Multiple Synapsin I Messenger RNAs Are Differentially Regulated during Neuronal Development

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Abstract. Synapsin I is a neuron-specific protein consisting of two isoforms Ia and Ib. It is thought to play a role in the regulation of neurotransmitter release. In this study the structure and expression of two classes of synapsin I mRNA have been examined. The two mRNA classes have molecular sizes of 3.4 and 4.5 kb, respectively. Both classes translate into synapsin I polypeptides and display a high degree of base sequence homology. Utilizing an oligonucleotide-directed RNase H assay we have shown that both mRNA classes have a common start site of transcription and differ from one another toward their 3′ ends. The expression of the two synapsin I mRNA classes is differentially regulated during the development of the rat brain and cerebellum. In the cerebellum the 4.5-kb transcript is expressed until postnatal day 7, after which it decreases to an undetectable level. The 3.4-kb mRNA is found throughout cerebellar development and in the adult. This suggests that the 3.4-kb mRNA class consists of messages which can encode both synapsin I polypeptides. Using quantitative Northern blot analysis a peak in the expression of this mRNA was observed at postnatal day 20. The maximum expression of the 3.4-kb class coincides with the period of synaptogenesis in the cerebellum. In addition to the developmental time course of synapsin mRNA expression a description of its spatial distribution throughout the cerebellum was performed using in situ hybridization histochemistry. From postnatal day 15 onwards, with a maximum at postnatal day 20, synapsin mRNA was localized in the internal granule cell layer of the cerebellum. On a cellular level, the granule cells, but not the neighboring Purkinje cells, express high levels of synapsin mRNA. These observations implicate developmentally coordinated differential RNA splicing in the regulation of neuron-specific gene expression and substantiate the correlation of synapsin gene expression with the period of synaptogenic differentiation of neurons.

THE nervous system offers an interesting system for the study of gene expression. It consists of a complicated network of highly specialized cell types whose development requires the precise regulation of the expression of subsets of genes. The phosphoprotein synapsin I, which is specifically expressed in neurons, is a good model for the investigation of neuron-specific gene expression. It is composed of two similar polypeptides of 74 and 78 kD, called synapsin Ia and Ib, respectively (Ueda and Greengard, 1977). Immunocytochemical studies have localized synapsin to the presynaptic terminal of virtually all neurons, where it is associated with the cytoplasmic surface of small synaptic vesicles (DeCamilli et al., 1983*a*, *b*; Navone et al., 1984). The protein is phosphorylated by both cAMP-dependent and Ca^{2+}/calmodulin-dependent protein kinases, and its state of phosphorylation is altered by conditions that affect neuronal activity (for a review, see Nestler and Greengard, 1984). Nerve cell depolarization has been shown to induce the phosphorylation of synapsin (Nairn et al., 1985). In vitro this phosphorylation leads to a reduction of the affinity between synapsin and synaptic vesicle membranes (Huttner et al., 1983; Schiebler et al., 1986). These and other data have led to the proposal that synapsin may play a role in neurotransmitter release. This hypothesis has been supported recently by the experiments of Llinas et al. (1985), in which the injection of synapsin or the Ca^{2+}/calmodulin-dependent protein kinase into the squid giant axon caused a change in postsynaptic potential.

Additional insight into the functional role of synapsin in the nerve terminal has come from studies that suggest that synapsin may interact with neuronal cytoskeletal elements. Baines and Bennett (1985) demonstrated that synapsin is immunologically related to the erythrocyte cytoskeletal protein 4.1 and is also a spectrin-binding protein. More recently synapsin has been shown to contain a potential actin-binding site (McCaffery and DeGennaro, 1986) and to bundle F-actin in a phosphorylation-dependent manner in vitro (Bähler and Greengard, 1987).

cDNA clones complementary to synapsin mRNA have

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been isolated and used to identify two synapsin mRNA species (Kilimann and DeGennaro, 1985), to determine the primary structure of synapsin (McCaffery and DeGennaro, 1986), and to localize the gene encoding synapsin to the human X chromosome (Yang-Feng et al., 1986). We report here the application of these clones as probes to study the molecular nature of the mRNAs encoding synapsin and the regulation of their expression during neuronal development. The relationship between multiple synapsin mRNA species has been investigated by the application of a technique involving oligonucleotide-directed cleavage of the mRNAs by RNase H. The rat cerebellum was chosen as a model system for the developmental study because it matures postnatally and has simple cellular architecture. We have used quantitative Northern blot analysis to assay changes in synapsin gene expression during the development of the cerebellum. In addition, in situ hybridization histochemistry was applied to provide further insight into the temporal and spatial appearance of the synapsin mRNA in individual neurons.

Materials and Methods

Isolation of RNA

Total RNA was isolated from brain and cerebella of rats of various ages using a guanidinium/cesium chloride method (Glisin et al., 1974). The cerebella of newborn pups were dissected under the microscope to avoid any contamination with other brain tissue. The tissue was homogenized in 6 M guanidinium isothiocyanate and centrifuged at 20°C, 35,000 rpm through a 5.7 M CsCl cushion. The resulting RNA pellet was resuspended in 10 mM Tris/HCl, pH 7.5, 5 mM sodium citrate, and 1% SDS. The RNA was further purified by butanol/chloroform extraction and ethanol precipitation. The final yield of RNA ranged between 0.25 and 0.50 μg RNA/mg tissue, wet weight.

Probes and Conditions for Hybridization

The probes for the RNA blot experiments were produced from restriction fragments 5E2 and 5E3 of the synapsin cDNA clone pSyn5 and from synapsin cDNA clone pSyn13 (Kilimann and DeGennaro, 1985). Labeling was performed with [^{32}P]dCTP by nick-translation to a specific activity of 1–2 × 10^8 cpm/μg of cDNA (Rigby, 1977). Filters were prehybridized for 3–5 h at 50°C in a solution containing 50% formamide, 5× Denhardt's, 4× SSPE (0.6 M NaCl, 40 mM NaH_2PO_4, pH 7.4, 4 mM EDTA), 0.1% SDS, and 100 μg/ml sonicated salmon sperm DNA. Hybridization was then carried out overnight at 50°C in the same solution, with the addition of 2–3 × 10^6 cpm/ml of heat-denatured, nick-translated probe. After hybridization the filters were washed three times for 15 min in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M NaCl sodium citrate), 0.1% SDS, and once for 15 min in 0.2× SSC, 0.1% SDS, all at 60°C, before exposure to x-ray film (Eastman Kodak Co., Rochester, NY).

