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Molecular Cloning of a 2′,3′-Cyclic Nucleotide 3′-Phosphodiesterase: mRNAs with Different 5′ Ends Encode the Same Set of Proteins in Nervous and Lymphoid Tissues

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Antibodies raised to a mixture of the 46 and 48 kDa rat CNS 2′,3′-cyclic nucleotide 3-phosphodiesterases (CNPs) recognized apparently identical proteins in peripheral nervous system (PNS), thymus, and circulating blood lymphocytes. These antibodies were used to identify, in a rat brain phage λ gt11 expression library, cDNA clones encoding β-galactosidase-CNP fusion proteins, some of which showed CNP activity. In RNA blots, the subcloned CNP cDNA inserts hybridized to mRNAs of ~2400 and ~2800 nucleotides (nts), and to a ~2500 nt mRNA from thymus. Several nonexpressing CNP cDNAs were identified by plaque hybridization, and the mRNA transcribed *in vitro* from one of these cDNAs (pCNP7) encoded a complete 46 kDa CNP polypeptide. Examination of the deduced amino acid sequence revealed an apparent homology to cAMP binding sites in several other proteins. A 373 bp segment from the 5′ end of this pCNP7 hybridized only to the 2800 nt nervous system mRNAs, thus revealing that not all CNP mRNAs share the same 5′-ends. Genomic DNA blots probed with CNP cDNAs suggest that there is a single gene which can be alternatively spliced to produce the various mRNA transcripts in the nervous and lymphoid tissues.

In the developing vertebrate nervous system, a sharp rise in 2′,3′ cyclic nucleotide 3′-phosphodiesterase (CNP; EC 3.1.4.37) activity, detectable even in crude brain homogenates, has been shown to be a reliable marker for the onset of rapid myelination (Kurihara and Tsukada, 1968; Olafson et al., 1969; Kurihara et al., 1970), and it is considered likely that the CNPs play a role in the formation of the myelin sheath (Sims and Carnegie, 1978). Polypeptides with CNP activity represent ~5% of the CNS myelin proteins and ~1% of the proteins in peripheral nervous system (PNS) myelin. The highest levels of CNP are found in the nervous system within oligodendrocytes and Schwann cells (Roussel et al., 1978, 1983; Nishizawa et al., 1985), but CNP activity is also detectable, albeit at much lower levels, outside the nervous system, most notably in lymphoid tissues (Weissbarth et al., 1981; Sprinkle et al., 1985). In purified rat CNS myelin, CNP activity has been ascribed to 2 polypeptides, CNPI (M_r = 46 kDa) and CNPII (M_r = 48 kDa) (Drummond and Dean, 1980; Sprinkle et al., 1980) whose amino acid sequences are not known. These yield almost identical peptide patterns after limited protease digestion (Drummond and Dean, 1980). Despite considerable attention in recent years, physiological roles for the CNPs have not been identified, and the relationship between the different enzymatic forms within myelin and other tissues has not been elucidated. A molecular biological approach to these problems may ultimately help clarify the precise functions of these polypeptides in all the tissues that express them.

Portions of this work have appeared in abstract form (Bernier et al., 1986).

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Materials and Methods

CNP antisera production. Twenty-five 30-d-old rats were killed by decapitation. The brains were rapidly dissected out, and myelin was purified according to the method of Norton and Poduslo (1973). Osmotically shocked myelin fragments were partially delipidated in cold acetone, washed in H_2O, and treated with acidified chloroform-methanol (2:1) (Folch-Pi and Lees, 1951). The residue from this extraction was washed with diethyl ether, followed by ethanol and water, dissolved in SDS-PAGE sample buffer, and subjected to electrophoresis. After light staining with Coomassie blue, the doublet that corresponded to CNPI and II was excised from the gel. Gel slices were placed in a test tube, pulverized by grinding with a glass rod, and then treated with 0.5% SDS at 95°C for 2 hr. A supernatant was prepared by centrifugation, and 10 vol of acidified acetone (−20°C) were added to precipitate the purified proteins, which were recovered by centrifugation. The pellet, consisting of CNPI and II, was dissolved in 0.5% SDS, and 25 μg of the CNP mixture was mixed with complete Freund's adjuvant and injected into surgically exposed popliteal lymph nodes of New Zealand rabbits (Coudrier et al., 1981) under pentobarbital anesthesia. Intradermal booster injections of 50 μg purified doublet in incomplete Freund's adjuvant were given every 2 weeks, and animals were bled weekly. Anti-CNP IgG was prepared by adsorption of CNP antisera on an Afigel (Biorad) matrix to which SDS-PAGE-purified CNPI and II had been covalently attached.

^{125}I-labeling of plasma membranes. Aliquots (1 μg protein) of pure CNS or PNS myelin were suspended in 25 μl of 50 mM Na borate buffer (pH 8) and frozen (−80°C) until used for iodination. Circulating lymphocytes extracted from blood (Keller et al., 1982) were aliquoted, pelleted, and frozen (−80°C) until used. Thawed lymphocytes (30 μg protein) were resuspended in 50 mM Na borate buffer, sonicated on ice, and the membranes pelleted (100,000 × g for 10 min). This procedure was repeated 4 times. The final pellet, consisting of washed lymphocyte membranes, was used for iodination. Lymphocyte and myelin membrane aliquots (1 μg protein) were iodinated with Bolton-Hunter reagent

(Bolton and Hunter, 1973) and the ^{125}I-labeled membranes were washed extensively (5 × 1 ml) in 400 mM Tris-HCl (pH 7.5) and pelleted prior to immunoprecipitation (Goldman and Blobel, 1978) with anti-CNP IgG. Briefly, samples were dissolved in 2% SDS, heated to 100°C, and cooled to room temperature. Four volumes of a nonionic detergent-containing buffer (2.5% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 8, 4 mM EDTA) were then added, followed by aliquots of antisera. After 8 hr of incubation at 4°C, immunocomplexes were recovered by adsorption to protein A Sepharose beads. Immunoprecipitates were analyzed by electrophoresis on 8–15% SDS polyacrylamide gels, and autoradiograms were prepared.

