NeuN, a neuronal specific nuclear protein in vertebrates

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Summary

A battery of monoclonal antibodies (mAbs) against brain cell nuclei has been generated by repeated immunizations. One of these, mAb A60, recognizes a vertebrate nervous system- and neuron-specific nuclear protein that we have named NeuN (*Neu*ronal *N*uclei). The expression of NeuN is observed in most neuronal cell types throughout the nervous sytem of adult mice. However, some major cell types appear devoid of immunoreactivity including cerebellar Purkinje cells, olfactory bulb mitral cells, and retinal photoreceptor cells. NeuN can also be detected in neurons in primary cerebellar cultures and in retinoic acid-stimulated P19 embryonal carcinoma cells. Immunohistochemically detectable NeuN protein first appears at developmental timepoints which correspond with the withdrawal of the neuron from the cell cycle and/or with the initiation of terminal differentiation of the neuron. NeuN is a soluble nuclear protein, appears as 3 bands ($46\text{-}48 \times 10^3\,M_\mathrm{r}$) on immunoblots, and binds to DNA in vitro. The mAb crossreacts immunohistochemically with nervous tissue from rats, chicks, humans, and salamanders. This mAb and the protein recognized by it serve as an excellent marker for neurons in the central and peripheral nervous systems in both the embryo and adult, and the protein may be important in the determination of neuronal phenotype.

Key words:DNA binding protein, neuronal nuclei, P19 cells, monoclonal antibody, mice.

Introduction

The complexity of the vertebrate nervous system is underscored by studies which estimate that there are 30,000 distinct mRNAs in the adult mammalian brain, of which 20,000 are brain-specific and low abundant messages (Sutcliffe, 1988). Many tissue-specific proteins must be directly involved in the development, differentiation and functioning of this system.Those localized in the nuclei of neuronal cells are of special interest since they may be involved in transcriptional regulation. Numerous strategies have been employed to identify and characterize such molecules. A highly popular and productive approach has been homologous screening of vertebrate nervous system cDNA libraries with probes to known or putative regulatory molecules of *Drosophila* and other species. The POU-domain-containing (He et al., 1989), homeobox-containing (Graham et. al., 1989; Holland and Hogan, 1988), MASH (Johnson et al., 1990), and many other putative vertebrate regulatory molecules have been identified with this approach.Homologous screening, however, is limited by design, since it will only detect those molecules within the same class as the probe itself. Entire classes of molecules may elude detection by this approach. Moreover, it is not unreasonable to suspect that vertebrate-specific classes of regulatory molecules may be necessary to generate the increased complexity of the nervous system of higher animals.

An alternative to homology screening is the production of monoclonal antibodies (mAbs) directed against complex mixtures of proteins in the nervous system. This approach has proved to be an effective means of identifying molecules with unique and interesting specificities (Fujita et al., 1982; McKay and Hockfield, 1982; Hockfield and McKay, 1985; Dodd et al., 1988; Keller et al., 1989; Sousa et al., 1990; Suzue et al., 1990).The immunological approach has the important advantage of being able to identify proteins which are present in low amounts such as nuclear regulatory factors (Bhorjee et al., 1983). Interesting mAbs may be used to detect the proteins in situ and to monitor purification of the protein. In addition, the mAb may be used to isolate the gene encoding the protein from expression cDNA libraries. The majority of nervous system-specific mAbs which have been described are directed against membrane or cytoplasmic proteins.

We have taken the approach of producing mAbs to mouse brain cell nuclei in an attempt to identify novel proteins of importance in the regulation of the neuronal phenotype. Our immunization procedure may have selected for rare antigens or those with low immunogenicity, as it includes long-term immunization with increasing host immune tolerance to common antigens. We have generated a number of interesting mAbs by immunizing BALB/c mice with cell nuclei isolated from the brains of a closely related species, *Mus caroli*. Hybridomas were screened immuno-

histochemically for nervous system immunoreactivity and neuronal cell type specificity.

In this paper we report on monoclonal antibody, mAb A60, whose cognate nuclear antigen is detected early in neurogenesis and is restricted to neurons. To our knowledge this is the first report of a monoclonal antibody against a neuron-specific nuclear antigen in vertebrates, although one has been reported for invertebrates (Bier et al., 1988). We suggest that this neuron-specific antigen is an early marker of neuronal differentiation and may be important in nervous system development and function. Since there is a neuronal cytoskeleton protein called A60 (Rayner and Baines, 1989), to avoid confusion, we have named the protein detected by our mAb A60 "NeuN" (*Neu*ronal *N*uclei).

Materials and methods

Brain nuclei isolation

Brain nuclei were isolated as described by Lovtrup-Rein and McEwen (1966) with minor modifications. Briefly, freshly dissected brain tissue was minced and manually homogenized with a loose-fitting pestle in 0.25% Triton X-100 in sucrose buffer (0.32 M sucrose, 1 mM $MgCl_2$, 1 mM potassium phosphate, pH 6.5). Homogenate was filtered through cheesecloth and nuclei pelleted by centrifugation at 1250 g for 10 minutes.Following two washes in sucrose buffer the nuclear fraction was resuspended in 2 M sucrose buffer and centrifuged through a 2.4 M sucrose cushion at 53,000 g for 75 minutes. The nuclear pellet was washed twice with 0.32 M sucrose buffer, resuspended in phosphate-buffered saline and nuclei counted with a hemocytometer.

Immunological techniques

BALB/c mice were immunized with the *Mus caroli* nuclei as shown in the schedule in Table 1. To suppress against common antigens, serum was collected from BALB/c mice that were immunized and boosted with BALB/c nuclei. This non-specific "anti-BALB/c serum" was then mixed with the *Mus caroli* nuclei for the last four boosts including the pre-fusion boost.This technique, which in principle may suppress the immune response to common or highly immunogenic proteins, is similar to the procedure used by Barclay and Smith (1986) with *Dictyostelium*. For the last boost, the nuclei (mixed with the anti-BALB serum) were injected intravenously and intrasplenically (Spitz et al., 1984).

Three days after the final boost, splenocytes were collected and fused with myeloma cell line P3X63-Ag8.653 and plated. Hybridoma supernatants were assayed on sections of both *Mus*

Table 1. *Immunization schedule*

Week	Treatment of nuclei	Route of injection
0	Emulsify with CFA	SC
9	Emulsify with IFA	SC
23	Emulsify with IFA	SC
32	Mix with anti-BALB serum	IP
33	Mix with anti-BALB serum	IP
36	Mix with anti-BALB serum	IP
37	Mix with anti-BALB serum	IV, IS, IP
37 + 3 days	Fusion	

CFA, complete Freunds adjuvant; IFA, incomplete Freunds adjuvant; anti-BALB serum, see text.SC, subcutaneous; IP, intraperitoneal; IV, intravenous; IS, intrasplenic.

caroli and BALB/c brains. Some mAbs generated by this technique were species-specific and stained *Mus caroli* but not BALB/c (Mullen and Cichocki, 1988), others such as mAb A60 described here, were not species-specific.

