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Molecular cloning of cDNAs for the nerve-cell specific phosphoprotein, synapsin I

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To provide access to synapsin I-specific DNA sequences, we have constructed cDNA clones complementary to synapsin I mRNA isolated from rat brain. Synapsin I mRNA was specifically enriched by immunoadsorption of polysomes prepared from the brains of 10–14 day old rats. Employing this enriched mRNA, a cDNA library was constructed in pBR322 and screened by differential colony hybridization with single-stranded cDNA probes made from synapsin I mRNA and total polysomal poly(A)$^+$ RNA. This screening procedure proved to be highly selective. Five independent recombinant plasmids which exhibited distinctly stronger hybridization with the synapsin I probe were characterized further by restriction mapping. All of the cDNA inserts gave restriction enzyme digestion patterns which could be aligned. In addition, some of the cDNA inserts were shown to contain poly(dA) sequences. Final identification of synapsin I cDNA clones relied on the ability of the cDNA inserts to hybridize specifically to synapsin I mRNA. Several plasmids were tested by positive hybridization selection. They specifically selected synapsin I mRNA which was identified by *in vitro* translation and immunoprecipitation of the translation products. The established cDNA clones were used for a blot-hybridization analysis of synapsin I mRNA. A fragment (1600 bases) from the longest cDNA clone hybridized with two discrete RNA species 5800 and 4500 bases long, in polyadenylated RNA from rat brain and PC12 cells. No hybridization was detected to RNA from rat liver, skeletal muscle or cardiac muscle.

Key words: brain/development/gene expression/mRNA/neurotransmitter vesicle

Introduction

The nervous system represents a particularly challenging subject for the study of the regulation of gene expression during ontogenetic differentiation. The vast variety of highly specialized cell types of which it is composed, and their formation in a definite time sequence during the development of the organism, must ultimately be based on the tissue-specific and cell type-specific expression of subsets of genes.

Synapsin I is a phosphoprotein which is specifically expressed in neurons, and is found throughout the central and peripheral nervous system (Greengard, 1981). It is composed of two polypeptides of 86 and 80 kd termed, respectively, synapsin Ia and Ib. These polypeptides exhibit many similar properties, including substantial immunological cross-reactivity (Ueda and Greengard, 1977; Browning *et al.*, 1982). Their amino acid sequences are not known. Immunohistochemistry and the preparation of highly purified synaptic vesicles have localized synapsin I, associated with the membranes of synaptic vesicles, to the synaptic terminal region of most, if not all, neurons (DeCamilli *et al.*, 1983a, 1983b; Huttner *et al.*, 1983). Synapsin I comprises 0.4% of the total protein of a cortical homogenate of rat brain and 6% of the total protein in synaptic vesicles (Huttner *et al.*, 1983; Goelz *et al.*, 1981). The protein is phosphorylated by both cAMP-dependent and Ca^{2+}/calmodulin-dependent protein kinases, and the state of phosphorylation is altered by conditions that affect neuronal activity (for a review, see Nestler and Greengard, 1984).

In addition to its tissue specificity, the biosynthesis of synapsin I is under developmental control. In developing cerebella of the rat and the chick, the appearance of immunohistochemically detectable synapsin I correlates temporally and topographically with the appearance of synapses (DeCamilli *et al.*, 1983a). The level of polysomal mRNA in rat brain that can direct the synthesis of synapsin I *in vitro* rises sharply during the first week of post-natal life and goes through a maximum between days 10 and 16 (DeGennaro *et al.*, 1984). This also correlates well with the time course of synapse formation during ontogenesis (Aghajanian and Bloom, 1967).

A full understanding of the tissue-specific and developmental regulation of synapsin I expression requires the analysis of the DNA encoding its gene and its RNA transcripts. As a first step in this analysis, we report here the identification and preliminary characterization of cDNA clones derived from rat synapsin I mRNA, and their use in the identification of synapsin I mRNA species.

Results and Discussion

Specificity of affinity purified anti-synapsin I immunoglobulin

The specificity of affinity-purified anti-synapsin I immunoglobulin was tested by protein immunoblotting. Duplicate aliquots of purified rat brain synapsin I (the antigen used to produce the immunogobulin), and a rat brain homogenate were electrophoresed in a 10% SDS-polyacrylamide gel. After electrophoresis the gel was divided and one half was fixed and stained with Coomassie blue. The proteins in the second half were electroblotted onto nitrocellulose and incubated with affinity-purified anti-synapsin I immunoglobulin and [^{125}I]Protein A as described in Materials and methods. In Figure 1, lanes 1–3 show the Coomassie blue staining of the purified synapsin I and the rat brain homogenate. Lanes 4 and 5 show the autoradiogram obtained from the immunoblot of duplicates of lanes 2 and 3. The amount of purified synapsin I loaded in lanes 2 and 4 has been adjusted to approximate the amount of synapsin I expected in the aliquot of rat brain homogenate.