Partial amino acid sequencing of bovine CNP. Bovine CNP was purified by a modification of the method of Suda and Tsukada (1980). Adult bovine white matter was homogenized in a buffer containing 0.5 M ammonium acetate, 10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT (pH 6.9), centrifuged at 40,000 × *g* for 30 min, and the pellet was re-extracted with the same buffer. After centrifugation, the pellet was treated with 1% Triton X-100, 1 M NH_4Ac, 10 mM Tris-HCl, 1 mM EDTA, and 1 mM DTT (pH 8.2). After centrifugation, CNP in the supernatant was purified by column chromatography on phenyl Sepharose and CM-Sepharose as described by Suda and Tsukada (1980). Column-purified CNP (approximately 90% pure) was electrophoresed on a 12.5% polyacrylamide gel, localized by Coomassie blue staining of adjacent lanes, and excised. Purified CNP was digested with staphylococcal V-8 protease (1 μg protease, 25 μg CNP) in 50 mM ammonium bicarbonate (37°C for 16 hr), and peptides were separated by electrophoresis on a 25% polyacrylamide gel. A 34,000 Da peptide was isolated by electroelution and sequenced as described by Dietzschold et al. (1987).

mRNA preparation, in vitro *translation, and hybridization selection.* RNA was extracted from brain and thymus using 6 M guanidinium-HCl (Liu et al., 1979), and poly(A)$^+$ mRNA was prepared as described by Aviv and Leder (1972). Rabbit reticulocyte lysates (NEN Products) or wheat-germ translation mixtures (Roman et al., 1978) containing ^{35}S-methionine were programmed with 4 μg/ml poly(A)$^+$ mRNA from rat brain or thymus. After translation for 3 hr at 28°C, samples (4 × 10^6 cpm) were immunoprecipitated (Goldman and Blobel, 1978) with affinity-purified CNP IgG and subjected to electrophoresis, followed by fluorography.

For hybridization selection, plasmid DNA containing CNP cDNA inserts was covalently bound to activated paper (DBM), and the hybridization selection was performed exactly as described by Traktman et al. (1984). Selected mRNA was used to program *in vitro* translations in the rabbit reticulocyte translation system as recommended (Traktman et al., 1984). It was necessary to immunoprecipitate the CNP polypeptides from all hybridization-selected CNP mRNA translation samples even though only 2 prominent bands, at 48 kDa and 46 kDa, were visible, because despite micrococcal nuclease treatment, the translations yielded an endogenous 46 kDa band that comigrated with ^{125}I-labeled CNP on SDS–PAGE. In control reticulocyte system translations in which no mRNA was added, this band was also detected, but could not be immunoprecipitated with CNP IgG.

cDNA library construction, screening, and restriction mapping of CNP cDNAs. Poly(A)$^+$ rat brain RNA (10 μg) was used as a template for cDNA synthesis. The first strand was synthesized using the protocol provided with M-MLV reverse transcriptase (Bethesda Research Laboratories), and second-strand synthesis was performed as described by Gubler and Hoffman (1983). Double-stranded (ds) cDNA (2 μg) was treated (20 min, 37°C) with mung bean nuclease (PL-Pharmacia) (5 units, in 50 mM NaCl, 30 mM Na acetate, pH 5.5, 1 mM $ZnCl_2$, and 3% glycerol; 100 μl final volume). The ds cDNA was methylated at internal EcoRI sites, and EcoRI linkers were attached in a standard overnight ligation reaction. Redundant linker sequences were excised with EcoRI enzyme (110 U/μl; Boerhinger-Mannheim). The ds cDNA was then size-fractionated on a Sepharose CL-4B (PL-Pharmacia) column (10 ml). Double-stranded cDNA larger than 1.5 kbase (as assessed by NaOH–EDTA agarose gel electrophoresis of aliquots of the column fractions) was ligated into phage λ gt11 arms (Stratagene), which had been cleaved and dephosphorylated at the single EcoRI site. The ligation mixture was packaged into bacteriophage with a commercial packaging extract (Stratagene). This library contained 3 × 10^6 independent recombinants.

Anti-CNP IgG was used to screen a portion (2 × 10^5 recombinants) of this library (Young and Davis, 1983). Eighteen expressing CNP clones were identified, of which the 3 largest (CNP15, 4, and 18) were subcloned into the EcoRI site of pGEM 1 (Promega Biotec) and mapped with restriction enzymes. In a subsequent screening of an additional 1.8 × 10^5 recombinants with nick-translated ^{32}P-labeled pCNP4, 30 additional CNP clones were detected by hybridization. Four of these, exemplified by pCNP7, were found to contain cDNA inserts of 2.8 kbase.

Detection of CNP activity in β-galactosidase–CNP fusion protein. Purified bacteriophage from CNP clones 15, 4, and 18 was used to infect *E. coli* Y1089, and liquid cultures were grown with no agitation at 32°C until they reached an O.D. of 550 = 1.0. The lysogenized bacteria were heat-shocked at 42°C for 30 min, treated with 10 mM isopropyl-β-D-thiogalactoside (IPTG) to induce the β-galactosidase promoter, and were incubated an additional 2 hr at 37°C. Bacteria from 30 ml of culture medium were pelleted (4000 × *g* for 10 min), dissolved in 600 μl of a buffer containing 10% SDS, 4 mM EDTA, 50 mM Tris-HCl (pH 8), sonicated, subjected to SDS–PAGE (6% acrylamide), and electroblotted onto nitrocellulose paper. The paper was treated using an agarose gel overlay containing the CNP reaction mixture prepared as described by Bradbury and Thompson (1984), except that 2% agarose was used, and the reaction carried out at 4°C for 10 hr. As a control, a portion of the reaction mixture in 2% agarose was also applied to an electroblot of purified rat CNS or PNS myelin that had been subjected to electrophoresis on a 10% SDS polyacrylamide gel. Immunoblotting was performed on all samples, as described (Towbin et al., 1979).

Expression of a full-length CNP cDNA. Restriction mapping of pCNP7 (see Fig. 2) in GEM 1 showed that the 5′ end of the insert was immediately downstream to the T7 promoter. mRNA was transcribed from 0.5 μg of pCNP7 in a 20 μl reaction mixture incubated for 1 hr (40°C), following the protocol recommended for the Riboprobe Gemini system (Promega Biotec). The reaction mixture also contained the nucleotide $m^7G(5')ppp(5')G$ (1 mM) to cap the synthesized mRNA. One microliter of this mixture was used to program a 25 μl wheat-germ translation mixture, which was incubated for 3 hr (25°C).