Animals and tissue preparation

The strains of mice used, BALB/c, C57BL/6J, C3H/HeN and *Mus caroli*, were raised in our animal facility. We have not detected any difference in the expression of the antigen in these different strains.However, all of the figures in this paper are of the BALB/c, C57 or C3H strains.

Neonatal and adult mice were perfused with paraformaldehyde-lysine-periodate (PLP) fixative (McLean and Nakane, 1974) for 10 minutes, the tissues then immersed in fixative for approximately 6 hours. The tissues were rinsed overnight in phosphate-buffered sucrose (5%), dehydrated and embedded in polyester wax (Polyethylene glycol 400 distearate, Ruger Chemical, Irvington, NJ) that had been treated as described by Feder (1976).7 or 8 μm sections were mounted on gelatin chrom-alum subbed slides. Alternatively, tissues were embedded in plastic (Methyl Methacrylate - Butyl Methacrylate Kit, Polysciences, Warrington, PA) and sectioned at 2 μm. The slides were deplasticized with xylene before immunohistochemistry. Except for Fig. 2A and B, which are plastic sections, all other figures are of 7-8 μm polyester wax sections.

For timed pregnancies, day of plug is considered embryonic day 0 (E0).As most embryos were collected around midday, they are designated as, for example, E12.5.Embryos were immersed in fixative, then processed as above.

Immunohistochemistry

Vecta ABC Elite Kit (Vector Labs, Burlingame, CA) was used for immunohistochemistry with 3,3 -Diaminobenzidine tetrahydrochloride (DAB) as the HRP substrate.For most studies the A60 antibody was diluted 1:100 and applied for 1-2 hours or overnight. Anti-GFAP antibody was obtained from Dako Scientific.No counterstain was used on any of the sections shown in the Figs.

Nuclear protein isolation

Isolated brain nuclei were sonicated with five, 15 second bursts at low pulse on a Fisher 300 Sonic dismembrator to liberate nuclear protein. The sonicated nuclei were centrifuged at 14,000 g to separate soluble and insoluble protein fractions. The vast majority of NeuN immunoreactivity was retained in the supernatant. Further analysis of the solubility of NeuN included centrifugation of the above supernatant at 100,000 g for 30 minutes in an airfuge. Again, essentially all of the immunologically detectable NeuN protein remained in the supernatant fraction following this high speed spin. Protein concentrations were determined using the Micro-BCA Protein Assay Reagent Kit (Pierce), according to supplier's instructions.

Immunoblot analysis

Transfer of brain nuclear protein from polyacrylamide gels to nitrocellulose membrane and immunodetection were performed as previously described (Harlow and Lane, 1988). For immunoblots, mAb A60 was diluted 1:40 in 3% normal horse serum (NHS). Various secondary antibody detections systems including the Vecta ABC kit (see above) and ^{125}I-labeled goat anti-mouse antibody (Amersham) have been used to detect the antigen with this technique.

Cell culture

Primary cultures of dissociated cerebellum were established as described by Messer (1989). Cell suspensions were made from trypsinized P6-7 cerebella and plated on poly-L-lysine coated cov-

erslips. Cytosine arabinoside is added after 24 hours in culture to remove mitotic cells. The resultant culture is predominantly composed of granule cell neurons and glia.

P19 embryonal carcinoma cells and the D3 derivative of these cells were cultured and induced to differentiate in the presence of retinoic acid (RA) as described by Rudnicki and McBurney (1987). P19 cells in suspension were treated with RA for 4 days, then plated on 0.1% gelatin-coated coverslips. PC12 cell culture was as previously described (Green and Tischler, 1976).

Chromatography

Heparin-Sepharose (CL-6B) and DNA-cellulose (Native DNA, calf thymus) were obtained from Pharmacia. 100-200 μg of soluble brain nuclear protein was bound to a 1 ml packed volume of each matrix using a batch procedure. Binding buffer was 25 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 1.0 mM DTT, 10 mM NaCl and 0.5 mM PMSF (phenylmethylsulfonyl fluoride). Following binding, the columns were packed and washed exhaustively in binding buffer. NeuN immunoreactivity was undetectable in the final washes under these conditions.Elutions were performed with increasing concentrations of NaCl (from 0.05 M to 1.0 M).Fractions were precipitated with an equal volume of acetone for 30 minutes on ice and the precipitated protein assayed for the presence of NeuN by mini-immunoblot analysis. NeuN reproducibly eluted from each column with 0.4 M to 0.5 M NaCl. The specificity of NeuN binding to DNA cellulose was demonstrated by the inability of the antigen to bind non-DNA linked cellulose and cellulose phosphate columns under the same conditions.

Results

Our initial screening with mAb A60 revealed that immunohistochemically the antibody stains the nuclei of most neuronal cell types in all regions of the adult brain including cerebral cortex, hippocampus, thalamus, caudate/putamen, cerebellum, etc. (Fig. 1A), as well as in the spinal cord (Fig. 1C). In Fig. 1A, the areas of olfactory bulb, cerebral cortex and cerebellum that show more intense staining is not a consistent finding and is probably an artifact**.** Although the antigen is detectable in the cytoplasm, the level of staining is usually markedly lower than that seen in the nucleus. For example, in the large pyramidal cells of the cerebral cortex the nucleus is intensely stained except for the nucleolus, while there is relatively light staining in the cytoplasm (Fig. 1B). However, in some cell types, such as cerebellar granule cells, we have observed a lack of immunoreactivity in the nuclei of some of the neurons although it does appear in the cytoplasm. This difference may be artifactual, perhaps involving penetration of reagents, as the extent of cytoplasmic and nuclear immunoreactivity demonstrates some fixation and section-to-section variability. It is also possible that this variation may indicate subpopulations within a particular cell type or different physiological states of those cells.

Despite the widespread distribution of this antigen in the brain, it is not detected in all neuronal cell types. In the cerebellum, for instance, A60 immunoreactivity is present at high levels in granule cells, Golgi type II neurons and cells in the deep nuclei, but it appears to be totally absent from Purkinje cells (Fig. 2A, B). The staining pattern in the granule cells suggests the antigen is localized in euchromatic, but not heterochromatic, regions.There are A60-pos-

itive cells in the molecular layer but their identity has not been determined. It is likely that at least some of the positive cells in the molecular layer are ectopic granule cells because in the heterozygous weaver mutant mouse, which has large numbers of ectopic granule cells, there are correspondingly large numbers of A60 immunoreactive cells in the molecular layer (data not shown). The olfactory bulb is similar to the cerebellum in that most neurons are A60 immunoreactive, with the exception of the mitral cells (Fig. 2C). In the retina, the antigen is detected in the ganglion cells and some cells in the inner nuclear layer, but is absent from the photoreceptor cells and most cells in the inner nuclear layer (Fig. 2D).As with the cerebellar granule cells discussed above, we do not know whether the absence or reduction in staining in some ganglion cells represents true variations in the level of the antigen. The number and position of stained cells in the inner nuclear layer suggests they may be amacrine cells. Preliminary observations suggest there is a population of large neurons in the dorsal cochlear nucleus that is also not stained by A60.