RNase H Digestion

The protocol of Donis-Keller (1979) was modified and applied for the RNase H digestion of poly(A)$^+$ RNA from 10-d-old rat brain, directed by a 19-base synapsin-specific oligonucleotide (see Results). Each reaction mixture (10 μl) contained 2–4 μg of poly(A)$^+$ RNA and 0.02–0.2 μg of oligonucleotide in 50 mM Tris/HCL, pH 8.0, 4 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.03 mg/ml BSA (RNase-free, Pharmacia Fine Chemicals, Piscataway, NJ). The reaction mixtures were heated to 50°C for 10 min. Mixtures were incubated a further 10 min at 37°C, 0.03 U of RNase H (*Escherichia coli* SK 780, Pharmacia Fine Chemicals), was added, and the reactions were allowed to proceed at 37°C for 30 min. The digestion was stopped by addition of 2 μl of 0.2 M EDTA. Control reactions contained poly(A)$^+$ RNA and RNase H, but no oligonucleotide, and were carried through the same procedure. The reaction volume was adjusted to 20 μl with sterile H_2O. The RNA was precipitated after the addition of 2 μl of 3 M sodium acetate (pH 5.5) and 65 μl of 95% ethanol by cooling in a dry ice-ethanol bath for 15 min. The precipitate was recovered by centrifugation at 12,000 rpm and 4°C. RNA pellets were dried briefly under vacuum, denatured by glyoxylation, and further analyzed by agarose gel electrophoresis and blotting as described by Thomas (1980). Sizes of the resulting RNA fragments were determined by comparison with the migration of ethidium bromide-stained, RNA size standards (Bethesda Research Laboratories, Gaithersburg, MD).

Quantitative Northern Blot Analysis

RNA was denatured by glyoxylation, electrophoresed on a 1% agarose gel, and blotted on nitrocellulose as described by Thomas (1980). Each gel contained a serial dilution of purified 28S and 18S ribosomal RNA from calf liver (Pharmacia Fine Chemicals) applied as standards adjacent to cerebellar RNA samples. After hybridization with the synapsin-specific probe and autoradiography, the blots were subsequently hybridized with an 18S ribosomal RNA-specific cDNA probe (Grummt et al., 1979) which had been labeled with [^{32}P]dCTP by nick-translation. The autoradiogram of the rRNA dilution series was scanned densitometrically (model DU-8 spectrophotometer, Beckman Instruments, Inc., Palo Alto, CA), and the peak areas were plotted vs. the amount of rRNA loaded, to give a standard curve. The actual amount of total cerebellar RNA loaded in each lane could be determined by comparing the densitometrically obtained value of 18S cerebellar rRNA with this standard. The signal obtained with the synapsin-specific probe was then corrected relative to the amount of total RNA loaded in each lane. Exposure times of the film were chosen to be within the linear range for densitometric scanning.

To confirm the reliability of the quantification based on hybridization with a 18S rRNA-specific probe, a second method using ethidium bromide was applied (Eiden et al., 1984; Biguet et al., 1986). Northern gels were run in duplicate with serial dilutions of rRNA standards (see above) and cerebellar total RNA samples. One-half of the gel was stained with ethidium bromide (30 μg/ml) for 30 min and washed extensively with 40 mM *N*-morpholinopropane sulfonic acid (MOPS), pH 7.0, 10 mM sodium acetate, 1 mM EDTA for several hours. The other duplicate of the gel was used for Northern transfer. The negative images of UV-transilluminated photographs of the ethidium bromide-stained gel were densitometrically scanned. The areas of the peaks from 28S and 18S rRNA bands of the cerebellar samples were compared with those of the serial dilution of standard rRNA. The actual amount of RNA loaded in the cerebellar samples could thus be calculated and used to standardize the synapsin specific signal.

In Situ Hybridization Histochemistry

Cerebella of young rats of various ages were fixed by immersion in Bouin's (picric acid, formaldehyde, acetic acid) solution for 4–12 h depending on the size of the tissue, cleared with 70% ethanol, dehydrated, and embedded in paraffin. Sections of 10 μm were cut, deparaffinized in xylene, and rehydrated. Pretreatment of the tissue was performed as follows: PBS (10 mM, pH 7.5) rinse twice for 5 min; 0.2 N HCl for 10 min at room temperature; PBS rinse twice for 3 min; 1 μg/ml proteinase K (Boehringer Mannheim Diagnostics, Houston, TX) in 20 mM Tris/HCl, pH 7.5, and 2 mM $CaCl_2$ at 37°C for 15 min; PBS with 2 mg/ml glycine twice for 3 min; postfixation with 4% buffered paraformaldehyde for 5 min at room temperature; and a final wash in PBS/glycine twice for 3 min. To reduce nonspecific binding, slices were acetylated according to Hayashi et al. (1978) for 10 min at room temperature, washed in H_2O, and dehydrated. Control sections were incubated for 30 min at 37°C with 100 μg/ml RNase A (Boehringer Mannheim Diagnostics) in 0.5 ml NaCl and 10 mM Tris/HCl, pH 7.5. Prehybridization (4–6 h) and hybridization (overnight) were carried out in the following buffer: 50% recrystallized deionized formamide, 2× SSC, 1 mM EDTA, 5× Denhardt's, 0.1 M DTT, 0.5 mg/ml salmon sperm DNA, 0.5 mg/ml heparin, 50 μg/ml tRNA from baker's yeast, and 5 μM unlabeled α-thio-UTP at 45°C in a moist chamber. Hybridization was performed in a volume of 8 μl containing 100,000 cpm of ^{35}S-labeled single-stranded cRNA probe (see below). During the hybridization the slices were covered with siliconized, heat-treated coverslips. After careful removal of coverslips, the slides were first washed with 2× SSC for 2 h at 50°C and then with 0.2× SSC at 50°C until radioactivity was no longer detectable with a Geiger counter. Sections were then treated with RNase A 10 μg/ml at 37°C for 30 min. Slices were dehydrated through a graded series of ethanol washes containing 0.3 M ammonium acetate, dipped in Kodak NTB-2 photoemulsion (diluted 1:2 in H_2O), exposed for 4-7 d, and finally developed in Kodak D 19 for 2 min. As a last step the sections were counterstained with thionin.