RNA and DNA blotting. For Northern blots, the total RNA (10 μg) extracted from brain, sciatic nerve, thymus, and liver was fractionated by electrophoresis in formaldehyde-containing 1.7% agarose gels (Genescreen Manual, NEN Products). The RNA was transferred to Genescreen membranes, which were then probed with nick-translated ^{32}P-labeled CNP cDNAs. The relative electrophoretic mobilities of each hybridizing mRNA species were calculated from RNA standards (BRL), which were coelectrophoresed on each blot. The blots were washed twice in 2 × SSC (Genescreen Manual) at room temperature (15 min) followed by 2 washes (2 × SSC, 1.0% SDS) at 65°C for 30 min each and a final wash (0.1 × SSC) at room temperature (15 min).

For Southern blots, high-molecular-weight rat liver DNA was prepared (Frischauf et al., 1983) and aliquots (10 μl) were digested to completion with restriction enzymes, subjected to electrophoresis on a 1.0% agarose gel, and electroblotted onto nitrocellulose. The blots were probed with nick-translated ^{32}P-labeled inserts from pCNP7. The blots were washed as above except that 0.1% SDS was used in all washes, and the final wash was performed at 65°C.

cDNA sequencing. Restricted fragments were directionally subcloned into compatible sites of M13 bacteriophages mp18 and 19 (Pharmacia) prior to sequencing by the dideoxy-chain termination method (Sanger et al., 1977). In 2 cases, oligonucleotide primers that had been synthesized in an Applied Biosystems DNA synthesizer were used. Parts of the sequence were obtained by analysis of overlapping deletions generated by the method of Dale et al. (1985), using the RDS cyclone system from IBI.

Computer analysis of the amino acid sequence. The protein database of the National Biomedical Research Foundation was screened for homologous protein sequences using the FASTP program (Lipman and Pearson, 1985).

Results and Discussion

In vitro *synthesis of CNPI and CNPII*

It has been reported that the sets of CNPs in the PNS, CNS, and in the extraneural tissues have significantly different electrophoretic mobilities (Sheedlo et al., 1984; Sprinkle et al., 1985). However, we found that antibodies raised against CNS CNPs recognized pairs of polypeptides of identical electrophoretic mobilites (46 and 48 kDa) in detergent-solubilized ^{125}I-labeled extracts of rat CNS and PNS myelin and lymphocyte membranes (Fig. 1, lanes a–c). This strongly suggests that the same proteins are synthesized in the rat tissues we examined. In ad-

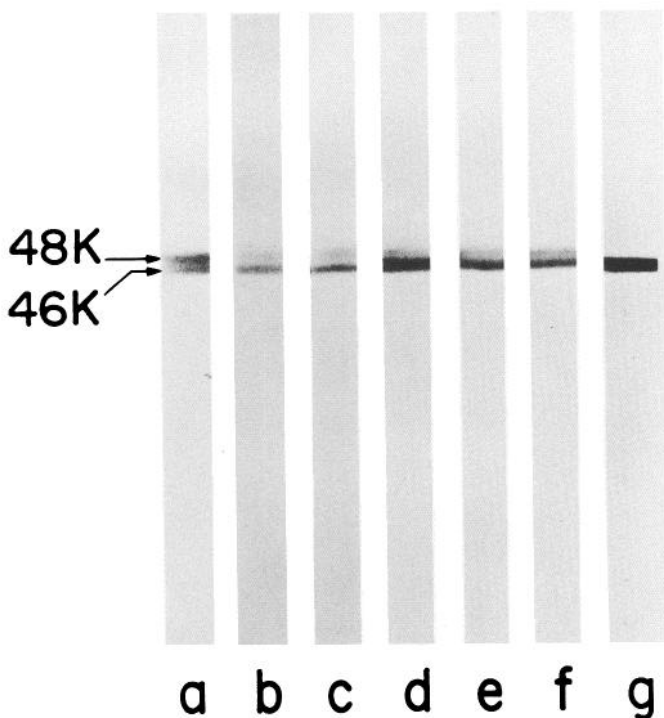

Figure 1. The set of CNP polypeptides is the same in brain, thymus, and blood lymphocytes. Lanes *a–c,* Immunoprecipitates of ^{125}I-labeled CNP doublet from membrane (10^6 cpm). *a,* CNS myelin; *b,* PNS myelin; *c,* lymphocyte membranes. Lanes *d* and *e,* Immunoprecipitates from ^{35}S-methionine-labeled *in vitro* translations programmed with (*d*) brain mRNA, (*e*) thymus mRNA, (*f*) mRNAs selected by clone pCNP4. Total translation products from mRNA transcribed *in vitro* from pCNP7 and translated in the wheat-germ system (*g*).

dition, pairs of polypeptides with the same electrophoretic mobilities were immunoprecipitated from translation mixtures programmed with brain (Fig. 1, lane d) or thymus (Fig. 1, lane e) mRNA. Since the relative mobilities of the *in vitro* synthesized and mature CNPs are identical, it can be concluded that the CNP polypeptides, which are not glycoproteins and are thought to be synthesized on free polysomes (Karin and Waehneldt, 1985), do not undergo co- or posttranslational proteolytic modifications during their incorporation into the myelin sheath or their association with the plasma membrane of the lymphocyte. It appears, therefore, that the only posttranslational modification that the CNPs may undergo is the well-known (Bradbury et al., 1984) phosphorylation *in vitro* of CNPII by a cAMP-dependent protein kinase that is apparently a normal constituent of myelin.

Molecular cloning of CNP cDNAs

Eighteen cDNA clones were initially recognized by affinity-purified CNP antibodies in a portion (2×10^5 recombinants) of the phage λ gt11 expression library (see Materials and Methods) and were subcloned into pGEM vectors. CNP inserts from these plasmids were used in hybridization-selection assays with total brain mRNA. In all cases, both the 46 and 48 kDa CNP polypeptides were recovered by immunoprecipitation from the translation mixtures programmed with the selected mRNA (for example, see Fig. 1, lane f). This demonstrates that these polypeptides are encoded by highly homologous mRNAs, which may be selected out of a mixture of total brain RNAs by hybridization to a single cloned cDNA insert. Several of these clones (e.g., pCNP15, pCNP4, and pCNP18) were characterized by restriction mapping (Fig. 2), and the 5′-3′ orientation of each insert was determined as described in Figure 2.