Lane 1 indicates the purity of the protein preparation used as antigen. The synapsin I is estimated to be 98–99% pure, containing only a small amount of synapsin I breakdown products. Lanes 4 and 5 show that the affinity-purified synapsin I immunoglobulin recognizes only synapsin I and a second protein band in a total homogenate of rat brain. We have tentatively identified the second band as Protein III (Huang *et al.*, 1982), another

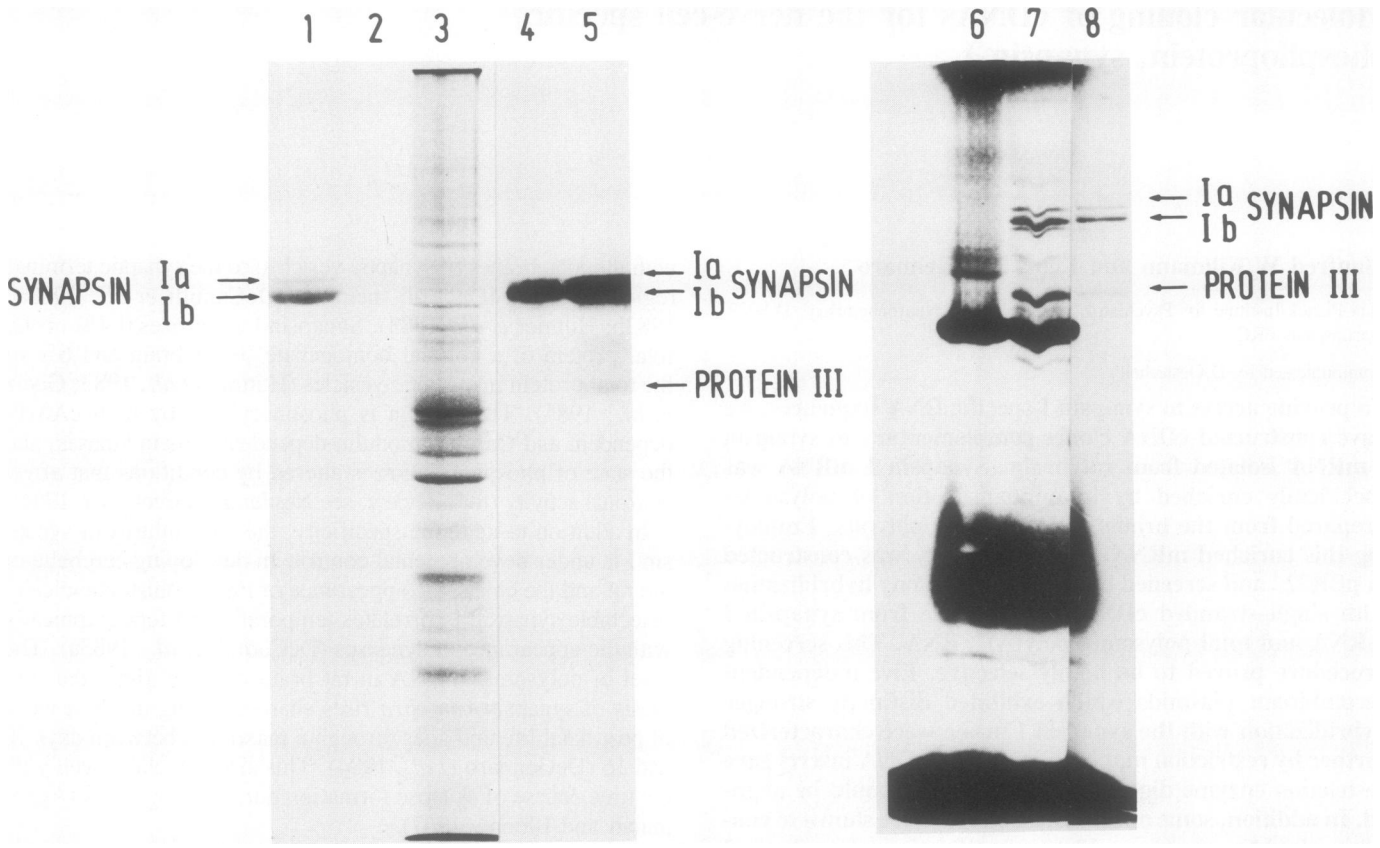

Fig. 1. Synapsin I immunoblotting and *in vitro* translation. **Lanes 1–3**: Coomassie blue staining of SDS-polyacrylamide gel. **Lane 1**: purified synapsin I (4 μg). **Lane 2**: purified synapsin I (100 ng). **Lane 3**: rat brain homogenate (50 μg). **Lanes 4** and **5**: autoradiogram of immunoblot of **lanes 2** and **3** probed with affinity-purified anti-synapsin I immunoglobulin. **Lanes 6–8**: autoradiogram of *in vitro* translation products. **Lane 6**: no RNA added, translation mixture applied directly to the gel. **Lane 7** and **8**: 1 ng of purified synapsin I mRNA was translated *in vitro*, half of the reaction mixture was applied directly to the gel (**lane 7**) and the other half applied after immunoprecipitation (**lane 8**). Exposure time of the autoradiogram was 3.5 days.

neuronal phosphoprotein to which anti-synapsin I antibodies often display cross-reactivity (Browning *et al.*, 1982). These data confirm the specificity of the affinity-purified anti-synapsin I immunogloublin.

