MOLECULAR AND CELLULAR BIOLOGY, Jan. 1985, p. 27–32 Vol. 5, No. 1
0270-7306/85/010027-06$02.00/0
Copyright © 1985, American Society for Microbiology

pp60$^{c\text{-}src}$ in the Developing Cerebellum

DANIEL W. FULTS,[1] ANDREW C. TOWLE,[2] JEAN M. LAUDER,[2] AND PATRICIA F. MANESS[3]*

Department of Neurosurgery, Bowman Gray School of Medicine, Winston-Salem, North Carolina 27103[1] and Departments of Anatomy[2] and Biochemistry,[3] University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

Received 12 July 1984/Accepted 19 September 1984

pp60$^{c\text{-}src}$ was localized in the cerebellum of developing chicken embryos by immunoperoxidase staining with antisera raised against bacterially expressed pp60$^{v\text{-}src}$. Immunoreactivity (IR) appeared in the cerebellum of the chicken embryos at the time of neuronal differentiation. pp60$^{c\text{-}src}$ IR was detected in regions of the developing cerebellum where processes of developing neurons and glia are located. In the early embryo (stage 17), pp60$^{c\text{-}src}$ IR was localized in the marginal zone of the cerebellar plate. By stage 40, pp60$^{c\text{-}src}$ IR was localized in the process-rich molecular layer of the cerebellum and between the cells of the developing internal granular layer. Cell bodies of cerebellar neurons did not show pp60$^{c\text{-}src}$ IR at any stage of development. Mitotically active neuroepithelial cells of the metencephalon did not express pp60$^{c\text{-}src}$ before the onset of differentiation in the early embryo, nor did proliferating cells of the external granular layer express pp60$^{c\text{-}src}$ at later stages. Although it is not possible to ascertain whether pp60$^{c\text{-}src}$ is localized in developing neurons or glia at the light microscope level, the time of its appearance and pattern of distribution in the molecular layer is suggestive of a localization within the developing neuronal processes which compose the bulk of this layer. Biochemical analyses of pp60$^{c\text{-}src}$ in the developing cerebellum by the immune complex protein kinase activity and sensitivity of the kinase to inhibition by P^1,P^4-di(adenosine-5′)tetraphosphate confirmed that the expression of pp60$^{c\text{-}src}$ coincided with the time of neuronal differentiation. We conclude from these results that in the central nervous system, pp60$^{c\text{-}src}$ may be more important in an aspect of cell differentiation or a mature neuronal function than in the proliferation of neuronal or glial precursors.

The oncogenes of acute transforming retroviruses are homologous to DNA sequences present in the genome of normal animal cells (1). These normal cellular oncogenes are highly conserved in vertebrate evolution and exist in a wide variety of animal species. The transforming gene of Rous sarcoma virus is closely related to the cellular oncogene c-*src* which encodes a 60,000-dalton phosphoprotein (pp60$^{c\text{-}src}$), a kinase (4, 29) specific for tyrosine residues (16). pp60$^{c\text{-}src}$ is expressed in elevated levels in neural tissues of both chickens (6, 25, 32, 33) and humans (18, 25) during organogenesis, but its function is unknown. Recently, we reported that pp60$^{c\text{-}src}$ in the chick neural retina is expressed in developing retinal neurons at the onset of differentiation at the time the cells cease proliferating (32, 33). Because high levels of pp60$^{c\text{-}src}$ are also present in the developing brain (6, 18, 25, 32, 33), we undertook the present study in an attempt to identify the specific cell types in the brain responsible for pp60$^{c\text{-}src}$ expression. The distribution of pp60$^{c\text{-}src}$ was examined in the chicken cerebellum at various stages of embryogenesis by immunocytochemical staining utilizing an antibody raised against bacterially expressed pp60$^{v\text{-}src}$, the product of the Rous sarcoma virus v-*src* gene. Because pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ have very close sequence homology (34), this antibody recognizes pp60$^{c\text{-}src}$ specifically in normal cells (11, 12). The cerebellum was chosen for this study because of its well-defined cytoarchitecture and pathways of cell migration occurring during neurogenesis (19). We found that pp60$^{c\text{-}src}$ was present chiefly in the developing molecular layer, which is composed of the processes of differentiating Purkinje cells and granule cells (30) as well as the radial processes of the Bergmann glia. A few neuronal cell bodies (basket and stellate cells) are also contained within this layer, but they are far less numerous than these neuropil components and appeared to be unstained.