Preparation of Single-stranded RNA Probes for In Situ Hybridization

Single-stranded RNA probes were produced by transcription from DNA

templates derived by insertion of the 5E2 fragment of synapsin cDNA into the plasmid vector pSP65 (Promega Biotec, Madison, WI). The two clones used contained the 5E2 fragment in opposite orientations with respect to the SP6 promoter. Transcription with SP6 polymerase yielded transcripts that were sense (identical to mRNA) and anti-sense (complementary to mRNA). Transcription reactions were performed with *Bgl II* (anti-sense)- and *Acc I* (sense)-linearized plasmids in the presence of [^{35}S]UTP, which yielded probes of 132 and 129 nucleotides, respectively. The specific activity of the ^{35}S-labeled RNA probes ranged from 1 to 3 × 10^8 cpm/μg template.

Hybridization with [^{3}H]Polyuridilate

To estimate the poly(A)$^+$ RNA content of the cerebellar neurons with respect to increasing age, hybridization with [^{3}H]polyuridilate ([^{3}H]poly(U))[1] was performed. The protocol of Griffin et al. (1985) was applied with slight variations. After fixation and pretreatment the sections were hybridized at 20°C overnight in 10 μl of hybridization solution (10 mM Tris/HCl, pH 7.6, 200 mM NaCl, 5 mM $MgCl_2$, 25% formamide, 500 μg/ml salmon sperm DNA, 100 μg/ml tRNA) containing 40,000 cpm [^{3}H]poly(U), 3 Ci/mmol (New England Nuclear, Boston, MA). Washing was done in 50 mM Tris/HCl, pH 7.6, 10 mM KCl, 1 mM $MgCl_2$ at 50°C for 1 h and at room temperature for 6 h. The sections were then further processed as described above.

Results

Two mRNA Classes Encode Synapsin

Previous experiments had demonstrated the existence of two synapsin mRNA classes in 10-d-old rat brain, 4.5 and 5.8 kb in length (Kilimann and DeGennaro, 1985). Using more accurate RNA standards (RNA ladder, Bethesda Research Laboratories) we now estimate the size of the two mRNA classes to be 3.4 and 4.5 kb (data not shown). The two transcripts will be designated by these newly determined sizes throughout the rest of this paper.

Several lines of experimental evidence demonstrate that both mRNA classes encode synapsin polypeptides. When mRNA is purified by immunoabsorption of rat brain polysomes with synapsin-specific antibodies, both the 3.4- and 4.5-kb mRNAs are isolated. This could only occur if both RNA classes were associated with polysomes bearing nascent synapsin polypeptides. Furthermore, in vitro translation reactions programmed by the immunoadsorbed mRNAs direct the synthesis of both synapsin polypeptides (Kilimann and DeGennaro, 1985). Finally, the data presented in Fig. 1 demonstrate a large extent of base sequence homology between the two synapsin mRNA classes. Hybridization probes were made by nick-translation of isolated segments of the synapsin cDNA clone pSyn5, which is a near full-length copy of the 3.4-kb synapsin mRNA (see below). When hybridized to Northern blots of total poly(A)$^+$ RNA from 10-d-old rat brains, each probe recognized both the 3.4- and 4.5-kb mRNAs. The similar ratio of intensity of the hybridization signals for each RNA class with each probe indicates substantial sequence homology between the two messenger RNA classes.

Structural Analysis of Synapsin mRNAs by Oligonucleotide-directed RNase H Cleavage

We have employed specific oligonucleotide-directed RNase H digestion, followed by RNA blot analysis of the resulting products, to address more carefully the characteristics of the

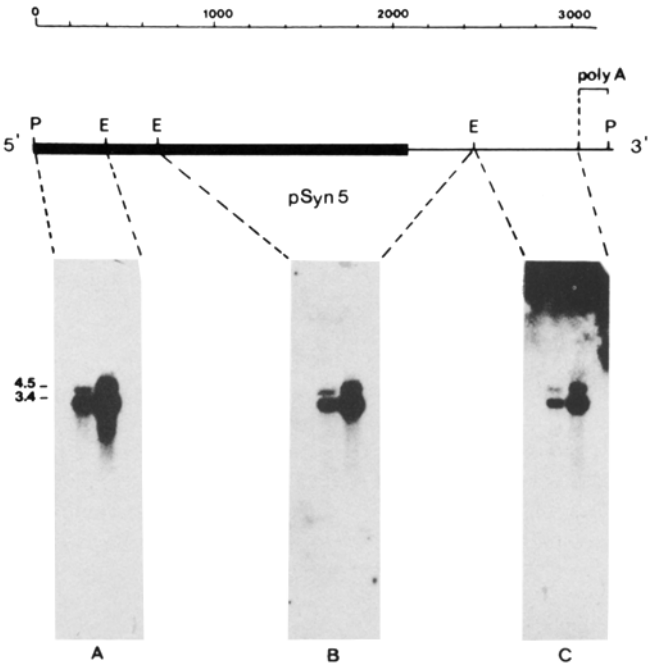

Figure 1. Homology between the two synapsin mRNA classes. (*A–C*) Autoradiograms of RNA blot hybridizations between total poly(A)$^+$ RNA of 10-d-old rat brain and nick-translated probes complementary to three regions of the synapsin-specific cDNA pSyn5. Scale at the top is in base pairs. Protein-coding region in pSyn5 is indicated by the heavy line. RNA blots and hybridizations were carried out as described in Materials and Methods. Each filter strip shows two lanes containing 2 and 4 μg of rat poly (A)$^+$ RNA, respectively. *P*, PstI cleavage sites; *E*, EcoRI cleavage sites.

two synapsin mRNA classes. An oligonucleotide complementary to synapsin mRNA was deduced from the synapsin cDNA pSyn5 sequence (bases 478–497, *arrow* in Fig. 2). We synthesized this oligonucleotide, hybridized it to synapsin mRNA present in the total poly(A)$^+$ RNA of 10-d-old rat brain, and cleaved the resulting hybrids with the RNA-DNA hybrid-specific endonuclease RNase H, as described in Materials and Methods. The resulting RNA fragments were denatured, displayed by agarose gel electrophoresis, and blotted onto nitrocellulose. These filters were hybridized with probes specific for the 5′ (E3 probe) or 3′ (E2 probe) products of the RNase digestion. Fig. 2 presents the results of this study. Fig. 2 *A* shows the mRNA digestion products detected by the 5′-specific probe. Lane *1* represents a control reaction containing RNA and RNase H but no oligonucleotide. It indicates the presence of both synapsin mRNA classes in the starting material. Lanes *2* and *3* show the products after digestion with RNase H in the presence of oligonucleotide with two different amounts of poly(A)$^+$ RNA in the digestion reactions. The band detected by the probe is ~800 bases in length. This experiment suggests that the 3.4- and 4.5-kb synapsin mRNAs have a common start site for transcription 800 bases upstream from the oligonucleotide binding site. This conclusion is supported by the mRNA digestion products detected by the 3′-specific probe, shown in Fig. 2 *B*. Lane *1* is a control lane indicating the presence of both synapsin mRNA classes. Lanes *2* and *3* show the fragments produced by the oligonucleotide directed RNase H cleavage. The 3′-specific probe detects two digestion products 2.6 and

1. *Abbreviations used in this paper*: PN, postnatal day; poly(U), polyuridilate.