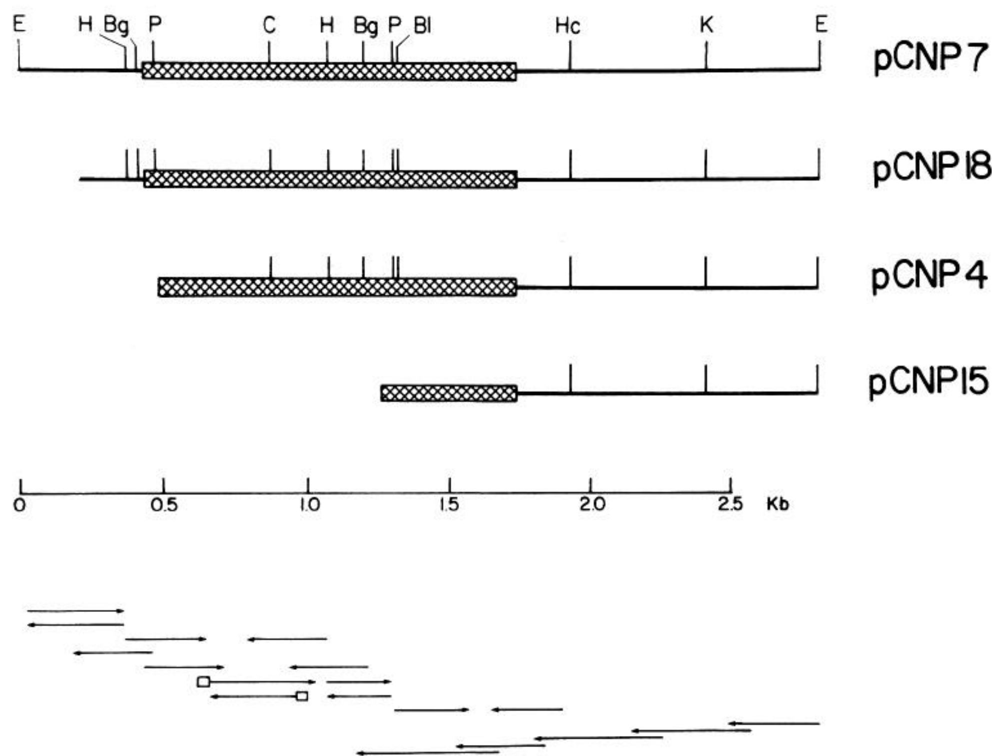

Figure 2. Restriction mapping of CNP cDNAs 15, 4, 18, and 7 and sequencing strategy. CNP cDNA inserts in pGEM were restricted with EcoRI and purified by agarose gel electrophoresis, eluted, and cut with various restriction enzymes. The 5′-3′ orientation of pCNP4 was determined by 3′ end-labeling with Klenow fragment at the artificial EcoRI site at each end of the cDNA insert, cutting the labeled insert with HindIII, and showing that only the smaller HindIII–EcoRI fragment hybridized to a brain RNA blot. The 5′ end of pCNP7 is at 0 kilobase. This orientation was confirmed by identifying poly(A$^+$) tails upon dideoxy sequencing of the 3′ end of each clone. *Hatched bars* represent coding regions. The direction of sequencing is shown by the *arrows.* An *open rectangle* at the beginning of an arrow indicates that a synthetic oligonucleotide primer was used. *Bg* = Bgl 11; *Bl* = Bal 1; *K* = Kpn; *H* = HindIII; *P* = PstI; *C* = ClaI; *Hc* = HincII.

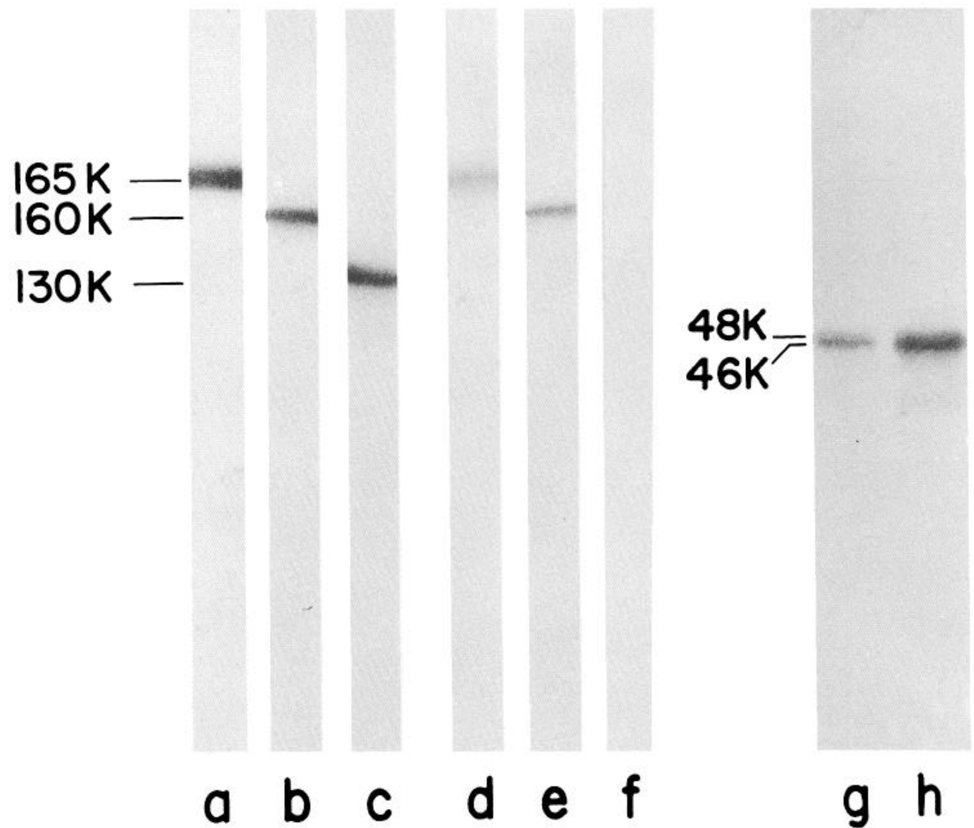

Figure 3. β-Galactosidase–CNP fusion proteins display CNP activity. Lanes *a–c,* Immunoblots of β-galactosidase–CNP fusion proteins (see Materials and Methods) from (*a*) CNP18, (*b*) CNP4, and (*c*) CNP15. Nitrocellulose blots were prepared from fusion proteins run on a 6% polyacrylamide gel. Goat anti-rabbit IgG conjugated to HRP was used as the second antibody. Lanes *d–f,* CNP activity in β-galactosidase–CNP fusion proteins from (*d*) CNP18, (*e*) CNP4, and (*f*) CNP15. Lanes *g* and *h,* CNP activity in highly purified rat (*g*) PNS and (*h*) CNS myelin, showing the specificity of the enzymatic reaction. The reaction products reveal the positions of CNPI and CNPII on the nitrocellulose blot, which was prepared from myelin samples run on a 10% polyacrylamide gel.

