strong expression of CHO1/MKLP1 mRNA at times corresponding to dendritic development is consistent with the proposed role of the protein in establishing the dendritic microtubule array. The fact that mRNA levels are very low in the adult brain is interesting, and raises several possibilities concerning how the neuron maintains the microtubule array of the dendrite. One possibility is that CHO1/MKLP1 protein levels are similarly diminished, and that the transport of new minus-end-distal microtubules from the cell body is no longer required once the non-uniform microtubule pattern of the dendrite has been established. If this is the case, new microtubules of this orientation may arise via fragmentation of existing minus-end-distal microtubules. In this regard, microtubule-stabilizing factors, e.g. MAP2d (Ferhat *et al*., 1996), may be essential for ensuring that the microtubules do not completely depolymerize during such fragmentation. On the other hand, perhaps only small numbers of minus-end-distal microtubules need be transported into the dendrites of adult neurons to maintain the non-uniform pattern, and the low levels of CHO1/MKLP1 expression may provide sufficient levels of the protein to accomplish this. It is also possible that CHO1/MKLP1 is expressed in transient bursts within individual neurons of the adult brain, and hence are difficult to detect in studies on brain slices. Some support for this possibility derives from the studies on cultured hippocampal neurons, in which neurons in the 14-day cultures displayed either very high or very low levels of expression, and the studies on cultured sympathetic neurons in which the levels of expression decreased at 8 days and then increased at 14 days. Yet another possibility is that the CHO1/MKLP1 mRNA that we are able to detect corresponds to a juvenile protein that is gradually replaced by expression of another motor protein that transports minus-end-distal microtubules into the dendrites of adult neurons. This protein may be a shorter splice-variant of CHO1/MKLP1 that is not detectable with our probe, or it may be an entirely different gene product with properties similar to CHO1/MKLP1. A final possibility is that CHO1/MKLP1 protein levels are actually high in the adult brain even though the mRNA levels are low. This may suggest that CHO1/MKLP1 is a relatively stable long-lived protein in the neuron.

Additional insights were provided by the studies on cultured neurons. The key results from the studies on hippocampal and sympathetic cultures were that CHO1/MKLP1 is expressed both in peripheral and central neurons that form dendrites, and that the levels of expression change during the development of individual neurons. Especially in the hippocampal cultures, the pattern of expression coincides well with dendritic differentiation. In the sympathetic cultures, the levels also undergo changes during neuronal development, but these changes are more difficult to interpret. As noted above, expression is initially high, decreases at day 8 after the dendrites have formed, and then increases again and stays high. The fact that expression does not decrease markedly in older cultures as it does in the adult brain may reflect a difference in peripheral versus central neurons, or may somehow relate to the fact that the sympathetic cultures are a regenerating system. It is also possible that this continued expression at later time points is an anomaly of cell culture. Also as noted above, some of the neurons in the older hippocampal cultures showed diminished levels of expression, and some did not.

Interestingly, a hybridization signal was detected in the proximal regions of the hippocampal dendrites, but not of the sympathetic dendrites, raising the possibility that different types of neurons may differ as to whether a portion of the CHO1/MKLP1 is synthesized locally within the dendrite. The key result from the studies on the sensory neurons of the dorsal root ganglia is that these neurons express only very low levels of CHO1/MKLP1. This result is consistent with the proposed function of CHO1/MKLP1 in dendritic development, given that both *in vivo* and *in vitro*, these neurons generate axons but not dendrites. The fact that CHO1/MKLP1 mRNA is expressed at all in these neurons may or may not suggest that the protein plays some additional role in neurons apart from participating in the differentiation of dendrites.

In conclusion, the present studies have documented that CHO1/MKLP1, once thought to be mitosis specific, continues to be expressed in postmitotic differentiated neurons and has a developmental pattern of expression consistent with its proposed role in dendritic differentiation. These results are also consistent with the results of our previous functional analyses, which strongly suggest that CHO1/MKLP1 transports minus-end-distal microtubules from the cell body to join plus-end-distal microtubules within developing dendrites, thus establishing the characteristic non-uniform microtubule polarity pattern of the dendrite (see Introduction). In addition, these results provide more evidence supporting the view that neurons do not invoke entirely novel mechanisms for organizing their postmitotic microtubule arrays, but instead rely on modifications of the same mechanisms that organize microtubules during mitosis in dividing cells. Specifically, it appears that both rely on the tightly regulated transport of microtubules by molecular motor proteins.

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Abbreviations

BCIP	bromochloroindolylyl phosphate
CHO	Chinese hamster ovary
DTT	dithiothreitol
MKLP1	Mitosis-specific Kinesin-Like Protein 1
MEM	minimum essential medium
mRNA	messenger ribonucleic acid
NBT	nitro-blue tetrazolium
NGF	nerve growth factor
PBS	phosphate-buffered saline

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