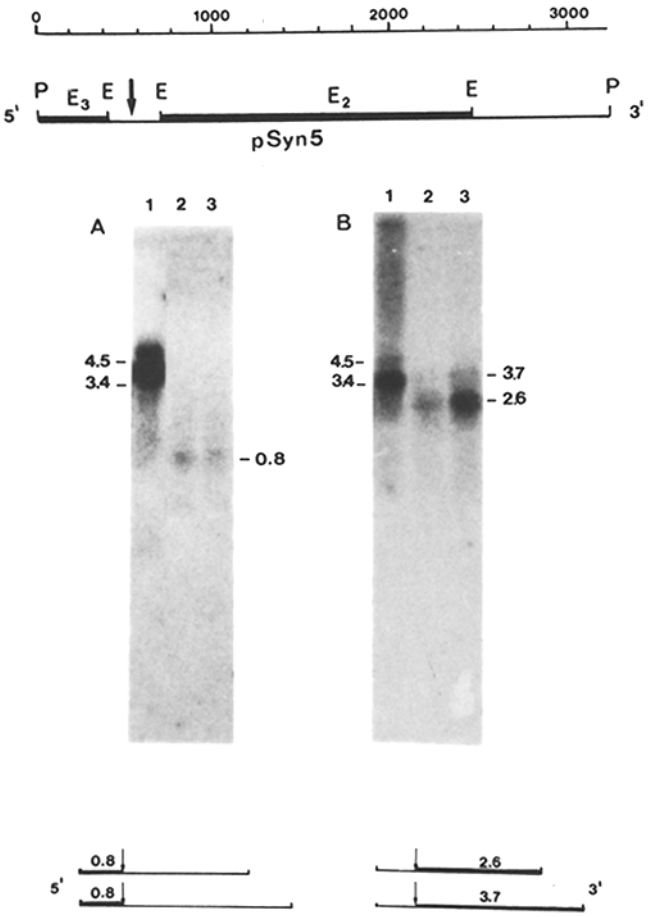

Figure 2. Oligonucleotide-directed cleavage of synapsin mRNA by RNase H. After oligonucleotide-directed cleavage of rat brain poly $(A)^+$ RNA by RNase H, as described in Materials and Methods, the resulting RNA fragments were displayed by agarose gel electrophoresis, blotted to nitrocellulose, and hybridized with nick-translated probes derived from synapsin cDNA clone pSyn5. Scale at the top is in base pairs. Heavy lines in the pSyn5 restriction map indicate position of fragments E3 and E2 used as probes; *P*, PstI cleavage sites; *E*, EcoRI cleavage sites. Arrow indicates site of oligonucleotide sequence in clone pSyn5. (*A*) Hybridization with probe E3; (B) hybridization with probe E2. Lanes *1*, *2*, and *3* contained 4, 2, and 4 μg of poly A^+ RNA, respectively. Diagram at the bottom of the figure depicts the interpretation of the experimental data (see text).

3.7 kb in length. This result indicates that the difference between the two synapsin mRNA classes occurs to the 3′ side of the oligonucleotide binding site. A summary of these conclusions appears in diagrammatic form at the bottom of Fig. 2. Note that the lengths of the observed RNA fragments sum to the expected mature RNA lengths. We believe that the relatively weak hybridization signal given by the 800-base RNA fragment is the result of poor retention of this fragment by the nitrocellulose filter during RNA transfer. The results reported above have been confirmed in several experiments and with the use of additional oligonucleotides which hybridize to alternate sites in the synapsin mRNAs (data not shown).

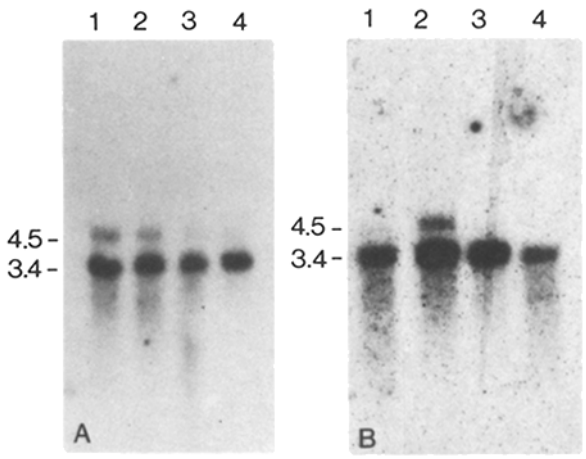

Figure 3. Differential regulation of synapsin mRNA expression. RNA blots of rat cerebellar RNA from various ages were performed as described in Materials and Methods and were hybridized with ^{32}P-nick-translated synapsin 5E2 cDNA fragment as probe. (*A*) Total RNA from rat cerebellum of various ages. Lane *1*, newborn; lane *2*, PN 7; lane *3*, PN 10; lane *4*, PN 15. (Exposure time: 2 d at −70°C.) Various amounts (1–2 μg) of RNA were used for the different stages to achieve equal intensity for the 3.4-kb band. This allowed a better comparison of the ratio of the 3.4- and 4.5-kb bands during development. (*B*) Northern blot of total RNA hybridized as in *A*. Lane *1*, adult rat brain minus cerebellum; lane *2*, 10-d-old rat brain minus cerebellum; lane *3*, adult rat cerebellum; lane *4*, adult rabbit cerebellum.