Restriction mapping (Fig. 2) indicated that the different inserts in the expressing clones CNP15, CNP4, and CNP18 share the same 3′ end, and we have confirmed this by nucleotide sequence analysis. Mapping experiments also demonstrated that these cDNAs extend to various lengths in the 5′ direction. These clones, therefore, encode fusion proteins (see Fig. 3, lanes a–c) consisting of successively longer carboxy-terminal segments of the CNP polypeptides linked at their NH_2 termini to β-galactosidase. It has been shown that the enzymatic activity of electrophoretically separated myelin CNPs can be regenerated by incubation with guanidinium hydrochloride (Bradbury and Thompson, 1984), and therefore we tested the capacity of similarly treated β-galactosidase–CNP fusion proteins to hydrolyze cyclic nucleotides. It was possible to show, in nitrocellulose blots of extracts of IPTG-induced lysogenized bacteria, that the fusion proteins generated by clones CNP18 and CNP4 possess CNP activity (Fig. 3, lanes d and e). On the other hand, the smaller fusion protein generated by CNP15 was enzymatically inactive (Fig. 3, lane f). By comparing on gels the sizes of the β-galactosidase–CNP fusion proteins with those of native β-galactosidase induced in *E. coli* infected with wild-type λ gt11, we estimated that fusion proteins from CNP18, 4, and 15 contained carboxy-terminal CNP peptide segments of approximately 48, 43, and 13 kDa, respectively.

CNP activity could also be detected in many antibody-positive plaques on the original filters used in screening the library. The general applicability of the use of enzymatic activities or ligand-binding properties to screen expression libraries (Sikela and Hahn, 1985; Kaufman et al., 1986), and even to delineate active sites in cloned polypeptides has yet to be fully explored.

Analysis of CNP cDNA clones

By comparing the size of each CNP cDNA insert in the expressing clones with the length of the nucleotide sequence we calculated to be necessary to encode the CNP portion of the corresponding fusion protein, we first estimated and later confirmed by sequence analysis that each CNP cDNA contained more than 1000 nucleotides of untranslated sequence at the 3′ end. Long 3′ noncoding regions seem to be a common feature among many brain mRNAs (Roach et al., 1983; Lewis et al., 1984; de Ferra et al., 1985; Lewis and Cowan, 1985; Milner et al., 1985; Mentaberry et al., 1986), but the functional significance of these sequences has yet to be determined.

RNA blots prepared from rat brain, peripheral nerve, thymus, and liver hybridized with ^{32}P-labeled CNP cDNA inserts from any CNP clone revealed bands of equal intensities at ~2800 and ~2400 nucleotides nts in the nervous tissue samples (Fig. 4, lanes a, b, and e), and a single band at ~2500 nt in the thymus (Fig. 4, lane c). There was no detectable hybridization to liver RNA (Fig. 4, lane d). It should be noted that the CNP polypeptides appear to differ by only 2000 Da (about 20 amino acids), which can be encoded by a nucleotide sequence of only about 60 bases. The presence or absence of this short sequence would not significantly affect the mobility of a CNP mRNA in the agarose gels we used for these experiments, and, in fact, the apparently single CNP mRNA species in thymus represents mRNAs that encode both polypeptides in this organ (see Fig. 1, lane e). The Northern blot analysis therefore suggested that the CNP mRNAs differ in size primarily because of differences in their noncoding regions.