Neuronal specificity

The antigen recognized by mAb A60 appears to be neuron-specific. No immunoreactivity has been observed in a variety of glial cell types, such as oligodendrocytes in the white matter of the spinal cord (Fig. 1C) and brain (Fig. 1B), and Bergmann glia in the cerebellum (Fig. 2A, B).Fig. 3 shows a region of the hippocampus double-labelled with mAb A60 and a rabbit anti-GFAP antibody (see Fig. 3 legend), both visualized with HRP/DAB. The nuclei of the hippocampal neurons show a typical A60 nuclear staining pattern while the cells with characteristic GFAP-positive processes (i.e. astrocytes) are devoid of immunoreactivity in their nuclei. To determine if there is A60 immunoreactivity in reactive glia, we examined the cerebellar molecular layer of the Purkinje cell degeneration (*pcd*) mutant mouse. In this mutant there is extensive proliferation of reactive glia in the molecular layer following the degeneration of the Purkinje cells (Mullen et al., 1976). No immunoreactivity was detected in this glial cell type. Further evidence of the neuronal specificity of this mAb comes from our tissue culture and biochemical studies described below.

A60 immunoreactivity is also found in the peripheral nervous system (PNS). Samples of PNS were collected from rats because outside of the CNS there is less of a background problem when examining rat tissues compared to mouse tissues.A60 immunoreactivity was observed in adult rat dorsal root ganglia, sympathetic chain ganglia and both the submucosal plexus (of Meissner) and myenteric plexus (of Auerbach) in the intestines (data not shown). Immunoreactivity was also found in the ciliary ganglion from an embalmed human cadaver. Our immunohistochemical analysis indicates that A60 immunoreactivity appears to be primarily confined to the nervous system. However, we have detected immunoreactivity in the adrenal medulla and the intermediate lobe of the pituitary.

Developmental expression

The expression of the antigen is developmentally regulated. In the neural tube, immunoreactivity appears as early as embryonic day 9.5 (E9.5) and even at this stage, expression
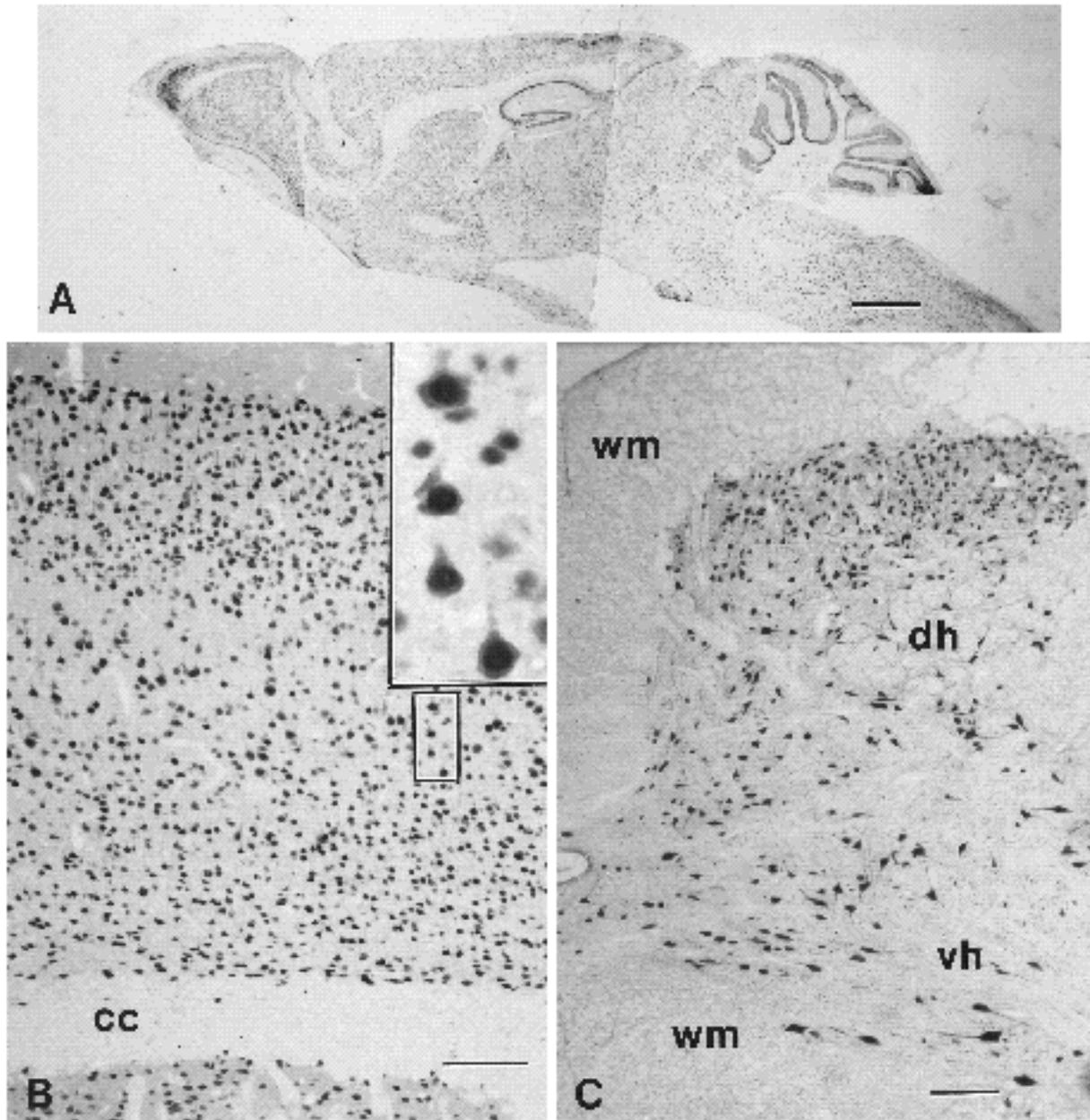
Fig. 1. Immunohistochemical staining of adult CNS with mAb A60.(A) Mid-sagittal section showing immunoreactivity in all regions of adult brain.(B) Sagittal section of cerebral cortex showing staining in all cortical layers.Note absence of staining in corpus collosum (cc). Inset shows intense nuclear staining in large pyramidal cells with less staining in the cytoplasm and no staining in nucleolus.(C) Cross section of spinal cord with cells staining in both the dorsal horn (dh) and ventral horn (vh), but no staining in the white matter (wm).Bars, 1 mm in A; 100 μm in B and C.

of the antigen is confined to the nervous system. Fig. 4A shows a representative section from an E10.5 embryo at the level of the mesencephalon.A60 immunoreactivity is absent from the mitotic ventricular zone and floor plate, but staining is clearly evident in the lateral margins of the basal plate. At this age, the more cranial regions of the spinal cord have more staining in the ventral regions than in the dorsal regions.The time of appearance and position of these positive cells coincides closely with the first-born neurons of the mouse neural tube (Nornes and Carry, 1978; McConnell, 1981). By embryonic day 12.5, there are large

numbers of A60 positive cells in the developing ventral horns and immunoreactivity is also detected in the developing dorsal horns as well as in the dorsal root ganglion (Fig. 4B, C). There is no staining in the ventricular zone, roof plate, floor plate or marginal zone (developing white matter).