Enrichment of synapsin I mRNA by immunoadsorption of polysomes

Total polysomes were prepared from the brains of 10–14 day old rats. Synapsin I-containing polysomes were selectively enriched from total rat brain polysomes by immunoadsorption using the immunogobulin described above. When poly(A)$^+$ RNA prepared from these enriched polysomes is translated in an *in vitro* translation system, synapsin I is found to be the main translation product with a relative abundance of at least 10% (Figure 1, lane 7). The newly synthesized synapsin I displays the typical doublet of polypeptides Ia and Ib, which co-migrate with authentic synapsin I (lane 1) and are immunoprecipitated with anti-synapsin I immunoglobulin (lane 8).

It appears that synapsin I mRNA is selectively enriched, while the main contaminants of the mRNA preparation correspond to the most abundant species in total poly(A)$^+$ RNA. A second mRNA species that is enriched in some preparations codes for a protein that we have tentatively identified as Protein III. We believe that this mRNA is enriched due to the cross-reactivity between anti-synapsin I immunoglobulin and Protein III described above.

Construction and screening of a cDNA library

cDNA was prepared from 400 ng of total poly(A)$^+$ RNA from 10–14 day old rat brain polysomes supplemented with 50 ng of enriched synapsin I mRNA, and was inserted into the *Pst*I site of pBR322. 16 000 bacterial clones were obtained and screened by differential colony hybridization (Figure 2). The hybridization probes were single-stranded cDNAs derived from enriched synapsin I mRNA, total polysomal poly(A)$^+$, and globin mRNA by oligo(dT)-primed reverse transcription. About 50 colonies were detected which gave distinctly stronger hybridization signals with the synapsin I probe than with the other two probes.

Restriction mapping of cDNAs

Twelve independent clones were selected from those identified by colony hybridization. Plasmid DNA was purified by cesium chloride gradient centrifugation and characterized by restriction mapping and dot-blot hybridization to ^{32}P-labeled oligo(dT)$_{12-18}$. The five longest cDNA inserts gave restriction enzyme digestion patterns which could be aligned (Figure 3). All plasmids except pSyn16 gave strong hybridization signals with the oligo(dT) probe (not shown). pSyn16, when compared with the other cDNA clones shown, lacks a DNA segment at the left side of the restriction map. We conclude that this region must contain the poly(dA) tract detected by the dot-blot analysis, and suggest that this poly(dA) tract probably represents the 3′-poly(A) sequence of the mRNA which was reverse transcribed to construct the clones. The fact that the majority of cDNAs contain poly(dA) tracts may result from our use of the cDNA synthesis method of Gubler and Hoffman (1983), which does not employ nuclease S1 and therefore selects for molecules with full-length