MATERIALS AND METHODS

Preparation of antisera. The antiserum used for immunocytochemistry was raised in rabbits immunized against pp60$^{v\text{-}src}$, purified from bacteria expressing a molecularly cloned Rous sarcoma virus *src* gene (11). Bacterially expressed pp60$^{v\text{-}src}$ was purified from bacterial lysates by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (12). Rabbit antiserum was obtained after four boosts ca. 12 weeks after the initial injection. For use in immunocytochemistry, a partially purified immunoglobulin G fraction was prepared by precipitating the antiserum with 37% saturated ammonium sulfate, followed by extensive dialysis in phosphate-buffered saline (PBS) and lyophilization. Serum obtained from rabbits before immunization was treated in an identical fashion to obtain a preimmune immunoglobulin fraction for use as a control in immunocytochemical experiments. The immune serum used in these studies did not recognize the pp90$^{gag\text{-}yes}$ oncogene product of Y73 virus, as determined from Western transfer analysis of extracts of chicken embryo fibroblasts transformed by Y73 virus. Y73 virus was kindly provided by H. Hanafusa (Rockefeller University).

Immunocytochemistry. Chicken embryos negative for lymphoid leukosis virus (SPAFAS; Norwich, Conn.) were staged according to the Hamburger and Hamilton classification (13). Whole early embryos (stage 12 to 32) or brains dissected from stage 40 embryos were fixed by immersion in 4% (wt/vol) paraformaldehyde–70 mM sodium phosphate (pH 7.4)–7% (vol/vol) saturated picric acid for 24 h at 4°C. Hatchling chicks were anesthetized with ether, perfused

* Corresponding author.

through the heart with ice-cold fixative, and decapitated. The brain was removed and immersed in the same fixative at 4°C for 24 h. The fixed tissues were dehydrated in an ascending series of alcohol and toluene and embedded in Paraplast-plus. Sections were cut at 10 μm, mounted on glass slides, and dried at 45°C for 1 week. After deparaffinization, sections were treated with 1% (vol/vol) H_2O_2 in PBS for 10 min, followed by 0.6 mg of bovine pancreatic trypsin (40 U/mg of protein; Boehringer Mannheim) per ml of PBS for 3 min. After two rinses with PBS and a 10-min incubation with 2% normal sheep serum, the sections were incubated with the antibody (10 to 20 μg/ml) for 48 h at 4°C. A modification (34a) of the avidin-biotin complex technique (15) was used to localize pp60^{c-src} immunoreactivity (IR). After washing in PBS, the sections were incubated with a 1:500 dilution of biotinylated goat antirabbit antibody (Vectastain; Vector Laboratories) for 2 h at room temperature, followed by a 2-h incubation at room temperature with a complex of avidin and biotinylated peroxidase. Sections were developed with 0.075% (wt/vol) diaminobenzidine–0.002% H_2O_2 (vol/vol) for 10 min at room temperature. All sections were counterstained with toluidine blue. To demonstrate competition, the diluted antibody (10 to 20 μg/ml) was preincubated with bacterially expressed pp60^{v-src} (25 μg/ml) for 12 h at 4°C before application to the sections. Competition was carried out with a different preparation of pp60^{v-src} than that used for immunization.

Preparation of chicken cerebellum and brain extracts. Cerebella dissected from stage 40 chicken embryos or brains from hatchling chicks were homogenized in 4 volumes of ice-cold lysis buffer (25 mM potassium phosphate [pH 7.1], 1.25 mM EDTA, 0.6% [wt/vol] sodium deoxycholate, 1.25% [vol/vol] Nonidet P-40, 12.5 mM sodium fluoride, 125 Kallikrein inhibitor units of aprotinin per ml, 2.5 mM dithiothreitol, 0.2 M potassium chloride) by 10 strokes of a Dounce tissue grinder fitted with a loose pestle and 10 strokes with a tight pestle. Homogenates were centrifuged at 100,000 × g for 1 h, and the supernatants were stored at −70°C. Protein was determined by the Lowry method (26).

Immune complex protein kinase assay. Immune complex protein kinase assays for pp60^{c-src} protein kinase were carried out as described by Collett and Erikson (4). Briefly, pp60^{c-src} was immunoprecipitated under conditions of antibody excess from whole brain extracts with tumor-bearing rabbit (TBR) serum, prepared by the method of Brugge and Erikson (2), that crossreacted with cellular pp60^{c-src}. Immune complexes were isolated from the extracts with protein A-Sepharose (Pharmacia), washed extensively, and incubated with [γ-^{32}P]ATP (2,900 Ci/mmol; New England Nuclear Corp.) for 10 min at 37°C. Immunoglobulin G heavy chains were isolated on sodium dodecyl sulfate-polyacrylamide gels, and the extent of phosphorylation was quantitated by counting dried gel slices for ^{32}P in Aquasol (New England Nuclear Corp.). This assay measures the extent rather than the rate of reaction since the antibody and the kinase do not dissociate and maximal phosphorylation of the antibody is reached within a few minutes of incubation (31). Under conditions of antibody excess, the amount of antibody phosphorylation is thus a measure of the amount of pp60^{c-src} rather than initial velocity. Autoradiography of the gels did not reveal phosphorylated proteins other than the 53,000-M_r immunoglobulin heavy chains, indicating the absence of other cellular tyrosine kinases which carry out autophosphorylation reactions in the immunoprecipitates. In addition, the TBR serum used in the kinase assay did not immunoprecipitate purified epidermal growth factor (EGF) receptor (25), in agreement with the results of others (3, 22).