In the remainder of the CNS the pattern of onset of A60 immunoreactivity is similar, with early differentiating cells being the first to express A60 immunoreactivity. In the E17 cerebral neocortex (Fig. 4D), A60 immunoreactivity is most intense in a population of cells whose positionsuggests that
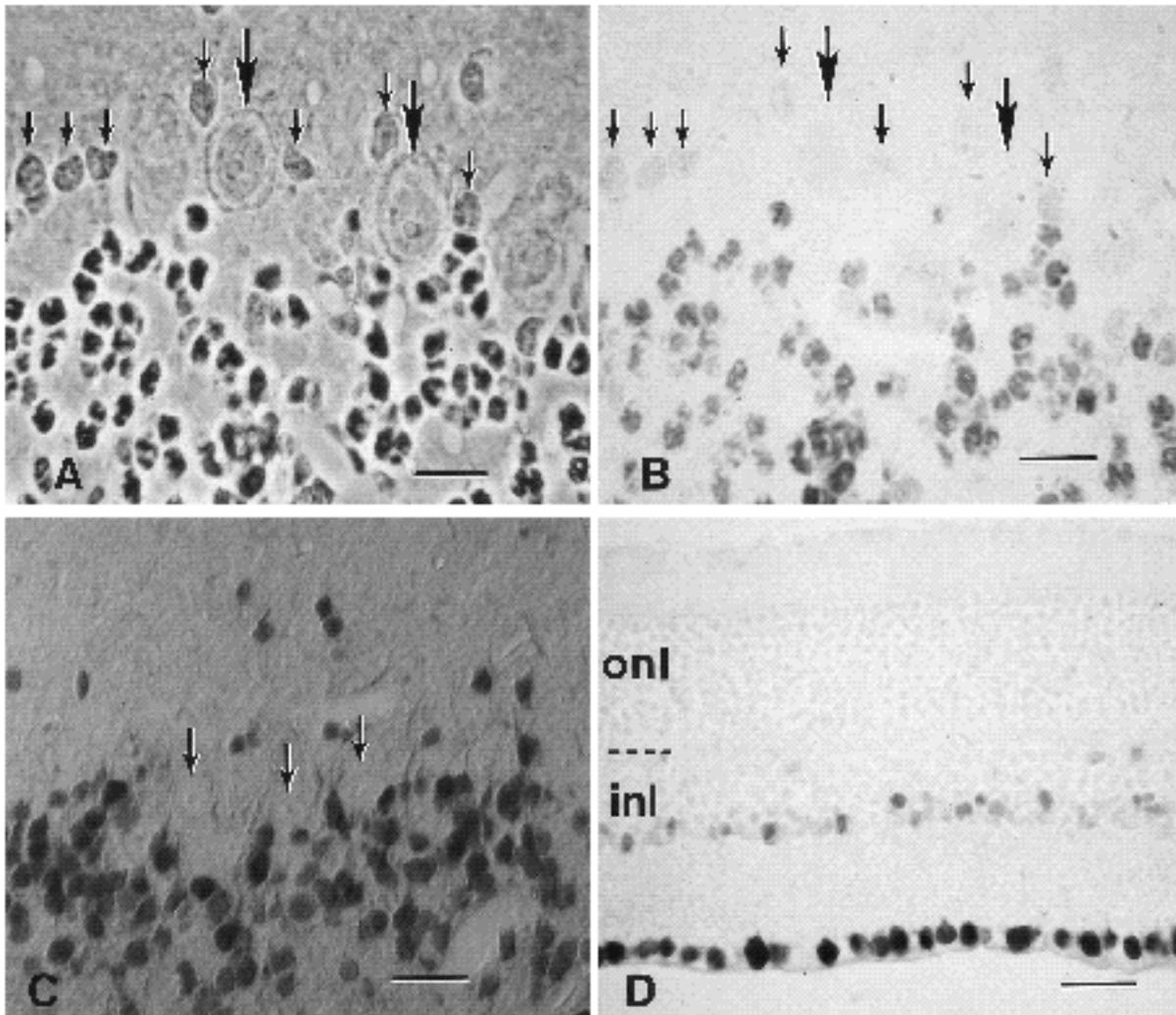
Fig. 2. Examples of CNS cell types lacking immunoreactivity.(A, B) Identical field of 2 μm plastic section of cerebellar cortex viewed by phase-contrast (A) and bright-field (B) illumination.The granule cells show intense nuclear immunoreactivity but there is none in the large Purkinje cells (large arrows) or in putative Bergmann glia (small arrows).The granule cells show a "moth-eaten" staining pattern typifying the distribution of euchromatin in this cell type.(C) Olfactory bulb viewed by Differential Interference Contrast microscopy.As in the cerebellum, there is intense nuclear staining in the granule cells but no immunoreactivity in the mitral cells (arrows).The small nucleoli are visible in the mitral cells as evidence that the section through these mitral cells did include the nuclei.(D) Section of neural retina with intense staining ofthe ganglion cells across the bottom of the field and staining of some cells along the inner margin of the inner nuclear layer (inl).However, the remainder of the cells in the inl and the photoreceptor cells in the outer nuclear layer (onl) are devoid of immunoreactivity.Bars, 100 μm in A and B; 25 μm in C and D.

they may be the cells of sublayer VIb (Valverde et al., 1989) which may be the rodent equivalent of the subplate cells of cats and monkeys (Luskin and Shatz, 1985). There are also some dark-staining cells in the marginal layer which may be the Cajal-Retzius cells. These VIb/subplate and Cajal-Retzius cells are among the first post-mitotic, and earliest differentiating neurons of the cortex (Luskin and Shatz, 1985; Valverde et al., 1989). In contrast to the neocortex, the subjacent piriform cortex, which is probably developmentally more advanced in this region (Gardette et al., 1982), shows intense staining across all layers.

In the developing granule cells of the postnatal day 3 (P3) cerebellar cortex, immunoreactivity is first clearly evident at the inner aspect of the transient external granule cell layer where the newly post-mitotic granule cell neurons of this region are differentiating to a bipolar morphology before they begin to migrate (Fig. 4E).The antigen is easily detected in the nuclei of these cells as they traverse the molecular layer en route to the permanent (inner) granule cell layer.