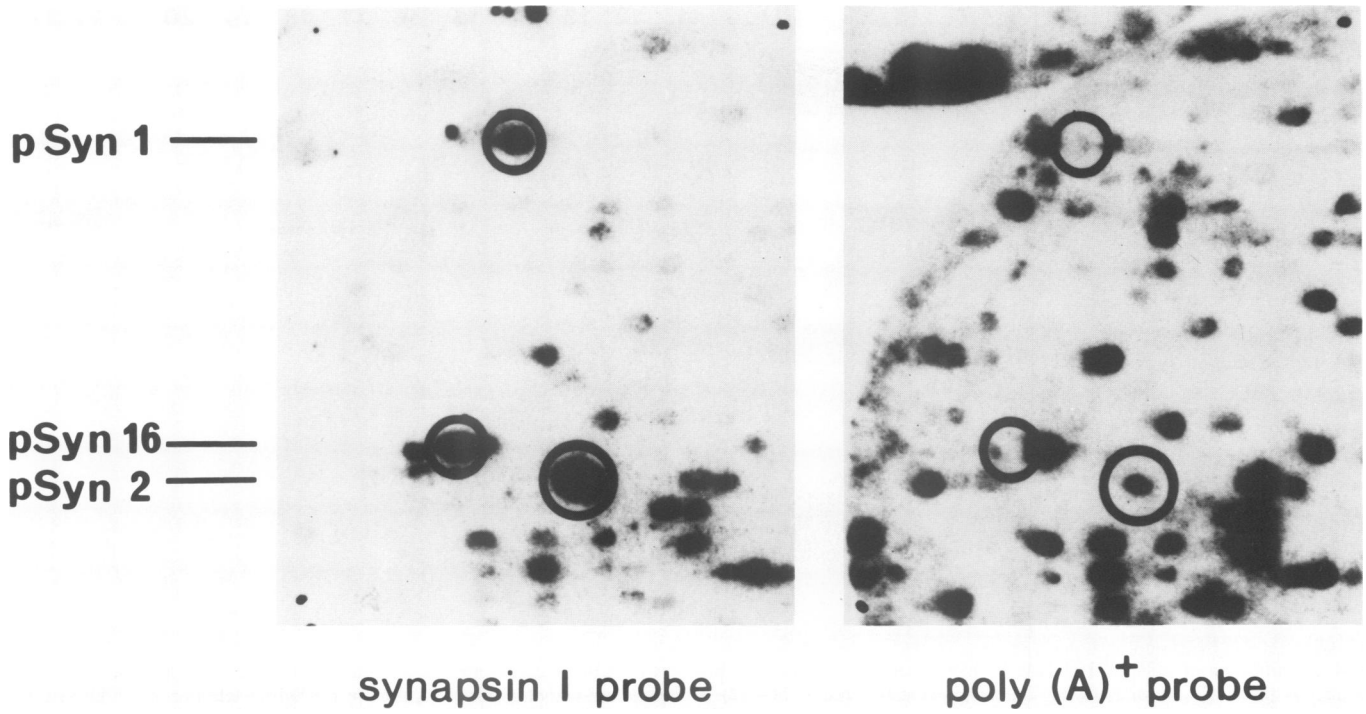

Fig. 2. Differential colony hybridization with single-stranded cDNA probes derived from purified synapsin I mRNA and total polysomal poly(A)$^+$ RNA from 10 – 14 day old rat brains. The three colonies in this figure that give stronger hybridization signals with the synapsin I probe were later designated as pSyn1, pSyn2 and pSyn16 (compare Figure 3). Autoradiograms were exposed for 3.5 days.

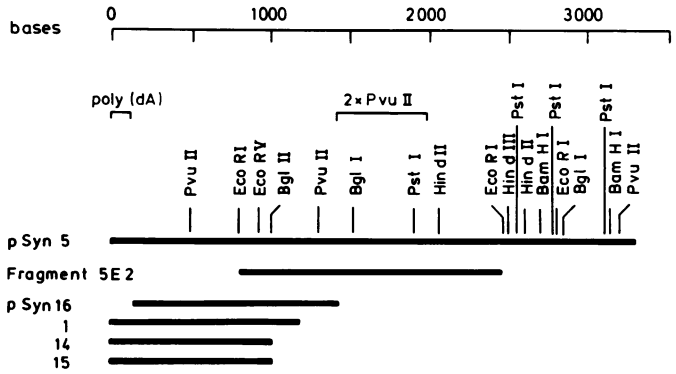

Fig. 3. Restriction maps of synapsin I cDNAs. Five synapsin I cDNAs and the *Eco*RI fragment 5E2 from pSyn5 are aligned by restriction enzyme analysis. pSyn5, 1, 14 and 15 contain poly(dA) tracts. pSyn16 does not. The following restriction endonucleases do not cut within the sequence covered by these cDNAs: *Cla*I, *Kpn*I, *Sal*I, *Xho*I. There is one unmapped site each for *Hpa*I, *Sma*I and *Apa*I above base 1200, and three unmapped *Acc*I sites between bases 700 and 2600.

second strands. The fact that all five cDNAs seem to represent the same mRNA is an indication of the high selectivity of the colony screening method.

Identification of synapsin I cDNAs by hybrid-selected translation

Plasmids pSyn1, pSyn5, pSyn13 [this plasmid has an insert size of ~500 bp, and lacks poly(dA) tracts, but is not mapped sufficiently well to be included in Figure 3], pSyn16, and the longest *Eco*RI fragment from pSyn5 (termed 5E2, cf. Figure 3), were used in hybrid selection experiments. Figure 4 shows that all of these molecules select, from total poly(A)$^+$ RNA, mRNA which directs the *in vitro* synthesis of synapsin I. Immunoprecipitation of this [^{35}S]methionine-labeled synapsin I is prevented by an excess of unlabeled authentic synapsin I (not shown). pSyn1 and pSyn5, which contain poly(dT) tracts, display some unspecific background retention of the complete spectrum of poly(A)$^+$ RNA species (lanes 3 and 4), whereas pSyn13, pSyn16 and fragment 5E2, which are free of poly(dT) tails, give essentially no background except that of the translation assay itself (lanes 5 – 7). Controls, employing pBR322 and various recombinant plasmids free of poly(dT) tracts, do not select synapsin I mRNA (lanes 1 and 2, 9 and 10).