EGF was obtained from Kor Biochemicals, insulin was from Sigma Chemical Co., and platelet-derived growth factor (PDGF) was the gift of J. Pledger (University of North Carolina). Ammonium P^1,P^4-di(adenosine-5′)tetraphosphate (Ap$_4$A) (Sigma Chemical Co.) was treated as previously described to hydrolyze any ATP or ADP present (27). Where indicated, Ap$_4$A was added to the immune complex before the addition of [γ-^{32}P]ATP.

RESULTS

Immunocytochemical localization of pp60^{c-src} in the developing cerebellum. To localize pp60^{c-src} IR in the cerebellum, we incubated paraformaldehyde-fixed paraffin sections of representative cerebellar tissues of the chicken embryo with antibody raised against bacterially produced pp60^{v-src} and visualized the immune complexes formed by immunoperoxidase staining, using a modification (Towle et al., in press) of the avidin-biotin method (15). Specific pp60^{c-src} IR was not present in any part of the metencephalon of the stage 12 (day 2 of incubation) chicken embryo (Fig. 1A). The dorso-lateral border of the metencephalon, which gives rise to the cerebellar plate, showed a small amount of staining at the ventricular surface (V) that appeared to be nonspecific, because it could not be blocked by competition with purified antigen. The metencephalon at this state consists chiefly of undifferentiated, actively proliferating neuroepithelial cells (37). At about stage 17 (day 2.5), pp60^{c-src} IR appeared for the first time in the marginal zone (M) of the metencephalon (Fig. 1B), which is mainly composed of the processes of proliferating neuroepithelial cells and glia. In contrast, cell bodies within the ventricular zone (V) (mainly proliferating neuroepithelial cells) did not exhibit pp60^{c-src} IR at this stage. pp60^{c-src} IR was completely blocked by preincubation of the antibody with purified pp60^{v-src}.

At about stage 23 (day 4), the dorso-lateral border of the metencephalon (the rhombic lip) gives rise to the cerebellar plate (23). By stage 40 (day 14) of development, a layer of proliferating cells (the external granular layer [EGL]) has accumulated on the external surface of the cerebellar plate by migration from the region of the rhombic lip. The undifferentiated cells of the EGL undergo a series of cell divisions followed by migration into the molecular layer to form the small interneurons of the cerebellum—the granule, stellate, and basket cells (14). The EGL is present from day 6, with the most active period of proliferation occurring between 12 and 14 days (10, 17). At no time during the period of EGL development was pp60^{c-src} IR observed in this germinal zone, as illustrated in the stage 40 (day 14) cerebellum (Fig. 1C and D). The process-rich molecular layer, however, continued to exhibit pp60^{c-src} IR throughout this period. At this stage, the molecular layer contains migrating granule cells and their axons (parallel fibers), differentiating basket and stellate cells, and immature dendritic processes of the Purkinje cells (28). It is not possible at this resolution to distinguish whether all processes or specific processes in the molecular layer are stained. However, only cell processes seem to be IR since cell bodies within this layer were unstained. Another component of the developing molecular layer is the Bergmann glial cell, which exhibits radially oriented processes extending from the cell bodies, located near the Purkinje cells, to the outer surface of the cerebellum (30). These cells may also have been stained by the pp60^{c-src} antibody, but no radial pattern of staining was discernible. This at least suggests that if such glial staining occurs, it does so in conjunction with the staining of neuronal processes in this layer, obscuring the radial pattern.