To confirm the neuronal specificity and to facilitate the characterization of this antigen we have examined its expression in several tissue culture systems.In primary cultures of dissociated P7 cerebellum, neurons express high levels of A60 immunoreactivity after seven days in culture (Fig. 5A). We have yet to examine cells at earlier stages of culture so we do not know whether the positive cells are those that were expressing the antigen when plated or if
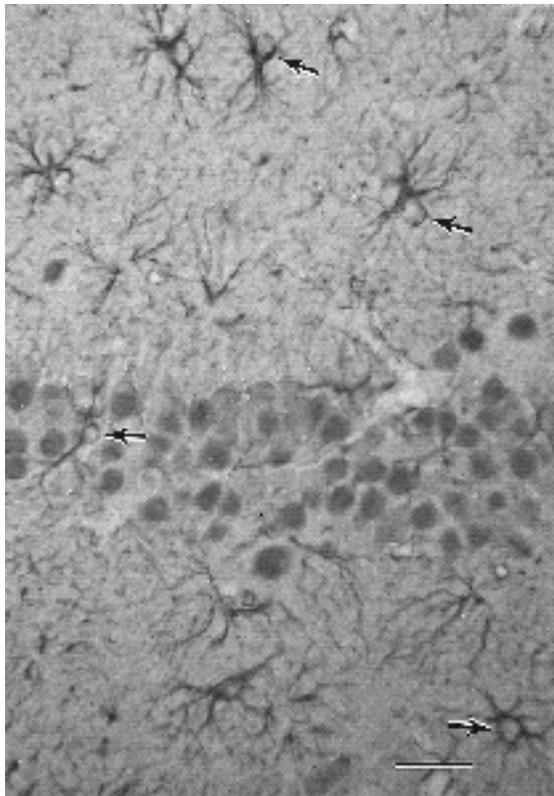
Fig. 3. Neuronal specificity of nuclear staining by mAb A60 in a section of hippocampus.The section was double-labeled with mAb A60 and an anti-GFAP antibody, both visualized with HRP/DAB.The anti-GFAP was a rabbit polyclonal detected by PAP staining.The nuclear staining by mAb A60 is clearly evident in the large hippocampal neurons across the center of the field.In contrast, the astrocytes (arrows), showing the typical GFAP staining of their processes, are devoid of nuclear staining.Note the smaller glia within the neuronal layer is also devoid of nuclear staining.Bar, 25 μm.

Fig. 4. Developmental regulation of the antigen recognized by mAb A60.(A) Cross section through the mesencephalon of an E10.5 embryo.There is no staining in theventricular zone (vz) where the mitotic cells reside adjacent to the lumen (l) of the neural tube.A60-positive cells, however, are seen at the margins of the neural tube in the areas where post-mitotic cells are found at this stage of development.(B)Cross section through a E12.5 embryo demonstrating immunoreactivity in the spinal cord and adjacent dorsal root ganglia.In the spinal cord immunoreactivity is clearly more advanced in the ventral regions, site of the developing motor neurons, than in the dorsal regions.Also note the continued absence of immunoreactivity in the ventricular zone and the floor plate (fp) demarcated by the dashed lines.(C) Higher magnification of the ventral region of the spinal cord section shown in B.(D) Coronal section through the cerebral hemisphere of an E17.5 embryo.A60-positive cells are seen throughout the hemispheres but in the neocortex (nc) the most intensely stained cells appear as a single laminar (arrows) whose position suggests these may be the subplate neurons, the first neocortical neurons to develop.Note how the staining pattern changes in the piriform cortex (pc), where A60 immunoreactivity is more uniformly distributed.The dashed line shows the approximate location of the rhinal sulcus.(E) Section through postnatal day 3 cerebellar cortex.A60-positive cells are evident along the inner margin of the external granular layer (eg), a region inhabited by post-mitotic granule cells before they migrate to the mature granular layer (g). Cells continue to express A60 immunoreactivity as they migrate (arrows).Note that many of the cells in the granular layer (g) exhibit more of a cytoplasmic staining pattern than a nuclear pattern.This is also seen in adult cerebellum but is not evident in the 2 μm section in Fig. 2.Bars, 25 μm in A, C and E; 100 μm in B and D.

expression was initiated while in culture. Morphologically undifferentiated cells, and cells with glial or fibroblast appearance do not express the antigen in these cultures. Similarly, immunoreactivity is robust following retinoic acid stimulation of the P19 embryonal carcinoma cell line (Fig. 5C), but no staining is evident in unstimulated P19 cells (Fig. 5B). A fraction of these cells differentiate into post-mitotic neuron-like cells following retinoic acid treatment (Jones-Villeneuve et al., 1982; McBurney et al., 1988) and it is only these neuron-like cells which display immunoreactivity following stimulation (Fig. 5B and C, and D. J. Morassutti and M. W. McBurney, personal communication). In two sublines of the pheochromocytoma line PC12, a model neuronal system (Green and Tischler, 1976), we find no detectable levels of antigen in either the presence or absence of NGF (data not shown).

Biochemistry

mAb A60 stains three bands of approximately $46\text{-}48 \times 10^3$ M_r on immunoblots from mouse brain nuclear protein separated by SDS polyacrylamide gel electrophoresis (PAGE)

and transferred to nitrocellulose filters (Fig. 6A).The lower two bands run as a tight doublet and usually are only distinguished on high resolution polyacrylamide gels. The same bands appear whether the nuclei are isolated from whole brain, cerebellum only, brain minus cerebellum, or whole embryos. The antigen is a relatively soluble nuclear protein as it is retained in the supernatant following mild sonication of total brain nuclear protein and centrifugation at 100,000 *g* for 30 minutes (Fig. 6B). These data confirm that mAb A60 recognizes a protein and suggest that the antigen is neither a nuclear matrix protein nor is it a protein tightly bound to the DNA.

Nervous system specificity (see above) was confirmed by immunoblot analysis of nuclear protein isolated from other mouse tissues. No antigen was detectable in a variety of tissues, even after long exposure times (Fig. 6C). We have not yet analyzed the adrenal medulla or pars intermedia by immunoblot to confirm the identity of this antigen in these non-neuronal tissues which exhibited immunohistochemical reactivity (see above).