Figure 4 demonstrates that the cDNAs described above select mRNA which, upon *in vitro* translation, gives rise to the typical polypeptide doublet of synapsin Ia + Ib. The relative intensities of the two protein bands are apparently the same in all cases. This seems to indicate that the two polypeptides are either encoded by the same mRNA species, or by two mRNAs with sufficient homology so that the selecting cDNAs do not discriminate between them. As stated above, anti-synapsin I antibody cross-reacts with protein III. However, protein III mRNA, which is present in the hybridizaton mixture (lane 21), does not hybridize to the synapsin I cDNA in pSyn16 (lane 22). This suggests that the similarity between the proteins breaks down at the level of their mRNA base sequences, at least for the sequence covered by this clone.

Northern blot analysis of rat RNA from different tissues

Poly(A)$^+$ RNA from various rat tissues was subjected to agarose gel electrophoresis, transferred to nitrocellulose and hybridized to nick-translated fragment 5E2 (Figure 5). This probe hybridized to two RNA species: a minor band, length 5800 bases, and a major band, length 4500 bases (glyoxalated rRNAs as mol. wt. standards).

These RNA species were detected in RNA from 10-day-old rat brain, prepared either from polysomes by phenol extraction or from whole tissue by extraction with guanidinium isothiocyanate (lanes 5 – 8), in approximately the same abundance. In immunopurified synapsin I mRNA they are enriched by more than two

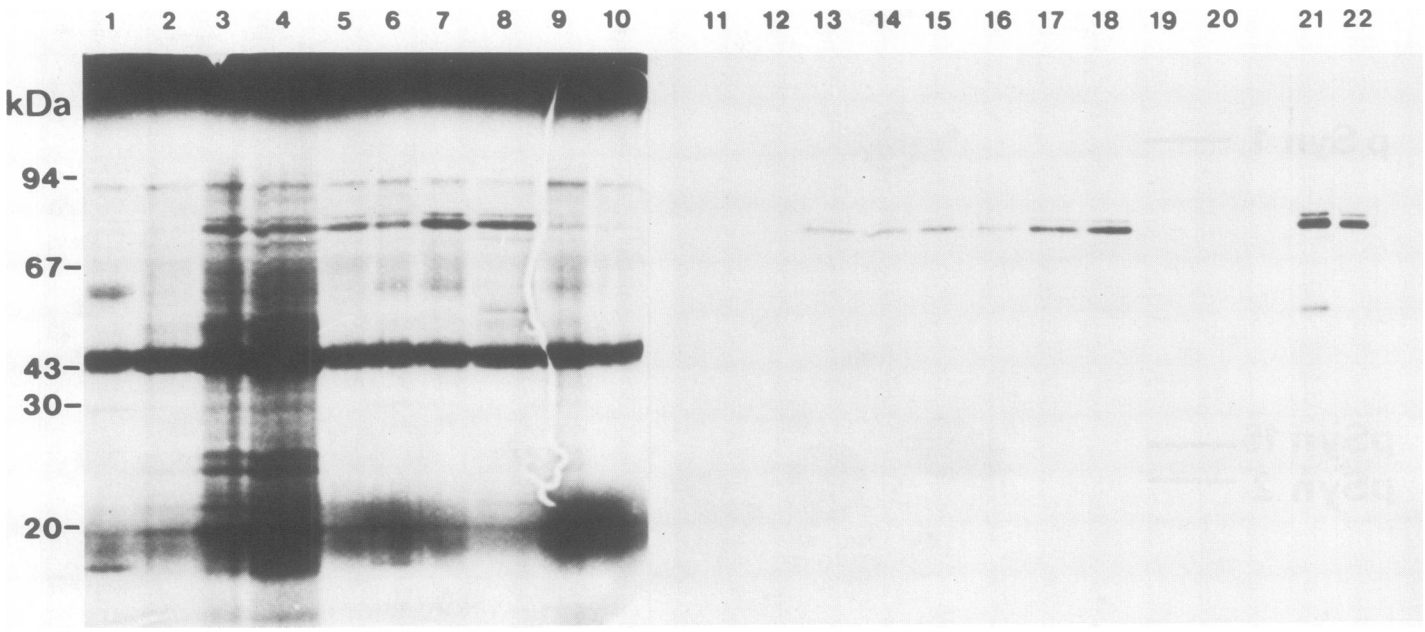

Fig. 4. Identification of synapsin I cDNAs by hybrid selection. Translation products of hybrid-selected mRNA were analyzed on SDS-polyacrylamide (10%) gels directly (**lanes 1–10**) or following immunoprecipitation (**lanes 11–22**). Nitrocellulose-immobilized DNAs used in the hybrid selection were: **lanes 1,11**: unidentified cDNA (600 bases) from rat brain in pBR322; **lanes 2,12**: a human procollagen cDNA (1800 bases) in pBR322 (11 μg); **lanes 3,13**: pSyn1; **lanes 4,14**: pSyn5; **lanes 5,15**: fragment 5E2 (2.5 μg); **lanes 6,16**: pSyn 13; **lanes 7,17**: pSyn16; **lanes 9,19**: unidentified cDNA (100 bases) from rat brain in pAT153; **lanes 10,20**: pBR322; **lanes 8,18**: translation products of 1 ng enriched synapsin I mRNA; **lane 21**: translation products of a 5% aliquot of a hybridization mixture (~1 μg poly(A)$^+$ RNA + 3 ng synapsin I mRNA), and **lane 22**: products of mRNA selected from this mixture by pSyn16, both after immunoprecipitation.