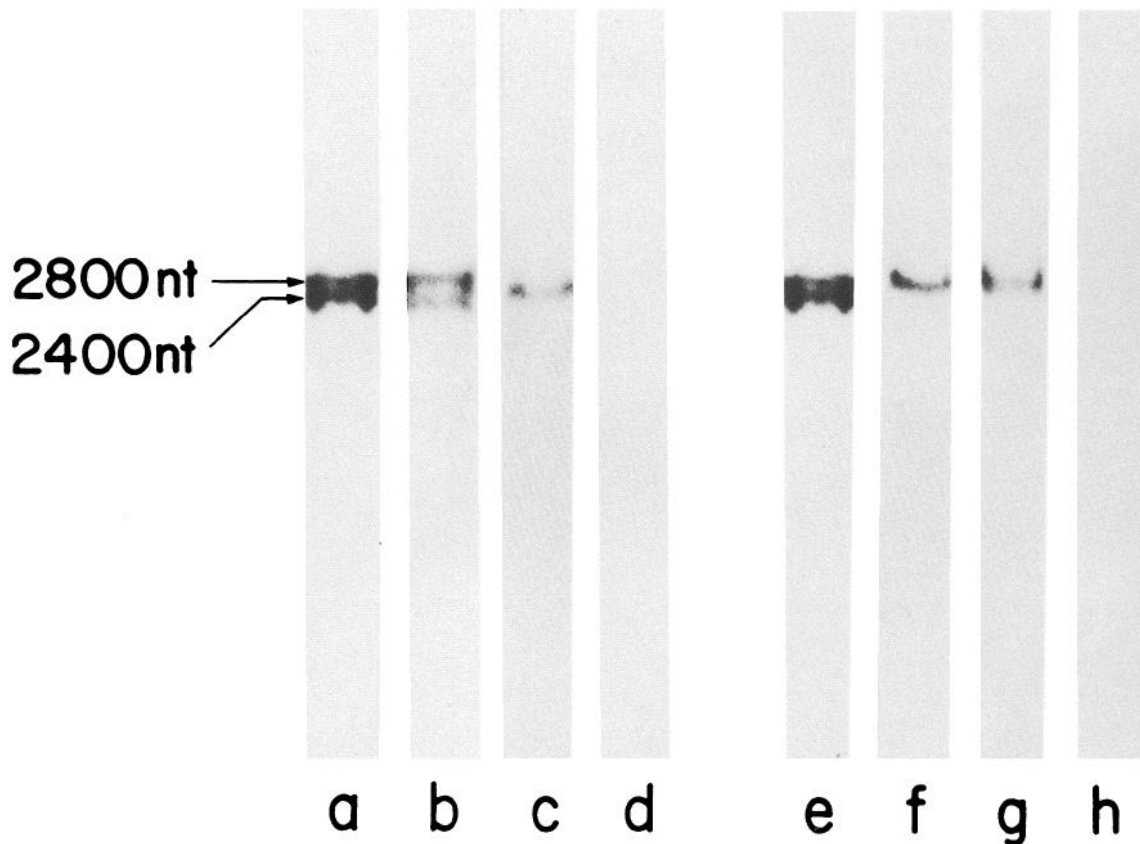

Figure 4. Northern blot analysis of CNP mRNAs. Lanes *a–d,* Total RNA from (*a*) brain, (*b*) sciatic nerve, (*c*) thymus, and (*d*) liver probed with ^{32}P-labeled nick-translated pCNP4. Lane *e,* Repeat of lane *a* for size comparison. Lanes *f–h,* Total RNA from brain (*f*), sciatic nerve (*g*), and thymus (*h*) probed with the ^{32}P-labeled, nick-translated 5′ EcoRI–HindIII fragment (373 bp) from pCNP7.

All of our expressing clones contained inserts that were smaller than the 2800 nt CNP mRNA detected in the nervous tissue RNA blots. In order to obtain full-length clones for these mRNAs, we screened an additional portion of the library with the ^{32}P-labeled 5′ HindIII–EcoRI fragment from pCNP4 (see Fig. 2). Several nonexpressing, apparently full-length cDNA clones containing 2.8 kKb inserts were obtained in this way, and restriction mapping (see pCNP7 in Fig. 2) showed that these CNP cDNAs extended about 450 bp upstream from the 5′ PstI site in pCNP4. When pCNP7 was used to program an *in vitro* transcription–translation system, the 46 kDa CNP polypeptide was synthesized (Fig. 1, lane g), demonstrating that this cDNA contained the natural initiation codon and entire coding sequence for CNPI.

In order to begin identifying sequences unique to the largest CNP mRNA species, we probed identical blots with restriction fragments from pCNP7. Restriction fragments (see Fig. 2) downstream from the 5′ HindIII site (HindIII–HindIII, PstI–PstI, ClaI–EcoRI) were found to hybridize to each CNP mRNA species

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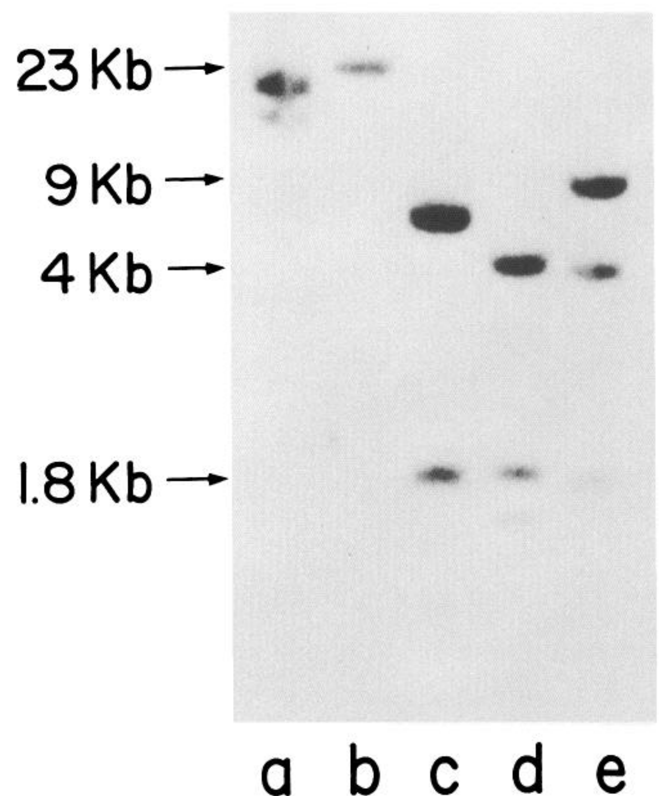

Figure 5. Southern blot analysis. High-molecular-weight rat liver DNA was prepared (see Materials and Methods), cut with restriction enzymes, run on a 0.5% agarose gel, and blotted onto nitrocellulose. The blot was probed with the entire ^{32}P-labeled, nick-translated insert from pCNP7. Lane *a,* BamHI; *b,* EcoRI; *c,* HindIII, *d,* PstI, *e,* NcoI.

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-420        -410        -400        -390        -380        -370
   C GGC TGA CTG GGA GCA GCA GGC ACT TGC TGC CCT TGC ATG CCC TGC CAC CAG GGT

-360        -350        -340        -330        -320        -310
GTT CTC TCT GAG GGG TAG CTG AAA GCC GGA GAG TAA GAG GTT TGA TAG CTT CAG TGA GGG

-300        -290        -280        -270        -260        -250
TCC TTC TAC TCA TAC AAC CCT GCT GTT GAA GAT ACG GAA GGA CCA GCT GGG AGG AAG AAA

-240        -230        -220        -210        -200        -190
AGC TAC CAT TCC TGC CGG GAG CTG GGA AAA GCG CCC ACG CAT TCA AGG GTC GTT CAA GTT

-180        -170        -160        -150        -140        -130
AGG CTC GGG GAT TCT GAA CTG ACA TCC GGT CCC CCT CTG GAG AAC TAG GCC AAA GAC AAG

-120        -110        -100        -90         -80         -70
GGT TTG GTG CTT GTG GGC TGG CTT TGA GGA GCC CGG GCT AAC ATC TCC TCC TCC CTT CTC

-60         -50         -40         -30         -20         -10
ACA TAG AGC ACA AGC TTT GCC CGA AAA AGC CAC ACA TTC CTG CCC AAG ATC TTC TTC AGA

1           10          20          30          40          50
AAA ATG TCA TCC TCA GGA GCT AAG GAC AAG CCA GAG CTG CAG TTC CCT TTC CTT CAA GAT
    Met Ser Ser Ser Gly Ala Lys Asp Lys Pro Glu Leu Gln Phe Pro Phe Leu Gln Asp

60          70          80          90          100         110
GAG GAC ACG GTG GCT ACG CTG CAC GAG TGC AAG ACG CTG TTC ATC CTG CGT GGC CTG CCG
Glu Asp Thr Val Ala Thr Leu His Glu Cys Lys Thr Leu Phe Ile Leu Arg Gly Leu Pro