Synapsin mRNA Expression Is Differentially Regulated during Neuronal Development

Northern blot analysis of synapsin mRNA expression during the development of the rat cerebellum was carried out using total RNA extracted from rat cerebella of various ages. The blots were hybridized with the ^{32}P-labeled E2 probe. Our data show that the two synapsin transcripts are differentially regulated during the development of the rat cerebellum (Fig. 3, *A* and *B*). The larger 4.5-kb transcript is easily detectable in cerebellar RNA at birth and through postnatal day 7 (PN 7); it then decreases rapidly to almost undetectable levels through PN 10 and 15, and in the adult. The smaller mRNA species (3.4 kb), however, is expressed throughout cerebellar development and in the adult. This phenomenon is not restricted to the cerebellum. Differential regulation of the two mRNA species can also be seen by comparing RNA from 10-d-old and adult rat brain minus cerebellum (Fig. 3 *B*). Furthermore, the 4.5-kb transcript is not detected in RNA from adult rabbit cerebellum (Fig. 3 *B*). These data indicate that differential regulation of the synapsin mRNAs occurs during development in both the rat brain and cerebellum and possibly in the cerebellum of at least one other species, the rabbit.

We next carried out a quantitative analysis of the 3.4- and 4.5-kb synapsin mRNA classes throughout postnatal development of the rat cerebellum. The mRNA levels were quantified by densitometric scanning of autoradiograms like those shown in Figs. 3 and 4. The 4.5-kb transcript is not visible in Fig. 4 because of the short exposure time chosen to ensure the linearity of the autoradiographic signal for the 3.5-kb mRNA. A graphic representation of the standardized data

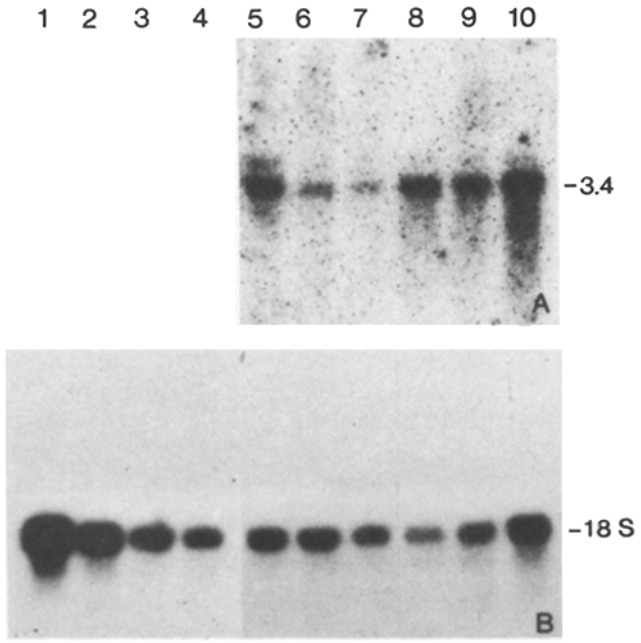

Figure 4. Quantitative Northern blot analysis of synapsin mRNA during rat cerebellar development. (*A*) Northern blot of total RNA from cerebella of various ages hybridized with ^{32}P-labeled 5E2 probe. Lane *5*, newborn; lane *6*, PN 5; lane *7*, PN 10; lane *8*, PN 20; lane *9*, PN 30; lane *10*, adult. (Exposure time: 3 d at −70°C.) (*B*) The same blot as in *A* was subsequently hybridized with a nick-translated 18S rRNA cDNA probe. Lanes 1–4, serial dilution of standard calf liver rRNA: 2, 1, 0.5, and 0.25 μg. Lanes *5–10*, the same as in *A*. (Exposure time 3 h, at −70°C.)

is shown in Fig. 5. The two synapsin mRNA classes are clearly differentially regulated. The 4.5-kb mRNA is present at birth but falls below the level of detection of this assay after PN 7. The level of the 3.4-kb mRNA species also changes

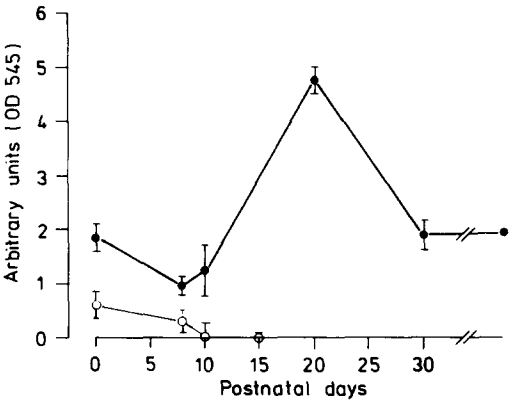

Figure 5. Quantification of synapsin mRNAs by densitometric scanning. Autoradiograms of the rRNA dilution series were scanned and the peak areas plotted vs. the amount of rRNA, to give a standard curve. Using this standard curve the actual amounts of total cerebellar RNA in each experimental lane were determined. The signal obtained with the synapsin specific probe was then corrected relative to the amount of total RNA loaded in each lane. The corrected values for the synapsin specific signal are shown. (○) 4.5-kb mRNA; (●) 3.4-kb mRNA. The values represent the mean ± SEM from at least three independent experiments.

during cerebellar development: in contrast to a relatively low level from birth to PN 10, there is a fivefold increase by PN 20. This peak of expression is followed by a decrease to the adult level.

Morphologic studies in the rat have shown that the axons of the cerebellar granule cells, the predominant neuronal cell type in the cerebellum, establish their synaptic connections with the dendritic trees of the Purkinje cells between PN 15 and PN 30 (Altman, 1972*a*, *b*). Therefore, the observed peak of synapsin mRNA expression at PN 20 coincides with the period of synaptogenesis of the granule cells.

High Levels of Synapsin mRNA Occur in Neurons Undergoing Synaptogenesis

Localization of synapsin mRNA in situ provided additional evidence for the temporal and spatial expression of the synapsin gene in the cerebellum. To address the question of temporal expression, in situ hybridization using a ^{35}S-labeled single-stranded synapsin cRNA probe was carried out on sections of the cerebellum from rats of various ages. In young cerebella (PN 0 and PN 5), no synapsin specific signal was observed (Figs. 6, *A* and *B*, and 7 *B*). At this stage of development the layered structure of the cerebellum has not yet formed and the undifferentiated granule cells still reside in the external granule cell layer. From PN 15 onward, however, synapsin gene expression was detectable in the internal granule cell layer, but not in the external granule cell layer (Fig. 6 *C*). The synapsin-specific signal increased sharply at PN 20 in the granule cell layer (Fig. 6, *E* and *F*). In control experiments in which an RNase A pretreatment of the sections was performed, the signal could be abolished (not shown). Control sections hybridized with the sense strand probe show a few grains homogeneously distributed over all layers of the cerebellum and confirm the specificity of the synapsin cRNA hybridization (Fig. 6 *D*).