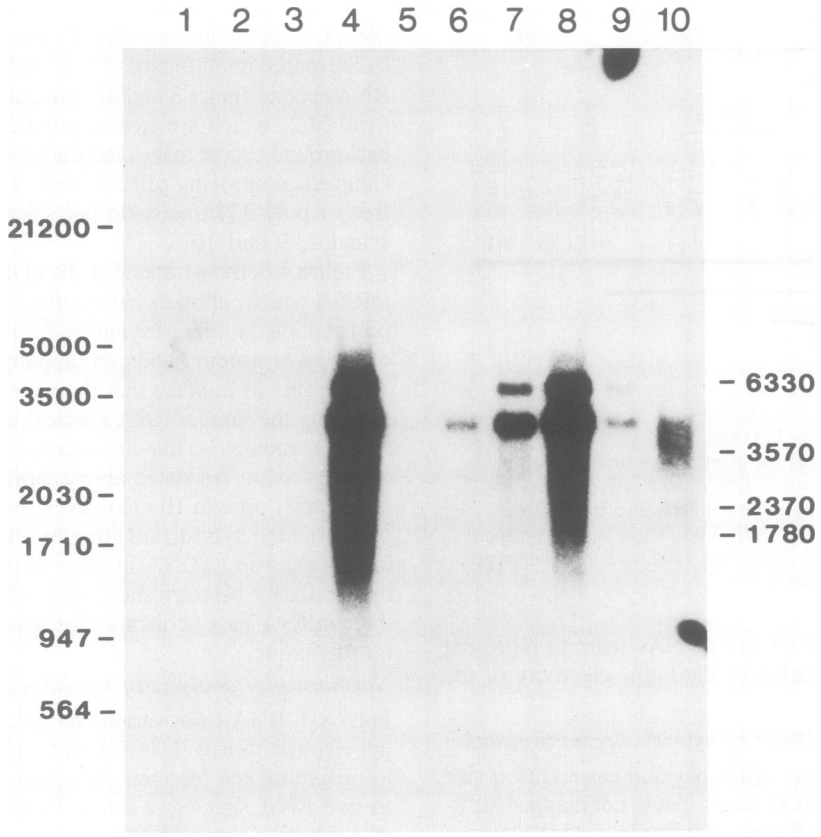

Fig. 5. Northern blot analysis of rat RNA. RNA samples were glyoxal-denatured, resolved on a 1% agarose gel, transferred to nitrocellulose (Thomas, 1980), and hybridized to nick-translated fragment 5E2. **Lane 1**: 10 μg poly(A)$^+$ RNA from heart ventricle. **Lane 2**: 10 μg poly(A)$^+$ RNA from skeletal muscle. **Lane 3**: 10 μg poly(A)$^+$ RNA from liver. **Lane 4**: 40 ng purified synapsin I mRNA. **Lanes 5–7**: polysomal poly(A)$^+$ RNA from 10–14 day old brain (**lane 5**: 40 ng; **lane 6**: 200 ng; **lane 7**: 1 μg). **Lane 8**: 3.75 μg poly(A)$^+$ RNA from whole brain tissue (10 day old). **Lane 9**: 2.5 μg poly(A)$^+$ RNA from PC12 cells. **Lane 10**: 10 μg poly(A)$^-$ RNA from 10–14 day old brain polysomes. The autoradiogram was exposed at −70°C for 2 h. Molecular size standards [in bases, values taken from Minster and Sealey (1983)] are given on the margins. Glyoxal-denatured rRNAs from rat and *E. coli* (right), and a non-denatured *Eco*RI/*Hind*III digest of bacteriophage λ (left) were run on the same gel and visualized by ethidium bromide staining.

orders of magnitude (lane 4). The two RNA species are also present in poly(A)$^+$ RNA from PC12 cells (which are derived from a rat pheochromocytoma), although less abundant by one order of magnitude (lane 9). Small amounts of what appears to be their degradation products are found in brain poly(A)$^-$ RNA [flow-through after two passages over oligo(dT)-cellulose (lane 10)]. The tissue specificity of the two RNA species was tested by hybridization of the probe to RNA from non-neuronal tissues. They cannot be detected in large quantities of poly(A)$^+$ RNA from liver, skeletal muscle and heart ventricle (lanes 1 – 3), even after exposure of the autoradiogram for a much longer time (18 h, not shown).