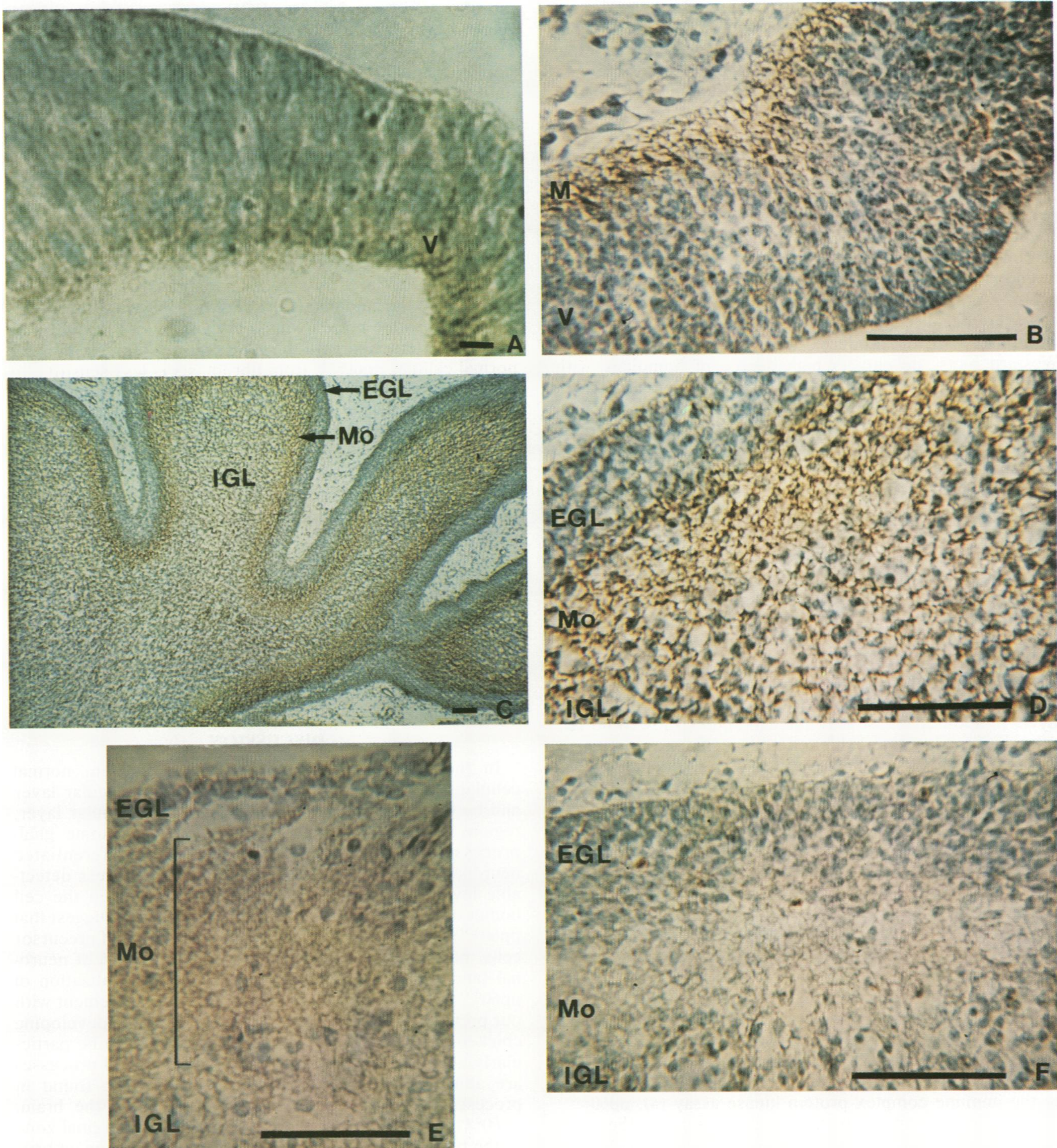

FIG. 1. Immunocytochemical staining of pp60$^{c\text{-}src}$ in the developing chicken cerebellum. (A) Stage 12 metencephalon, dorso-lateral region, transverse section. Dorsal surface at right. 10 μg of antibody per ml. (B) Stage 17 metencephalon, sagittal section. 20 μg of antibody per ml. (C) Stage 40 cerebellum, sagittal section. 13 μg of antibody per ml. (D) Stage 40 cerebellum, sagittal section. 13 μg of antibody per ml. (E) Adjacent section to (D) but with antibody preabsorbed with bacterially expressed pp60$^{v\text{-}src}$ (25 μg/ml) before application to the section. (F) Hatchling cerebellum, transverse section. 10 μg of antibody per ml. Sections were counterstained with toluidine blue. V, Ventricular zone; M, marginal zone; Mo, molecular layer; P, Purkinje cell layer; IGL, internal granular layer. Bars, 20 μM.

The developing internal granular layer of the stage 40 cerebellum also showed pp60$^{c\text{-}src}$ IR, which appeared to be contained in cellular processes surrounding the granule cell bodies (Fig. 1D). This staining, lighter in intensity than that found in the molecular layer, extended throughout the entire width of the internal layer (Fig. 1C). At this stage of development, the processes that are located in the internal granular layer consist chiefly of climbing fibers, mossy fibers, granule cell dendrites, Golgi cell axons and dendrites, and Purkinje cell axons. Like the cell bodies in the EGL and

molecular layer, those of the internal granular layer did not appear to exhibit pp60$^{c\text{-}src}$ IR. However, possible staining of the plasma membrane of the cell bodies in the internal granular layer could be obscured by the processes there.