The same bands appear in the crude "cytoplasmic" fraction as in the purified nuclear fraction (Fig. 6D). However, since some nuclei are undoubtedly lysed during the initial homogenization procedure, it cannot be stated with certainty that the proteins in the cytoplasmic fraction are identical to those represented by the 2-3 bands in the purified nuclear fraction. Suffice it to say that this data suggests, but does not prove, the identity of the proteins recognized in cytoplasmic and nuclear fractions.
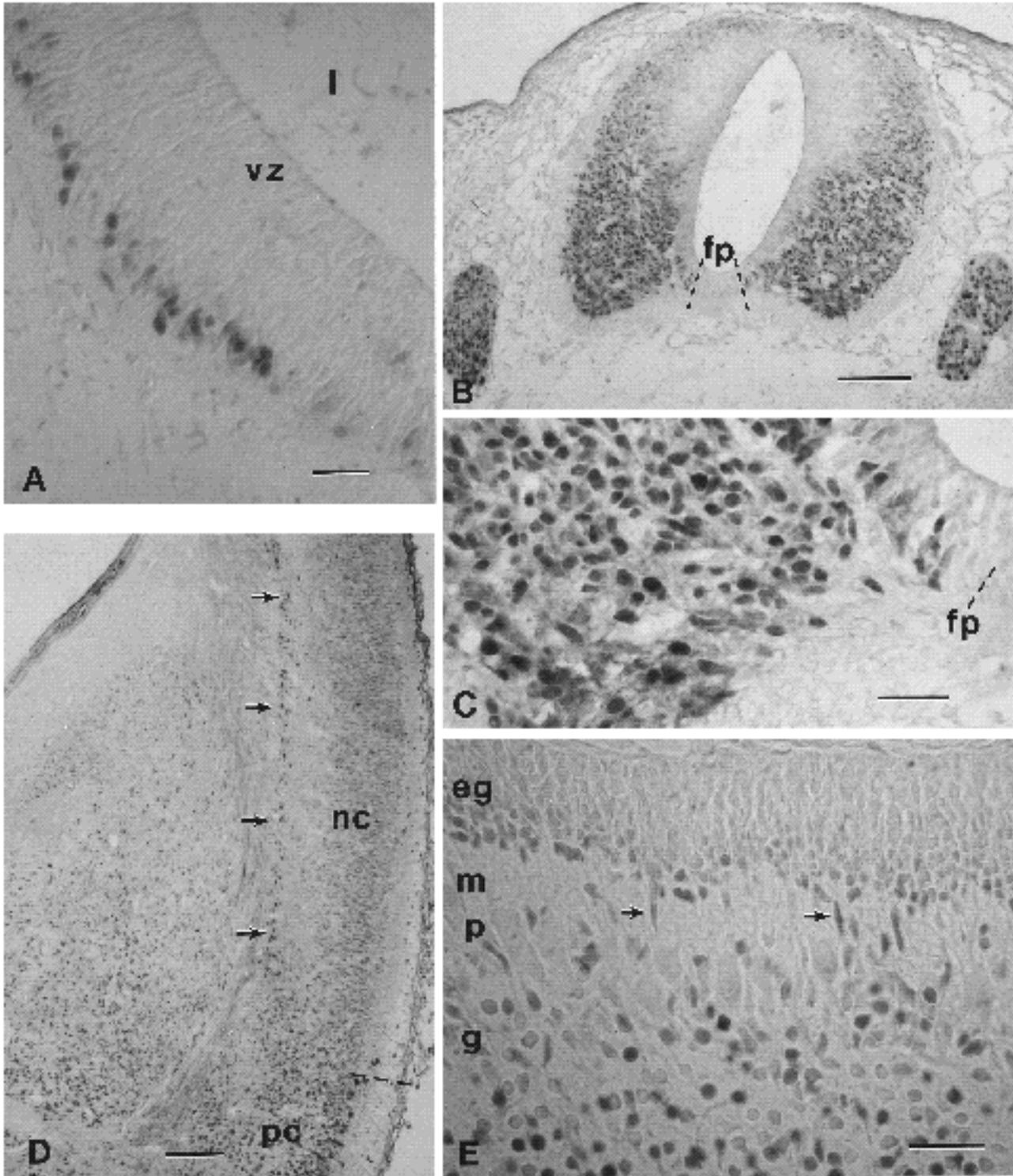
The murine epitope recognized by mAb A60 appears to be conserved among vertebrates as it crossreacts immuno-histochemically with rat, chicken and human (data not shown) and with salamanders (B. Davis, personal communication). Immunohistochemical analysis did not reveal any crossreactivity in adult *Drosophila melanogaster* central nervous system (only eyes and heads were examined).Also, immunoblots of larval, pupal and embryonic *Drosophila*

protein failed to demonstrate A60 crossreactivity (data not shown).

The nuclear localization and solubility properties of the antigen suggested to us that this protein may interact with DNA. To determine if this antigen can bind DNA in a specific manner, mouse brain nuclear protein was subjected to native DNA-cellulose column chromatography. The columns were eluted with a salt gradient and fractions were
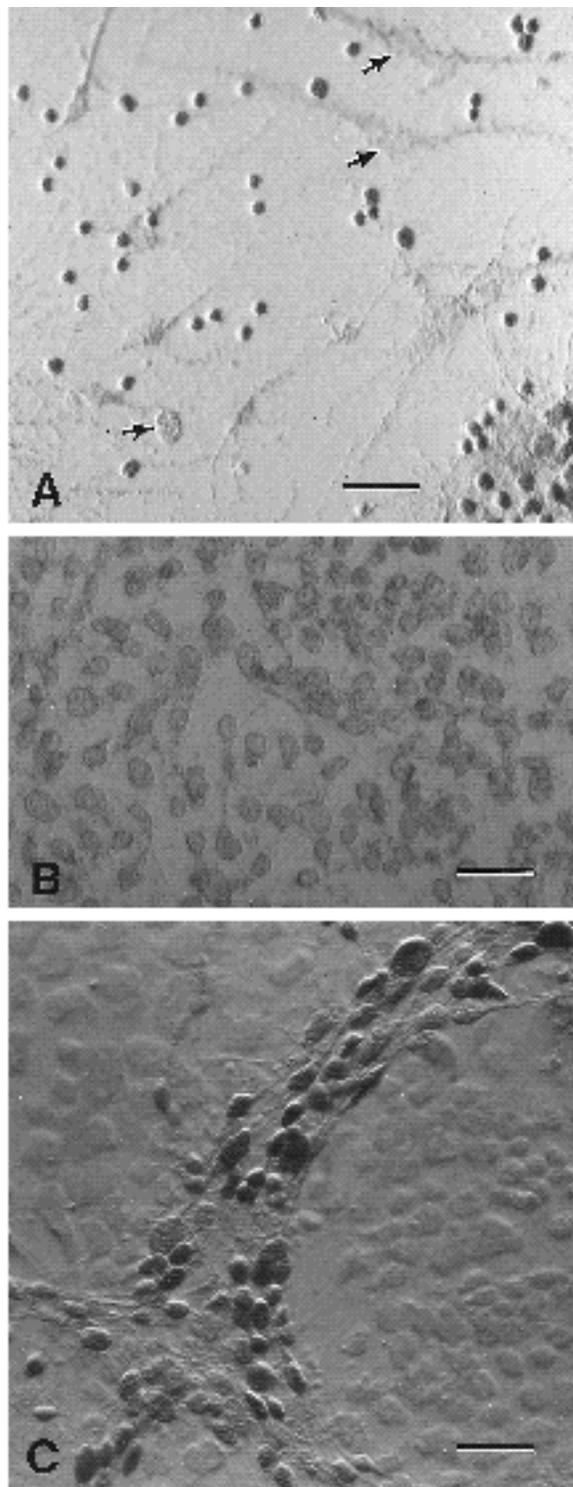
Fig. 5. Expression of the antigen recognized by mAb A60 in tissue culture systems.(A) Primary cerebellar culture stained with mAb A60 viewed by Differential Interference Contrast microscopy.The numerous small granule cells are A60-positive but the other non-neuronal appearing cells lack nuclear immunoreactivity (arrows). (B, C) P19 embryonal carcinoma cells before (B) and after (C) stimulation with retinoic acid.There is no immunoreactivity in unstimulated P19 cells (B) but 4 days after plating stimulated cells, nuclear immunoreactivity is intense in the neuronal-like cells but not in the non-neuronal cells in the background (C).Bars, 50 μm in A; 25 μm in B and C.
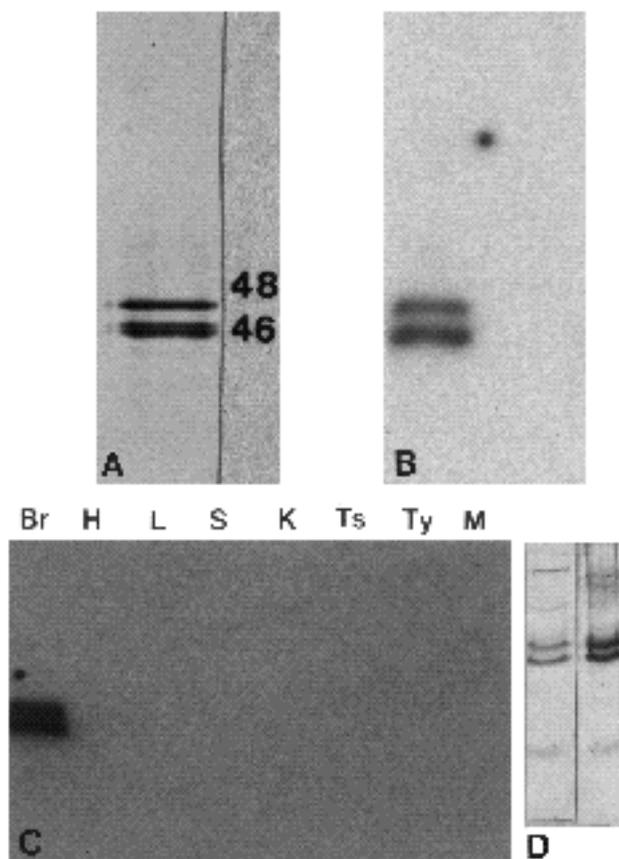