120         130         140         150         160         170
GGC AGC GGC AAG TCC ACT CTG GCT CGG CTC AAT CCG TGG AGA AGT ACC ACA ACG GCA CCA
Gly Ser Gly Lys Ser Thr Leu Ala Arg Leu Asn Pro Trp Arg Ser Thr Thr Thr Ala Pro

180         190         200         210         220         230
AGA TGG TGT CTG CTG ATG CTT ACA AGA TCA TTC CTG GCT CTC GGG CAG ACT TCT CCG AGG
Arg Trp Cys Leu Leu Met Leu Thr Arg Ser Phe Leu Ala Leu Gly Gln Thr Ser Pro Arg

240         250         260         270         280         290
GAG TAC AAG CGT CTG GAC GAG GAC CTG GCT GGC ATA CTG CCG CGG GAC ATC AGG GTT CTT
Glu Tyr Lys Arg Leu Asp Glu Asp Leu Ala Gly Ile Leu Pro Arg Asp Ile Arg Val Leu

300         310         320         330         340         350
GTG CTC GAT GAC ACC AAC CAT GAG CGG GAA CGG CTG GAC CAG TTT TTT GAA ATG GCA GAC
Val Leu Asp Asp Thr Asn His Glu Arg Glu Arg Leu Asp Gln Phe Phe Glu Met Ala Asp

360         370         380         390         400         410
CAG TAC CAG TAC CAG GTG GTG CTG GTG GAG CCC AAG ACG GCG TGG CGA CTA GAC TGT GCC
Gln Tyr Gln Tyr Gln Val Val Leu Val Glu Pro Lys Thr Ala Trp Arg Leu Asp Cys Ala

420         430         440         450         460         470
CAG CTC AAG GAG AAG AAC CAA TGG CAG CTG CTC GCT CGC ATC GAT GAC CTG AAG AAG CTG
Gln Leu Lys Glu Lys Asn Gln Trp Gln Leu Leu Ala Arg Ile Asp Asp Leu Lys Lys Leu

480         490         500         510         520         530
AAG CCC GGG CTG GAG AAG GAC TTC CTG CCA CTT TAC TTT GGC TGG TTC CTG ACC AAA AAG
Lys Pro Gly Leu Glu Lys Asp Phe Leu Pro Leu Tyr Phe Gly Trp Phe Leu Thr Lys Lys

540         550         560         570         580         590
AGT TCC GAG ACC CTC CGA AAA GAC AGC CGG CCA GGA TCA TTT CAG TGG AAG CTG GGA AAT
Ser Ser Glu Thr Leu Arg Lys Asp Ser Arg Pro Gly Ser Phe Gln Trp Lys Leu Gly Asn

600         610         620         630         640         650
CAC AAG GCT TTA AGA AAG AGC TTC GAC ACT TCA CTT TAC ATG GGG ATG AAC CCA AGG AGA
His Lys Ala Leu Arg Lys Ser Phe Asp Thr Ser Leu Tyr Met Gly Met Asn Pro Arg Arg

660         670         680         690         700         710
AGC TTG CCT GTC AGC TAC TTT GGC AAG AGA CCT CCA GGC GTG CTG CAC TGT ACA ACC AAA
Ser Leu Pro Val Ser Tyr Phe Gly Lys Arg Pro Pro Gly Val Leu His Cys Thr Thr Lys

720         730         740         750         760         770
TTC TGT GAC TAC GGG AAG GCC ACC GGG GCA GAA GAA TAT GCC CAA CAG GAT GTG GTG AGG
Phe Cys Asp Tyr Gly Lys Ala Thr Gly Ala Glu Glu Tyr Ala Gln Gln Asp Val Val Arg

780         790         800         810         820         830
AGA TCT TAC GGC AAG GCC TTC AAA CTG TCC ATC TCT GCC CTC TTT GTG ACA CCC AAG ACA
Arg Ser Tyr Gly Lys Ala Phe Lys Leu Ser Ile Ser Ala Leu Phe Val Thr Pro Lys Thr

840         850         860         870         880         890
GCT GGG GCC CAG GTG GTG CTT AAT GAG CAG GAG CTG CAG TTG TGG CCA AGT GAC CTG GAC
Ala Gly Ala Gln Val Val Leu Asn Glu Gln Glu Leu Gln Leu Trp Pro Ser Asp Leu Asp

900         910         920         930         940         950
AAG CCC TCA TCC TCC GAG AGC CTG CCC CCA GGG AGC CGA GCT CAC GTC ACC CTA GGC TGT
Lys Pro Ser Ser Ser Glu Ser Leu Pro Pro Gly Ser Arg Ala His Val Thr Leu Gly Cys

960         970         980         990         1000        1010
GCA GCC GAC GTG CAG CCA GTG CAG ACA GGC CTT GAC CTC TTA GAG ATT TTA CAG CAG GTG
Ala Ala Asp Val Gln Pro Val Gln Thr Gly Leu Asp Leu Leu Glu Ile Leu Gln Gln Val

1020        1030        1040        1050        1060        1070
AAG GGG GGC AGC CAA GGT GAG GAG GTG GGT GAG CTC CCC CGG GGC AAG CTC TAT TCC TTG
Lys Gly Gly Ser Gln Gly Glu Glu Val Gly Glu Leu Pro Arg Gly Lys Leu Tyr Ser Leu

1080        1090        1100        1110        1120        1130
GGC AAA GGG CGT TGG ATG CTG AGC TTG GCG AAG AAG ATG GAA GTC AAG GCC ATC TTC ACG
Gly Lys Gly Arg Trp Met Leu Ser Leu Ala Lys Lys Met Glu Val Lys Ala Ile Phe Thr

1140        1150        1160        1170        1180        1190
GGT ACT ATG GGA AGG GCA AAC CTG TAC CGT ACA CGG CAG CCG AAG GGG GTT GCC ATG CAG
Gly Thr Met Gly Arg Ala Asn Leu Tyr Arg Thr Arg Gln Pro Lys Gly Val Ala Met Gln

1200        1210        1220        1230        1240        1250
ATC TGC ACC ATC ATC TAG GGT TCA CGC CAC TGT GCC ACC GCT GTG GAC ACA GCA ACA TGC
Ile Cys Thr Ile Ile ---