At every stage examined, pp60$^{c\text{-}src}$ IR staining was blocked by preincubation of the antibody with the antigen, purified bacterially expressed pp60$^{v\text{-}src}$. Figure 1F demonstrates competition of the pp60$^{c\text{-}src}$ IR shown in Fig. 1D, an adjacent section through the stage 40 cerebellum. The virtually complete competition of immunostaining with low amounts of purified pp60$^{v\text{-}src}$ (25 μg/ml) suggests that the immunocytochemical staining specifically recognizes pp60$^{c\text{-}src}$ in the cerebellum. Because the antiserum was raised against denatured pp60$^{v\text{-}src}$, it is possible that a protein closely related to pp60$^{c\text{-}src}$ could also be recognized. This appears unlikely, however, because the antiserum used did not cross-react with pp90$^{gag\text{-}yes}$, the product of the *yes* gene of Y73 virus on Western blots, despite its high degree of homology with pp60$^{v\text{-}src}$, particularly in the tyrosine kinase domain (21).

At hatching, the chick cerebellum begins to assume a more adult morphology, except for remnants of the EGL which soon disappear. pp60$^{c\text{-}src}$ IR was still apparent in the widening molecular layer of the hatchling chick cerebellum (Fig. 1E), where the density of neuronal processes has increased considerably and synaptogenesis is under way (28). It is noteworthy that the intensity of staining in the molecular layer of the cerebellum of the chick at the hatchling stage was considerably lighter than that observed at stage 17 or 40. This was always observed even when sections from each of the three stages were processed simultaneously for staining. No staining was observed in the undifferentiated cells remaining in the EGL or within the cell bodies of differentiating granule cells, either present in the internal granular layer or migrating through the molecular layer. In addition, most Purkinje cell bodies showed no IR, but occasionally some exhibited nonspecific staining which could not be blocked by competition with purified pp60$^{v\text{-}src}$. Not shown is the central white matter of the hatchling cerebellum, which consists primarily of climbing fibers, mossy fibers, Purkinje axons, and myelin-forming oligodendrocytes. In contrast to IR in the molecular layer, which was completely blocked with antigen, the central white matter exhibited nonspecific staining, lighter in intensity than that of the molecular layer, which could not be blocked and may be attributable to nonspecific staining of myelin sheaths developing at this stage around some of the axons.

Efforts to demonstrate immunoreactive pp60$^{c\text{-}src}$ in the cerebellum of the adult chicken were unsuccessful.

pp60$^{c\text{-}src}$ protein kinase activity in the developing cerebellum. The presence of pp60$^{c\text{-}src}$ in the developing cerebellum was also demonstrated by immunoprecipitation from cerebellar extracts and measurement of protein kinase activity by the immune complex protein kinase assay (4). pp60$^{c\text{-}src}$ was immunoprecipitated from detergent lysates of the cerebellum of stage 40 chicken embryos and hatchlings with TBR serum under conditions of antibody excess. The phosphorylation of antibody heavy chains by pp60$^{c\text{-}src}$ was carried out in the immunoprecipitates. Because this assay measures the extent rather than the rate of reaction, the amount of phosphorylation of antibody heavy chains is related to the amount of pp60$^{c\text{-}src}$ kinase in the immunoprecipitate rather than its initial velocity (31). The amount of pp60$^{c\text{-}src}$ kinase relative to total protein in the stage 40 cerebellum (100 fmol of phosphate incorporated into immunoglobulin G heavy chains per mg of extract protein) was about sevenfold higher than in the hatchling cerebellum (15 fmol of phosphate per mg of extract protein). The EGF receptor (35), the PDGF receptor (9), and the insulin receptor (20), which have related tyrosine kinase activities that are activated upon hormone binding, did not appear to contribute to the TBR antibody phosphorylation observed in these assays. The addition of EGF (164 nM), PDGF (72 nM), or insulin (86 nM) to immunoprecipitates from stage 36 chicken embryo brain extracts did not stimulate antibody phosphorylation: no addition (40 fmol of phosphate per mg of extract protein); EGF (37 fmol/mg); PDGF (45 fmol/mg); insulin (40 fmol/mg).