Fig. 6. (A) Immunoblot of mAb A60.Nuclear protein from brain run on a high resolution 5-15% gradient SDS polyacrylamide gel and incubated with A60 antibody and detected using an HRP ABC Vectastain Kit.Note that the lower relative molecular mass band appears to be composed of two bands.(B) Isolated brain nuclei were briefly sonicated and centrifuged at 100,000 *g* for 30 minutes.Immunoblot from SDS-PAGE of supernatant (left lane) and pellet (right lane) was incubated with mAb A60 and detected with ^{125}I-labeled anti-mouse secondary antibody.(C) Immunoblot using mAb A60 and detected with ^{125}I-labeled anti-mouse secondary antibody.Blot shows the tissue specificity of mAb A60 for its cognate antigen.Equal amounts of nuclear protein from several tissues were run.Br, brain; H, heart; L, liver; S, spleen; K, kidney; Ts, testis; Ty, thymus; M, muscle (skeletal).(D) Immunoblots of "cytoplasmic" and nuclear fractions detected with an HRP ABC Vectastain Kit.The "cytoplasmic" fraction (left lane) is a 1:10 dilution of the supernatant from the crude homogenate.It would include soluble cytoplasmic proteins plus soluble proteins from lyzed nuclei.The nuclear fraction (right lane) is the soluble protein from purified nuclei.The two major bands appear the same in the two fractions.The lighter bands above and below the major bands are artifacts, appearing even in the absence of primary antibody.

assayed for antigen by immunoblot analysis. The antigen eluted from the column with 0.4 M NaCl (Fig. 7C). Specificity of antigen-binding to DNA is evident by the inability of the antigen to bind to either cellulose (Fig. 7A) or to cellulose phosphate (Fig. 7B) matrices alone**.** The specific binding and elution profiles of this antigen in vitro are similar to previously described DNA-binding proteins such as
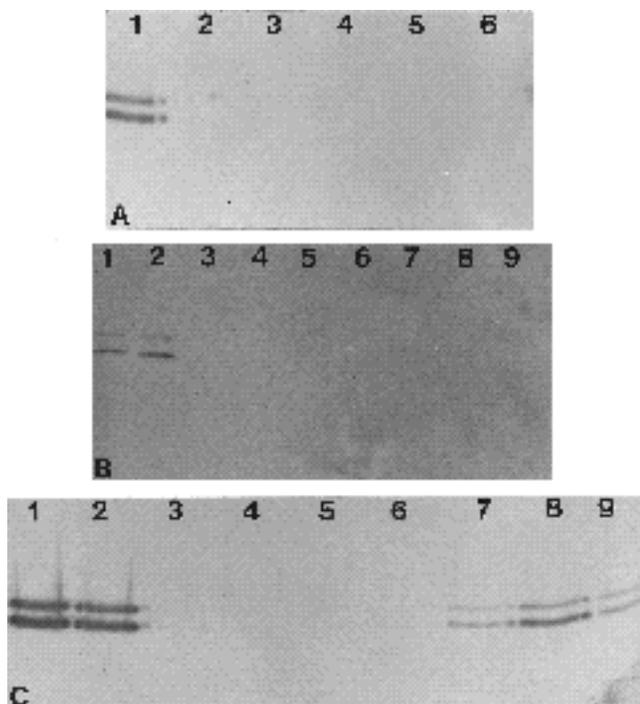
Fig. 7. 1 ml columns were loaded with 100 μg of soluble brain cell nuclear protein and eluted with increasing concentrations of NaCl.Fractions were precipitated, resolublized, run on SDS-PAGE and analyzed onimmunoblots using mAb A60.(A) Column prepared with cellulose (Sigmacell Type 100).Fractions from left to right: 1, flow through; 2, 50 mM; 3, 200 mM; 4, 400 mM; 5, 500 mM; 6, 1.0 M.This immunoblot indicates that no detectable antigen binds to cellulose.(B) Column prepared with cellulose phosphate (Sigma cation exchange 2383).Fractions from left to right: 1, flow through; 2, first wash; 3, final wash; 4, 50 mM; 5, 100 mM; 6, 200 mM; 7, 400 mM; 8, 500 mM; 9, 1.0 M. No detectable antigen binds to the cellulose phosphate.(C) Column prepared with DNA-Cellulose.Fractions from left to right: 1, flow through; 2, first wash; 3, final wash; 4, 50 mM; 5, 100 mM; 6, 200 mM; 7, 400 mM; 8, 500 mM; 9, 1.0 M.The bound antigen begins to elute at 400-500 mM NaCl.

c-fos (Sambucetti and Curran, 1986), and support the suggestion that A60 antigen is also a DNA-binding protein. Soluble protein from brain cell nuclei was also loaded onto a heparin-sepharosecolumn and eluted with increasing concentrations of NaCl. The elution profile for the antigen (data not shown) was similar to that with the DNA-cellulose column.