The detection of two RNA species by the single probe, 5E2, further supports the hypothesis that the two polypeptides, synapsin Ia and Ib, might be encoded by two discrete but homologous mRNA species. It remains to be determined whether these mRNAs are derived from two genes or, by differential splicing, from a single gene. Both RNAs are clearly long enough to accommodate the coding sequence required for either polypeptide (2000 – 2500 bases), and, in addition, seem to contain long untranslated sequences. The apparent length difference between the two RNA molecules (1300 bases) far exceeds the additional coding sequence (150 bases) required to account for the observed difference in mol. wt. between the two synapsin I polypeptides (6000 daltons). This data confirms for a specific, well-characterized neuronal protein, the general observation that brain-specific mRNAs tend to display exceptional nucleotide length (Milner and Sutcliffe, 1983).

The isolation of cDNA sequences encoding synapsin I will now allow direct determinations of synapsin I mRNA concentrations in different tissues and at different stages of development. Such sequences will also make it possible to deduce the primary structure of synapsin I, and open up approaches for the further characterization of its function. Finally, they are a prerequisite for the isolation of the synapsin I gene, and for the study of the molecular basis of the developmental control and the tissue-specificity of its expression.

Materials and methods

Purification of proteins and RNA

Synapsin I was purified from rat brain by a modification (Huttner *et al.*, 1981) of the original procedure (Ueda and Greengard, 1977). Anti-synapsin I antisera were raised in rabbits by two successive injections of 3 mg of purified synapsin I. The first administration consisted of a 1 ml suspension of synapsin I in complete Freund's adjuvant (1 volume synapsin I, 1 volume of adjuvant), which was injected at multiple sites. The second administration was 2 weeks after the first and contained Freund's incomplete adjuvant. Rabbits were allowed to rest for 6 weeks, then serum was collected by cardiac puncture. Affinity-purified anti-synapsin I antibody was prepared from sera according to the procedure of Gough and Adams (1978) employing an affinity column of purified synapsin I immobilized on CNBr-activated Sepharose (Pharmacia). For Western blot characterization of the immunoglobulin, rat brain homogenates were prepared as described (Goelz *et al.*, 1981). Protein was transferred to nitrocellulose by the procedure of Vaessen *et al.* (1981). After transfer the nitrocellulose filters were incubated in blocking buffer (8% bovine hemoglobin, 0.01 M phosphate buffer, 0.3 M NaCl, 0.05% Tween-20, pH 7.2) for 2 h at room temperature. Affinity-purified anti-synapsin I immunoglobulin (2 μg/ml in blocking buffer) was incubated with the filters for 12 h at 4°C, and the filters were then washed with wash buffer (0.01 M phosphate buffer, 0.3 M NaCl, 0.05% Tween-20, pH 7.2) 3 x 10 min at room temperature. [^{125}I]Protein A (10 μCi, Amersham) in blocking buffer was incubated with the washed filters for 60 min at room temperature, the filters were then washed with wash buffer as above and autoradiographic exposures were made.

Polysomes were prepared from the brains of 10 – 14 day old rats using the Mg^{2+} precipitation method (Palmiter, 1974). Synapsin I mRNA was enriched by immunoadsorption of polysomes (Shapiro and Young, 1981). A typical yield from 30 g of brains was 100 – 200 ng synapsin I mRNA. Total poly(A)$^+$ RNA was prepared from whole brain by extraction with guanidinium isothiocyanate (Chirgwin *et al.*, 1979) or from polysomes by phenol extraction, and subsequent passage over an oligo(dT)$_{12-18}$ cellulose column. Globin mRNA was from an *in vitro* translation kit (NEN).

In vitro translation and immunoprecipitation

In vitro translation in the rabbit reticulocyte lysate system was carried out for 3 h at 37°C using commercial kits from NEN or BRL, and [^{35}S]methionine was the label. The reaction was terminated by the addition of RNase A to 20 μg/ml and incubation at 37°C for 10 min. The immunoprecipitation procedure was essentially that described by Ivarie and Jones (1979). After termination, the translation reaction mixture was adjusted to 400 mM NaCl, 12.5 mg/ml bovine serum albumin, 0.5% Nonidet P-40, 25 mM Tris, pH 7.4 (200 μl). It was pre-cleared for 10 min with 10 μl *Staphylococcus aureus* suspension (Pansorbin from Calbiochem), followed by centrifugation to remove the *S. aureus*. The pre-cleared supernatant was incubated with affinity-purified anti-synapsin I antibody for 20 min and then with 10 μl *S. aureus* for 10 min (all at 0°C). Bacteria-bound immunocomplexes were collected by centrifugation and washed three times with 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, 50 mM Tris, pH 7.4. Gel electrophoresis was carried out according to Laemmli (1970) and the translation products visualized by fluorography.

cDNA synthesis, cloning and screening

cDNA was prepared according to the method of Gubler and Hoffman (1983), using oligo(dT)-primed reverse transcription for the synthesis of the first strand and DNA polymerase I together with RNase H and DNA ligase for the second strand. The cDNA was oligo(dC)-tailed, inserted into commercially available oligo(dG)$_{9-12}$ tailed pBR322 (from NEN), and introduced into *Escherichia coli* C600 using the method of Hanahan (1983). Tetracycline-resistant colonies were replica-plated on nitrocellulose filters, amplified with chloramphenicol (340 μg/ml), lysed (5 min each on filter paper saturated with 0.5 M NaOH; 1.5 M NaCl, 0.5 M Tris pH 7.4; and 2 mM NH_4OAc) and hybridized with ^{32}P-labeled single-stranded cDNA probes.