To provide confirmation that the enzyme activity measured in the cerebellar extracts was due to pp60$^{c\text{-}src}$, the sensitivity of the pp60$^{c\text{-}src}$ protein kinase to the dinucleotide Ap_4A was compared in cerebellar extracts of the stage 40 embryo and the hatchling chick. Previously, we showed that Ap_4A inhibits the protein kinase activity of viral pp60$^{v\text{-}src}$ by apparent interaction with the ATP binding site (27) and that normal cellular pp60$^{c\text{-}src}$ from fibroblasts is less sensitive to inhibition than pp60$^{v\text{-}src}$ (24). pp60$^{c\text{-}src}$ from the stage 40 and hatchling cerebellar extracts was immunoprecipitated with TBR serum, and phosphorylation of TBR antibody heavy chains in the immunoprecipitate was measured in the presence of various concentrations of Ap_4A and 50 nM ATP. The concentration of Ap_4A required for 50% inhibition of protein kinase activity was found to be the same (15 μM) for the stage 40 and hatchling cerebellar kinases. These values were significantly greater than the concentration of Ap_4A required for 50% inhibition of pp60$^{v\text{-}src}$ protein kinase encoded by the virus (2 μM), which was immunoprecipitated in parallel with the same TBR antiserum from extracts of chicken embryo fibroblasts transformed by Rous sarcoma virus (Schmidt-Ruppin D strain). Thus, the cerebellar enzyme, like pp60$^{c\text{-}src}$ from fibroblasts, is less sensitive to inhibition by Ap_4A than the viral enzyme.

DISCUSSION

In the cerebellum of the developing chicken, normal cellular pp60$^{c\text{-}src}$ IR was found within the molecular layer and between the cell bodies of the internal granular layer, regions which contain mainly neuronal (and some glial) processes; whereas mitotically active, undifferentiated neuronal recursor cells in the EGL did not express detectable levels of pp60$^{c\text{-}src}$, nor was staining seen in the cell bodies of differentiating neurons. These findings suggest that pp60$^{c\text{-}src}$ does not participate in the proliferation of precursor cells, but could be involved in the differentiation of neuronal (and possibly glial) cell processes. The localization of pp60$^{c\text{-}src}$ in processes of cerebellar cells is in agreement with our previous work on pp60$^{c\text{-}src}$ expression in the developing chicken neural retina, which showed pp60$^{c\text{-}src}$ to be particularly abundant in plexiform layers where neural processes are abundant (32, 33). pp60$^{c\text{-}src}$ has also been found in processes of cells located in other regions of the brain: pp60$^{c\text{-}src}$ IR was localized in the process-rich marginal zone of the telencephalon and optic nerve of the chicken embryo (33) and in fiber tracts of the optic tectum of the stage 40 embryo (P. F. Maness, unpublished data). The appearance of pp60$^{c\text{-}src}$ within processes of these embryonic brain cells suggests that pp60$^{c\text{-}src}$ is a component of developing neuronal processes. However, pp60$^{c\text{-}src}$ appears not to be restricted to nerve processes, since it was also present in the cell bodies of retinal ganglion and amacrine cells (33). This cell body localization does not appear to be the case for most parts of the nervous system.

The expression of pp60$^{c\text{-}src}$ in the molecular layer of the developing chicken cerebellum correlated with neuro-

nal differentiation rather than proliferation. In the retina, pp60$^{c\text{-}src}$ expression also occurred at the time when the first neuronal cells ceased dividing and began to differentiate. The conspicuous absence of IR pp60$^{c\text{-}src}$ from the primitive neuroepithelial cells of the metencephalon and the cerebellar EGL, both of which are actively proliferating, indicates that pp60$^{c\text{-}src}$ is not likely to be required for cell division in these brain regions. It is interesting to note that somatomedin C (a growth factor for fibroblasts) is expressed in the cell bodies and processes of differentiating neurons in culture (V. Han personal communication). A role for other cellular oncogenes in cell proliferation is suggested by recent studies demonstrating homology between the EGF receptor and the v-*erb*-B sequence (8), and between PDGF and the v-*cis* sequence (7, 36). If pp60$^{c\text{-}src}$ plays any role in neuronal stem cell proliferation, it could conceivably regulate the transition from proliferation to differentiation, which occurs at the time of pp60$^{c\text{-}src}$ expression. Alternatively, activation of pp60$^{c\text{-}src}$ expression at the onset of neuronal differentiation may occur in preparation for its role in a mature neuronal function.

An important unanswered question is whether expression of pp60$^{c\text{-}src}$ in the developing nervous system decreases per cell during the embryonic to adult transition. pp60$^{c\text{-}src}$ IR in the hatchling cerebellum was considerably lighter than in the stage 40 cerebellum, and IR was not detectable in the adult. Furthermore, the amount of pp60$^{c\text{-}src}$ kinase activity relative to total protein in the hatchling and adult cerebellum was markedly lower than in the stage 40 cerebellum. Because pp60$^{c\text{-}src}$ kinase activity was quantitated relative to total protein, the lower amount of pp60$^{c\text{-}src}$ kinase in the hatchling and adult could reflect a developmental decrease in pp60$^{c\text{-}src}$ synthesis, the induction of synthesis of other proteins during differentiation, or the emergence of a population of cells, such as glia, which may not express high levels of pp60$^{c\text{-}src}$. Similarly, several explanations can be offered to account for the decreased intensity of IR staining in the cerebellum from stage 40 to hatching. The density of pp60$^{c\text{-}src}$ in processes of embryonic neurons may decrease as they grow longer during development, resulting in progressively lighter staining; pp60$^{c\text{-}src}$ IR may decrease as a result of an interaction with new proteins or lipid components in the mature neuron; or the synthesis of pp60$^{c\text{-}src}$ may decrease as a result of developmental changes in gene expression.