The developmental time course of synapsin mRNA expression described by in situ hybridization corresponds well with the result of the Northern blot analysis described above. It confirms that the peak of synapsin gene expression is correlated with the synaptogenesis of cerebellar granule cells. Synapsin mRNA in young cerebella (PN 0, 5, and 10), demonstrated by Northern blot analysis, was not visualized in situ. This suggests that it lies below our present level of detection.

We exploited the potential for higher resolution offered by ^{35}S-labeled probes to define the cell type expressing synapsin mRNA. High-magnification photomicrographs revealed heavy accumulation of silver grains over granule cells and only a small number of grains over Purkinje cells (Fig. 7 *D*). Thus the expression of synapsin mRNA was demonstrated specifically in the population of granule cells undergoing a particular phase of differentiation, i.e., synaptogenesis.

To show that the variation in synapsin mRNA expression was not due to selective loss of poly(A)$^+$ RNA from particular cell types during the processing of the tissue, hybridization experiments were carried out using [^{3}H]poly(U). This method has been applied previously to quantify total poly(A)$^+$ mRNA in tissue sections. It was found that the abundance of autoradiographic grains from [^{3}H]poly(U)/poly(A)$^+$ RNA hybrids is directly proportional to the total poly(A)$^+$ RNA in cells (Angerer and Angerer, 1981; Hecht et al.,

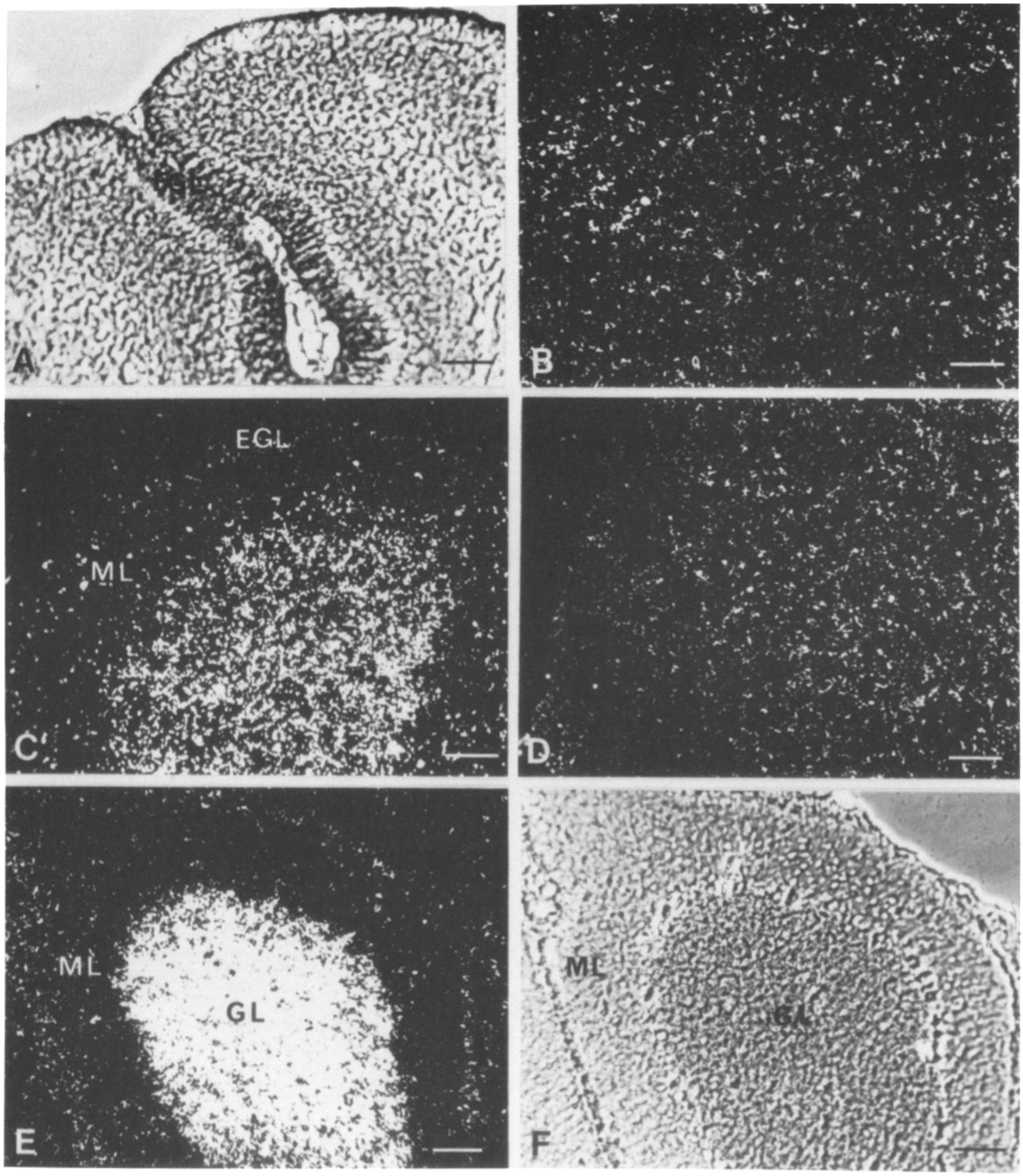

Figure 6. Hybridization of single-stranded ^{35}S-labeled synapsin cRNA probe to 10-μm sections from the rat cerebellum. (*A* and *F*) Phase-contrast photomicrographs. (*B–E*) Dark field photomicrographs; silver grains of the photoemulsion appear white. (*A* and *B*) Section from PN 5 cerebellum; homogenous distribution of silver grains over the tissue reflecting only background hybridization. (*C*) PN 15 cerebellum showing accumulation of grains over the GL but not the EGL. (*D*) Hybridization with control synapsin cRNA (same sequence as mRNA) to PN 15 cerebellum; background hybridization only. (*E*) PN 20 cerebellum; high accumulation of silver grains over GL. (*F*) Phase-contrast picture corresponding to *E*. *GL*, granule cell layer; *ML*, molecular layer; EGL, external granule cell layer. Exposure time: 6 d. Bars, 25 μm.