Purification of plasmids and hybrid selection

Plasmids were purified using alkaline lysis (Birnboim and Doly, 1979) and CsCl gradient centrifugation (Maniatis *et al.*, 1982). For hybrid selection, 20 μg of plasmid DNA was nicked and denatured by boiling for 90 s in 100 mM NaOH, neutralized by bringing to 1.5 M NH_4OAc and applied to 9 mm nitrocellulose discs. Before the first use, filters were soaked in pre-hybridization buffer overnight, boiled 3 min in water and washed extensively. Pre-hybridization and hybridization were carried out for 3 h each at 53°C in 67.5% formamide, 30 mM Hepes (pH 6.6), 350 mM NaCl, 1.25 mM EDTA, 0.2% SDS, 100 μg/ml calf liver tRNA (Boehringer, phenol-extracted). The hybridization buffer also contained 200 U/ml placental RNase inhibitor, 20 μg poly(A)$^+$ RNA (from 10 – 14 day old rat brain polysomes) and 60 ng of enriched synapsin I mRNA. Filters were washed at 65°C 10 times in 120 mM NaCl, 12 mM sodium citrate (pH 7.0), 0.5% SDS and three times in the same buffer without detergent. RNA was eluted by boiling in 200 μl H_2O and concentrated by ethanol precipitation in the presence of 8 μg calf liver tRNA. Samples were translated *in vitro*. Anti-synapsin I antibody immunoprecipitates were prepared from one half of the reaction mixture and were subjected to gel electrophoresis along with the other half which was run directly.

Probes and conditions for hybridization

Single-stranded cDNA probes for colony hybridization were prepared by oligo(dT)$_{12-18}$ primed reverse transcription of mRNAs (Gubler and Hoffman, 1983) in the presence of [^{32}P]dATP (10 μM). The RNA was then hydrolyzed in 0.3 M NaOH at 70°C for 30 min. Typically, hybridization was carried out with 5 x 10^5 c.p.m. of probe (derived from 10 – 20 ng of mRNA) at 55°C for 40 h. The hybridization buffer was 50% formamide, 5 x Denhardt's solution (1 x Denhardt's solution is 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 0.6 M NaCl, 40 mM NaH_2PO_4 (pH 7.4), 4 mM EDTA, 0.1% SDS, 100 μg/ml herring sperm DNA (sonicated + boiled) and 100 μg/ml poly(A) (thermally fragmented by boiling 2 min in 50 mM Tris, pH 9.5). Pre-hybridization was in the same buffer at 55°C overnight.

Oligo(dT)$_{12-18}$ probe for the detection of poly(dA) tracts in plasmids was labeled with [γ-^{32}P]ATP and polynucleotide kinase. Typically, 5 x 10^6 c.p.m. of probe were used. Pre-hybridization and hybridization (>4 h each) were carried out at 4°C in 50% formamide, 5 x Denhardt's solution, 750 mM NaCl, 50 mM sodium citrate (pH 7.0), 0.1% SDS and 100 μg/ml herring sperm DNA. Filters were washed 3 x 5 min in 300 mM NaCl, 30 mM sodium citrate (pH 7.0) at room temperature and then for 7 min at 120 mM NaCl, 12 mM sodium citrate (pH 7.0) at 35°C.

Fragment 5E2, the probe used in blot-hybridization experiments, was labeled with [α-^{32}P]dCTP by nick-translation (Rigby *et al.*, 1977). Pre-hybridization and hybridization were each carried out overnight at 50°C in 50% formamide, 5 x Denhardt's solution, 0.6 M NaCl, 40 mM NaH_2PO_4 (pH 7.4), 4 mM EDTA, 0.1% SDS, 200 μg/ml herring sperm DNA and 50 μg/ml phenol-extracted calf

liver tRNA. Hybridization was done with 3 x 10^7 c.p.m./ml (3 x 10^8 c.p.m./μg) of nick-translated probe. The filters were washed 3 x 15 min in 300 mM NaCl, 20 mM NaH_2PO_4 (pH 7.4), 0.2 mM EDTA, 0.1% SDS, and once for 15 min in 30 mM NaCl, 2 mM NaH_2PO_4 (pH 7.4), 0.2 mM EDTA, 0.1% SDS, all at 60°C.

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