At this time, the functional role of pp60$^{c\text{-}src}$ in the developing nervous system is unknown. Its possible involvement in differentiating neurons raises questions as to the role of oncogenes in the control of differentiated functions in all cells, since the neuron is but one example in which proliferation is clearly separate from the differentiation process at the time of oncogene expression. It is possible, therefore, that the developing nervous system, with its well-studied cytoarchitectonics, may provide a good model system for the study of roles for oncogenes in proliferation and differentiation-related cell functions generally.

ACKNOWLEDGMENTS

We thank Laurie K. Sorge for her generous assistance and helpful advice. Tona Gilmer is gratefully acknowledged for providing the stock of bacteria containing a plasmid with the molecularly cloned v-*src* gene. The expert technical assistance of David Hillsgrove is gratefully acknowledged.

This work was supported by National Science Foundation grant PCM-8203857 (P.F.M.), American Cancer Society grant BC-416 (P.F.M.), and Public Health Service grant NS-15706 (J.M.L.) from the National Institutes of Health.

LITERATURE CITED

1. **Bishop, J. M., and H. Varmus.** 1982. RNA tumor viruses, 2nd ed., p. 999–1108. Cold Spring Harbor Laboratory, Cold Spring, N.Y.
2. **Brugge, J. S., and R. L. Erikson.** 1977. Identification of a transformation specific antigen induced by an avian sarcoma virus. Nature (London) **269:**346–348.
3. **Chinkers, V., and S. Cohen.** 1981. Purified EGF receptor-kinase interacts specifically with antibodies to Rous sarcoma virus transforming protein. Nature (London) **290:**516–519.
4. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. U.S.A. **75:**2021–2024.
5. **Collett, M. S., E. Erikson, A. F. Purchio, J. S. Brugge, and R. L. Erikson.** 1979. A normal cell protein similar in structure and function to the avian sarcoma virus transforming gene product. Proc. Natl. Acad. Sci. U.S.A. **76:**3159–3163.
6. **Cotton, P. C., and J. S. Brugge.** 1983. Neural tissues express high levels of the cellular *src* gene product pp60$^{c\text{-}src}$. Mol. Cell. Biol. **3:**1157–1162.
7. **Doolittle, R. F., M. W. Hunkapiller, L. E. Hood, S. G. DeVare, K. C. Robbins, S. A. Aaronson, and H. N. Antoniades.** 1983. Simian sarcoma virus *onc* gene, v-*sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science **221:**275–277.
8. **Downward, J., Y. Yarden, E. Mayes, G. Scrace, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger, and M. D. Waterfield.** 1984. Close similarity of epidermal growth factor receptor and v-*erb*-B oncogene protein sequences. Nature (London) **307:**521–526.
9. **Ek, B., B. Westermark, A. Wasteson, and D. Heldin.** 1982. Stimulation of tyrosine-specific phosphorylation by platelet-derived growth factor. Nature (London) **295:**419–420.
10. **Fujita, S.** 1964. Analysis of neuron differentiation in the central nervous system by tritiated thymidine autoradiography. J. Comp. Neurol. **122:**311–328.
11. **Gilmer, T. M., and R. L. Erikson.** 1981. Rous sarcoma virus transforming protein, pp60^{src} expressed in *E. coli*, functions as a protein kinase. Nature (London) **294:**771–773.
12. **Gilmer, T. M., and R. L. Erikson.** 1983. Development of anti-pp60$^{c\text{-}src}$ serum antigen produced in *Escherichia coli*. Cell **46:**249–257.
13. **Hamburger, V., and H. L. Hamilton.** 1951. A series of normal stages in the development of the chick embryo. J. Morphol. **88:**49–92.
14. **Hanaway, J.** 1967. Formation and differentiation of the external granular layer of chick cerebellum. J. Comp. Neurol. **131:**1–14.
15. **Hsu, S. M., L. Raine, and H. Fanger.** 1981. Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unrelated antibody (PAP) procedures. J. Histochem. Cytochem. **29:**577–580.
16. **Hunter, T., and B. Sefton.** 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. **77:**1311–1315.
17. **Ishida, H.** 1973. Fine structure of the embryonal granular cells of the chick cerebellum. Tohoku J. Exp. Med. **109:**261–280.
18. **Jacobs, C., and H. Ruebsamen.** 1983. Expression of pp60$^{c\text{-}src}$ protein kinase in adult and fetal human tissue: high activities in some sarcomas and mammalian carcinomas. Cancer Res. **43:**1696–1702.
19. **Jacobson, Marcus.** 1978. Developmental neurobiology, 2nd ed., p. 76–91. Plenum Publishing Corp., New York.
20. **Kasuga, M., Y. Zick, D. L. Blithe, M. Crettaz, and C. R. Kahn.** 1982. Insulin stimulated tyrosine phosphorylation of the insulin receptor in a cell-free system. Nature (London) **298:**667–669.
21. **Kitamura, N., A. Kitamura, K. Toyoshima, Y. Hirayama, and M. Yoshida.** 1982. Avian sarcoma virus Y73 genome sequence and structural similarity of its transforming gene product to that of Rous sarcoma virus. Nature (London) **297:**205–208.
22. **Kudlow, J. E., J. E. Buss, and G. N. Gill.** 1981. Anti-pp60^{src} antibodies are substrates for EGF-stimulated protein kinase.