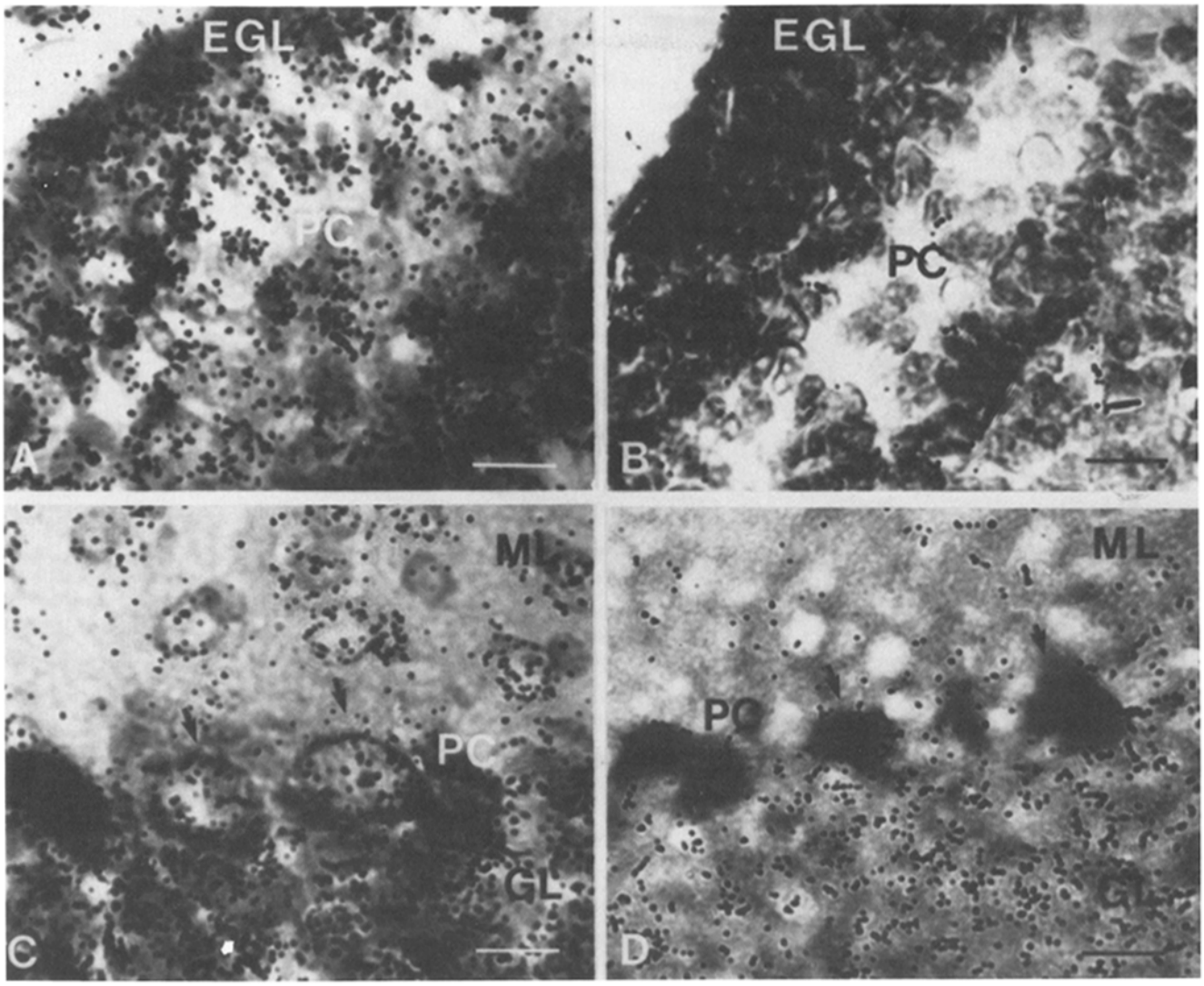

Figure 7. Cellular localization of poly(A)$^+$ RNA and synapsin mRNA in the rat cerebellum. (*A* and *C*) Hybridization with [^{3}H]poly(U), 9-d exposure. (*B* and *D*) Hybridization with [^{35}S]synapsin-cRNA probe, 6-d exposure. (*A*) Section of PN 5 cerebellum; accumulation of silver grains over neurons in EGL, PC, and deeper layers of the immature cerebellum reflecting the poly(A)$^+$ RNA content of the cells. (*B*) PN 5 cerebellar section, no synapsin specific signal. (*C*) Section of PN 20 cerebellum, as in *A*, high poly(A)$^+$ content of interneurons in ML, PC, and GL. (*D*) PN 20 cerebellum; accumulation of silver grains over GL indicating synapsin-specific signal, but not over PC (*arrows*). The dark color of the PC results from incomplete destaining after thionin counterstaining. *GL*, granule cell layer; *ML*, molecular layer; *PC*, Purkinje cell layer. Bars, 5 μm.

1981). When we applied this technique to cerebellar slices of different ages, poly(A)$^+$ RNA was detectable in high amounts in the various cell types of the cerebellum at all stages studied. (Data for two of these are shown in Fig. 7, *A* and *C*). This result further demonstrates the specificity of the synapsin mRNA hybridization.

Discussion

Nature of the Mature Synapsin mRNA Species

Two synapsin mRNA classes have been described in RNA prepared from 10-d-old rat brain (Kilimann and DeGennaro, 1985), both of which actively encode synapsin protein in vivo, as described in Results. In this report we have further confirmed the sequence homology between them using RNA blot hybridizations with synapsin cDNA probes (Fig. 1).

The experiments described in Fig. 2 demonstrate that both mRNA classes have the same transcription start site, and that the difference between the messages occurs to the 3′ side of the oligonucleotide used in the RNase H study. The following conclusions can be drawn from this data. The synapsin-specific cDNA pSyn5 represents a near full-length copy of the 3.4-kb synapsin mRNA, lacking only about 300 bases of 5′ untranslated sequence. At the level of analysis performed, all of the sequences contained in pSyn5 seem also to be present in the 4.5 kb mRNA.