Discussion

Monoclonal antibody A60 immunohistochemically stains the nuclei of the vast majority of neuronal cell types in both the central and peripheral nervous systems. With minor exceptions (i.e. adrenal medulla and intermediate pituitary) the expression of the antigen is nervous system- and neuron-specific, based on the following observations: (1) immunohistochemistry and immunoblots of a variety of tissues revealed immunologically detectable antigen only in

nervous tissue; (2) cell nuclei of the white matter (oligodendrocytes) are negative for A60 immunoreactivity; (3) Bergmann glia and reactive glia are negative; (4) staining of brain sections with a mAb specific for glial fibrillary acidic protein (GFAP), the major intermediate filament of glia, is in no case coincident with A60 immunoreactivity; and (5) only the neuron-like cells in primary cerebellar culture and in stimulated P19 cell culture are immunoreactive with mAb A60. We cannot formally rule out the possibility that negative cells simply express the antigen at a level which is below the limit of our detection system. However, this is unlikely owing to the unusually strong binding of this monoclonal. mAb A60 hybridoma supernatant can be diluted to 1:500 with little loss of specific signal (mAb A60 ascites fluid can be diluted 1:10,000). Thus, if unstained cells do express this antigen, it is at very low levels or, alternatively, the protein lacks the specific epitope in these cells.

Although most neuronal cell types express the antigen, there are some major cell types that do not. Purkinje, photoreceptor and mitral cells which are all devoid of staining, are also the same cells which degenerate in the Purkinje cell degeneration (*pcd*) mutant mouse (Mullen et al., 1976). It has been recently reported that the cartwheel neurons of the dorsal cochlear nucleus (DCN) also degenerate in *pcd* (Berrebi et al., 1990). Consequently, we examined the DCN for A60 immunoreactivity. Indeed, in the sections that clearly transect the DCN, there is a population of large neurons that fail to stain with mAb A60 but we have not positively identified these as cartwheel neurons. This may be further evidence of a relationship between these neuronal cell types.

Aside from the staining of neurons, A60 immunoreactivity has been detected in only two other cell types, the chromaffin cells of the adrenal medulla and the cells in the intermediate lobe of the pituitary. Both of these are neuroendocrine cells. The adrenal chromaffin cell is a typical neural crest-derived "paraneuron". In view of the fact that these adrenal medulla cells exhibit immunoreactivity,it was interesting to find that NGF-stimulated PC12 cells do not. There are a number of possible explanations for this. We have examined only two PC12 cell lines and it could be a peculiarity of those cell lines; also, PC12 is derived from a rat and we have not examined rat adrenals for immunoreactivity.We have not definitively identified the adrenal antigen on immunoblots so it may be a different protein that crossreacts.Finally, a more interesting possibility is that this antigen is only expressed in cells that have "terminally" differentiated, and PC12 cells will lose their neuronal properties if NGF is withdrawn.

Taken together, our findings on the developing nervous system suggest that this nuclear antigen is first expressed (or becomes immunologically detectable) in neurons when they have become post-mitotic and are initiating cellular and morphological differentiation. The observation that some cell types seem to express high levels of antigen shortly after exiting the cell cycle (e.g. ventral motoneurons and cerebellar granule cells) while other cell types exhibit a delay in expressing high levels of the antigen (e.g. neocortex neurons), suggests that the expression of high levels of the antigen may correspond to the state of differ-

entiation or level of functioning of the neuron, rather than simply to withdrawal from the cell cycle.

Although we have focused primarily on the nuclear staining by mAb A60, we do want to emphasize that the cytoplasm is also immunoreactive, though to a lesser extent.For the most part the cytoplasmic staining is concentrated in the soma although it does extend a short distance into some of the processes (mostly dendritic processes). We have no evidence as to whether the antigen has a function in the cytoplasm or whether it is merely synthesized there before being transported to the nucleus. We have not detected any difference on immunoblots between protein isolated from purified nuclei and whole brain extract (i.e. nuclei and cytoplasm).

It has been amply demonstrated that nuclear factors control the expression of tissue-specific genes involved in the development, differentiation and functioning of a variety of tissues. In recent years there has been a great deal of interest in putative vertebrate gene regulatory molecules (transcription factors).A number of families of such factors sharing common sequence motifs have been identified, including the leucine zipper (Landschulz et al., 1988), zinc finger (Passananti et al., 1989), basic helix-loop-helix (Murre et al., 1989; Davis et al., 1990), POU domain (He et al., 1989; Rosenfeld, 1991), helix-turn-helix (Harrison and Aggarwal, 1990), and lin domain (Freyd et al., 1990) families of transcription factors.Members of virtually all of these families have been identified that display a tissue-specific distribution in vertebrates. These factors may interact with specific DNA regulatory sequences and/or with each other to direct the expression of both ubiquitous and tissue specific genes. In addition, other nuclear proteins which have regulatory roles distinct from direct transcriptional control, such as the scaffold-associated region (SAR) topoisomerases (Gasser and Laemmli, 1987) and the SnRNP-associated proteins (McAllister et al., 1988), may also be important to the determination of tissue specific traits.

The complexity of the vertebrate nervous system suggests that a vast array of regulatory mechanisms must interact to control the development and differentiation of this tissue. Thus, in addition to the expression of proteins specific to neuronal function, there must be a number of nervous system-specific nuclear regulatory proteins.Indeed, several nervous system-specific putative transcription factors have been identified (Milbrant, 1987; Chowdury et al., 1988; He et al., 1989; Korner et al., 1989; Wilkinson et al., 1989; Johnson et al., 1990; Treacy et al., 1991) which are members of the above classes of transcription regulatory molecules.Additional nervous system-specific molecules as well as additional classes of regulatory molecules are likely to exist.

We are giving the name NeuN (*Neu*ronal *N*uclei) to the protein detected by mAb A60.The nuclear localization, solubility and in vitro DNA-binding properties of NeuN are consistent with the idea that it may represent a nervous system-specific nuclear regulatory molecule. If NeuN is a regulatory molecule, the fact that its expression is associated with neuronal differentiation and persists throughout life, may indicate that it is a permanent regulator of neuron specific traits. However, the absence of expression in a few

neuronal cell types argues that other such molecules may regulate these traits in NeuN-negative neurons.

Without sequence data, we cannot exclude the possibility that NeuN may be a member of one of the known families of nuclear proteins. The distribution, developmentally regulated pattern of expression and relative molecular mass of NeuN, however, indicate that it is not identical to any previously reported protein. In addition, a number of monoclonal antibodies specific to the nervous system have also been described. As with NeuN itself, we have no evidence to suggest that mAb A60 is identical to any other mAb of which we are aware.

Our analysis of the NeuN protein warrants the following summation:(1) NeuN is expressed almost exclusively in the nervous system. (2) It appears early in development and persists in the adult. (3) Nervous system expression of NeuN is confined to neurons and is neuronal cell type-specific.(4) It is expressed in primary neuronal cultures and neuronal-like cells in retinoic acid stimulated P19 cells. (5) Native DNA-cellulose chromotography of brain nuclear protein suggests that NeuN protein is capable of DNA binding. Whether or not NeuN binds to DNA in vivo, remains to be determined**.**

Confirmation of our hypothesis that NeuN is a candidate for a regulatory molecule directly involved in neuronal differentiation and/or function awaits further biochemical characterization, purification and eventual cloning of the gene encoding this antigen. These efforts are underway. Meanwhile, mAb A60 has proved to be an early and robust marker of most maturing and differentiated neurons both in vivo and in vitro.

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