Nature (London) **209**:519–521.

23. **Larsell, O.** 1948. The development and subdivisions of the cerebellum of birds. J. Comp. Neurol. **89**:123–189.
24. **Levy, B. T., L. K. Sorge, C. C. Drum, and P. F. Maness.** 1983. Differential inhibition of cellular and viral pp60^{src} kinase by P^1,P^4-di(adenosine-5′)tetraphosphate. Mol. Cell. Biol. **3**: 1718–1723.
25. **Levy, B. T., L. K. Sorge, A. Meymandi, and P. F. Maness.** 1984. pp60^{c-src} kinase in embryonic tissues of chick and human. Dev. Biol. **104**:9–17.
26. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
27. **Maness, P. F., M. C. Perry, and B. T. Levy.** 1983. P^1-P^4-Di(adenosine-5′)tetraphosphate inhibits phosphorylation of immunoglobulin G by Rous sarcoma virus pp60^{src}. J. Biol. Chem. **251**:4055–4058.
28. **Mugnaini, E.** 1969. Ultrastructural studies on the cerebellar histogeneis. II. Maturation of nerve cell populations and establishment of synaptic connections in the cerebellar cortex of the chick, p. 749–782. *In* R. Llinás (ed.), Neurobiology of cerebellar evolution and development. American Medical Association Education and Research Foundation, Chicago.
29. **Opperman, H., A. D. Levinson, H. E. Varmus, L. Levintow, and J. M. Bishop.** 1979. Uninfected vertebrate cells contain a protein that is closely related to the product of the avian sarcoma virus transforming gene (src). Proc. Natl. Acad. Sci. U.S.A. **76**:1804–1808.
30. **Palay, S., and V. Chan-Palay.** 1974. The cerebellar cortex. Springer-Verlag, Berlin.
31. **Richert, N. D., D. L. Blithe, and I. Pastan.** 1982. Properties of the *src* kinase purified from Rous sarcoma virus rat tumors. J. Biol. Chem. **257**:7143–7150.
32. **Sorge, L. K., B. T. Levy, T. M. Gilmer, and P. F. Maness.** 1984. pp60^{c-src} expression in embryonic nervous tissue: immunocytochemical localization in the developing chick retina, p. 117–122. *In* A. J. Levine, G. F. Vande Woude, W. C. Topp, and J. D. Watson (ed.), Cancer cell I: the transformed phenotype. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Sorge, L. K., B. T. Levy, and P. F. Maness.** 1984. pp60^{c-src} is developmentally regulated in the neural retina. Cell **36**:249–257.
34. **Takeya, T., and H. Hanafusa.** 1982. DNA sequence of the viral and cellular *src* gene of chickens. II. Comparison of the *src* genes of two strains of avian sarcoma virus and of the cellular homolog. J. Virol. **44**:12–18.

34a. **Towle, A. C., J. M. Lauder, and T. H. Joh.** 1984. Optimization of tyrosine hydroxylase immunocytochemistry in paraffin sections using pretreatment with proteolytic enzymes. J. Histochem. Cytochem. **32**:766–770.

35. **Ushiro, H., and S. Cohen.** 1980. Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes. J. Biol. Chem. **255**:8363–8365.
36. **Waterfield, M. D., G.T. Scrace, N. Whittle, P. Stroobant, A. Johnsson, A. Wasteson, B. Westermark, C.-H. Heldin, J. S. Huang, and T. F. Deuel.** 1983. Platelet-derived growth factor is structurally related to the putative transforming protein p28^{sis} of simian sarcoma virus. Nature (London) **304**:36–39.
37. **Yurkewicz, L., J. M. Lauder, M. Marchi, and E. Giacobini.** 1982. ^{3}H-thymidine long survival autoradiography as a method for dating the time of neuronal origin in the chick embryo: the locus coeruleus and cerebellar Purkinje cells. J. Comp. Neurol. **203**:257–267.