The recent localization of the synapsin gene to the human and murine X chromosome (Yang-Feng et al., 1986) and Southern blot data of rat genomic DNA (Carroll, D. P., and

L. J. DeGennaro, unpublished observations) provide strong evidence for the existence of a single copy of the synapsin gene in the haploid genome. Preliminary data derived from the analysis of the rat synapsin gene suggest that additional sequences occur in the 3′ untranslated region of the 4.5 kb mRNA (Carroll, D. P., C. A. Haas, and L. J. DeGennaro, unpublished observations). Therefore, the origin of the two classes of mature transcripts from this gene can now be explained. We conclude that the 4.5-kb mRNA is produced by alternate splicing of the primary synapsin gene transcript, to include additional sequences totaling ~1,000 bases. This means that the developmentally coordinated decrease in the expression of the 4.5-kb RNA that we have described results from an alteration in the processing of the primary transcript of the synapsin gene. The biological function of the alteration in splicing is unclear. The possibility that the alternate mRNA classes represent fetal and adult forms, or that they are expressed in different neuronal cell types, awaits investigation with hybridization probes specific for each class.

We proposed earlier (Kilimann and DeGennaro, 1985) that each of the mRNA classes might encode one of the two synapsin polypeptides Ia and Ib. Our Northern blot analysis, however, clearly demonstrates differential regulation of the two synapsin mRNA classes during the development of rat cerebellum and brain such that only the 3.4-kb transcript is continuously expressed from birth into adulthood. By contrast, the synapsin proteins Ia and Ib appear to be synthesized in a constant ratio at all stages of rat brain development, including adult (DeGennaro et al., 1984). The different developmental profiles of the two synapsin mRNAs and proteins now lead us to the hypothesis that the 3.4-kb band alone can give rise to both proteins. In order to explain this, we suggest that the 3.4-kb band consists of more than one mRNA species of similar length, which are not resolved by the agarose gel system used. Because the two synapsin polypeptides Ia and Ib differ only by 4–6 kD, i.e., ~40 amino acids, the putative mRNAs encoding them need only differ by 120 nucleotides. The existence of synapsin mRNA variants has been shown previously by DNA sequence analysis of synapsin-specific cDNA molecules (McCaffery and DeGennaro, 1986), and a similar heterogeneity within brain-specific mRNAs was reported recently by Tsou et al. (1986). Understanding the origin and function of the synapsin mRNA and protein microheterogeneity will require extensive analysis of the synapsin gene and protein sequences.

Developmental Regulation of Synapsin Gene Expression

The developing rat cerebellum offers the following advantages for the study of neuron-specific gene expression: a simple cytologic architecture, a limited number of neuronal cell types, and a well-characterized postnatal development (Altman, 1972*a*, *b*, *c*). Thus we chose it as a model system to study the expression of the synapsin gene during development.

Quantification of the 4.5-kb mRNA class shows that between birth and PN 10 levels of this mRNA decrease to below the level of detection of our RNA blot assay (Figs. 3, 4, and 5). By contrast, quantification of the 3.4-kb mRNA species shows that a fivefold increase occurs between PN 7 and 20. This time course suggests a strong correlation with the synaptogenesis of granule cell axons on Purkinje cell dendrites, the major event in cerebellar development at that time (Altman, 1972*a*, *b*). A similar correlation has been found at the protein level, showing that the amount of synapsin increases to a maximum during the period of synaptogenesis in brain (Lohmann et al., 1978; DeGennaro et al., 1984). The peak level of the 3.4-kb mRNA species decreases to an adult level which is still quite high, indicating a constitutive synthesis of synapsin mRNA in neurons of adult animals. The correlation between high levels of synapsin gene expression and synaptogenesis is not surprising given that the synapsin protein is highly concentrated in virtually all nerve terminals of the peripheral and central nervous system (DeCamilli et al., 1983). The persistence of elevated levels of synapsin mRNA in the adult cerebellum may support the hypothesis that synaptic connections undergo constant remodeling (for a review, see Cotman and Nieto-Sampedro, 1984). The process might involve the degradation of synapsin in old terminals and its *de novo* synthesis for new nerve terminals in the adult.

Correlation of Synapsin Gene Expression and Granule Cell Synaptogenesis

The localization of synapsin mRNA during development by in situ hybridization provides additional insight into the temporal and spatial appearance of synapsin mRNA in relation to the state of differentiation of a particular neuronal cell type (Figs. 6 and 7). As a model for a developing neuron we chose the granule cell of the cerebellum. We could localize the synapsin mRNA from PN 15 onward in the internal granule cell layer of the cerebellum. This coincides with the maximum level of synapsin mRNA demonstrated by quantitative Northern blot analysis. The lower levels of expression of synapsin mRNA present in the cerebellum from birth until PN 10 could not be visualized in situ, indicating the limit of detection. Using ^{35}S-labeled probes synapsin mRNA localization could be extended to the cellular level. Thereby it was possible to define the cell population responsible for the strong increase in synapsin mRNA expression during development as the granule cells of the internal granule cell layer. The accumulation of silver grains over the cell bodies of these neurons reflects synapsin mRNA production during the period of synaptogenesis of the parallel fibers on the Purkinje cell dendrites. In other cell populations of the cerebellum, such as the germinal matrix of external granule cells, the interneurons of the molecular layer or the Purkinje cells, synapsin mRNA levels were below the limit of detection. These findings are also in good agreement with the immunocytochemical localization of the synapsin protein in the molecular layer of the cerebellum during the same period of development (DeCamilli et al., 1983; Mason, 1986). The specificity of the in situ hybridization procedure has been demonstrated by RNase A treatment of the tissue prior to hybridization; by the use of the nonhybridizing synapsin cRNA as a negative control and by demonstrating the poly(A)$^+$ RNA content by hybridization with a ^{3}H-labeled poly(U)-probe.

In summary, the application of an oligonucleotide directed RNase H cleavage assay, quantitative Northern blots and in situ hybridization histochemistry has allowed the detailed analysis of the nature of the mature messenger RNAs encoding the neuron-specific protein synapsin and the description of changes in their expression at the cellular level during development. The results have yielded the intriguing finding that the expression of the gene for synapsin, reflected by steady-state levels of mature synapsin mRNA, is regulated in

at least two ways. First, a developmentally coordinated decrease in the production of one of two synapsin mRNA classes appears to be the consequence of a change in splicing of the primary transcript of the gene. Secondly, the overall level of expression of the synapsin gene is further regulated in a fashion coordinate with the major period of synaptogenesis. Finally, a direct demonstration of the presence of high levels of synapsin mRNA in cells undergoing synaptogenesis has been demonstrated by in situ hybridization histochemistry. This opens the possibility of detailed mapping of the brain with a probe which identifies the cell bodies of neurons in the process of synaptogenesis.

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