1260        1270        1280        1290        1300        1310
CTT CCA CTT GAT TTT TTA AGA TTT TTT TTT TTT ACT CAA AGC TAA TCT ACC TAT AAC TTT

1320        1330        1340        1350        1360        1370
TAG AAG TCT GTT AAA ATA ACC ATC AGT TCC TTC ACC CAC CCT CTT CCC CTT AAC ACT CAG

1380        1390        1400        1410        1420        1430
CTA ACA CAG GCA GTG GGG CAG AGG AAG ACA CCA TTC AGG AAC CTG GAC CAG AGG TGG TAA

1440        1450        1460        1470        1480        1490
TTA AAG GGC TGG GTC ATT GGC CTT GGA GCG GCC ACC TAG ATG CAC CCA GGA CCT TGT TGA

1500        1510        1520        1530        1540        1550
CCC TCC CTT AGC CGC TGC CCA TGC AGA GCT GGG CAC ACA CAC CTC AAG CCA CCA CAT CAT

1560        1570        1580        1590        1600
GCA AGG GAC AAA TGC TAC TGG CAC AGA AAA CAG TGT AGC TTT AA
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Figure 6. Nucleotide and amino acid sequences of rat CNPI. The amino acid sequence derived from N-terminal analysis of a 34,000 Da peptide prepared by V8 digestion is *underlined.* The 3′ untranslated sequence is incomplete; 900 bp are not shown.

on Northern blots. However, when these blots were probed with the EcoRI–HindIII fragment from the 5′ end of pCNP7, only a 2800 nt nervous system mRNA was detected (Fig. 4, lanes f and g), revealing that the nonhybridizing species have significantly different 5′ ends than does the mRNA from which pCNP7 was derived. This restriction fragment (373 bp) is approximately the same length as the size difference observed between the 2 nervous system CNP mRNA species, and therefore probably

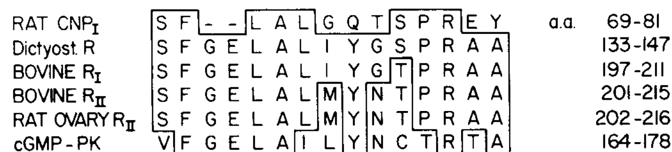

Figure 7. CNP contains a potential cyclic nucleotide binding site. The amino acid sequence 69–81 of rat CNS CNPI is homologous to the cyclic nucleotide binding sites of the cAMP-dependent protein kinase regulatory subunit of *Dictyostelium discoideum* R (Mutzel et al., 1987), bovine skeletal muscle RI (Titani et al., 1984), bovine heart RII (Takio et al., 1984a), rat ovary RII (Jahnsen et al., 1986) and bovine lung cGMP-dependent protein kinase (Takio et al., 1984b).

represents the major structural difference between them. The fact that all the thymus CNP mRNAs are about 100 nt larger than the 2400 nt mRNA species suggests that yet other differences between the primary nucleotide sequences of the nervous system CNP mRNAs and their extraneural counterparts must also exist. Although the precise structural basis for the 5′ heterogeneity between the various CNP mRNAs remains to be elucidated, genomic DNA blots probed with either pCNP7 or 4 reveal only a few bands (Fig. 5) consistent with the existence of a single CNP gene from which the various mRNA transcripts may be derived, probably by the splicing of different 5′ exonic regions onto a set of 3′ exons common to all CNP mRNAs. Recent precedents for this have been reported by Ben-Neriah et al. (1986) for the cellular abl proto-oncogene and by Nabeshima et al. (1984) and Robert et al. (1984) for 2 myosin light chains.

Amino acid sequence of CNPI

Sequence analysis of pCNP7, which we found in transcription–translation experiments to encode CNPI, revealed a protein of 404 amino acids, including the initiator methionine (Fig. 6). The calculated molecular weight was 45,592 Da, in excellent

agreement with the estimated molecular weight for CNPI (46 kDa) as assessed by SDS–PAGE. The deduced amino acid composition of CNPI closely corresponds to the experimentally determined composition of rat CNP reported by Sprinkle et al. (1980). The mature protein has a N-terminal serine that we believe is blocked *in vivo,* probably by acetylation, a common modification of this amino acid.

Partial digestion of bovine CNS CNP with staphylococcal V8 protease generated several peptides, one of which (34,000 Da) was purified by electroelution for N-terminal amino acid sequencing (see Materials and Methods). The N-terminal sequence revealed 29 consecutive amino acids, which, when compared with the amino acid sequence deduced from the nucleotide sequence obtained from pCNP7, indicated that the protease-generated N-terminus corresponded to amino acids 117–145 in the rat CNPI protein. The only difference observed was a leucine in bovine CNP that substitutes for methionine (amino acid 117) in the rat sequence. This difference could have arisen from a single base substitution in the CNP gene.

A search of the protein database of the National Biomedical Research Foundation revealed no extensive homologies to other proteins. However, we did identify a sequence in CNP (amino acids 69–81) that is homologous to known sites for cyclic nucleotide binding in other proteins (see Fig. 7). That this site might be functional in CNP is suggested by the observation that 3′5′ cAMP is known to inhibit CNP activity (Sims and Carnegie, 1978).

Although CNPI is not known to be phosphorylated *in vivo* or *in vitro,* some potential sites for phosphorylation were revealed in the sequence analysis. These include a potential phosphorylation site for protein kinase C at amino acids 185–189 (RKD*S*R). In addition, the sequence TGAEE*Y* at amino acids 247–252 could possibly be a substrate for a tyrosine protein kinase.

The CNP cDNA clones that we have now isolated should prove to be useful tools in investigations into the functional role(s) of the CNPs in the various tissues in which they are found. The fact that we can generate fusion proteins containing incomplete CNP portions that still retain CNP activity may lead to the precise localization of the active site in these molecules and eventually to the identification of the natural substrate(s) for these enzymes.

Note added in proof: Just prior to publication of this paper, Kurihara et al. (1987) published the nucleotide sequence for a bovine brain.

References

Aviv, H., and P. Leder (1972) Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid–cellulose. Proc. Natl. Acad. Sci. USA *9:* 1408–1412.

Ben-Neriah, Y., A. Bernards, M. Paskind, G. Q. Daley, and D. Baltimore (1986) Alternative 5′ exons in c-abl mRNA. Cell *44:* 577–586.

Bernier, L., F. Alvarez, M. Norgard, D. D. Sabatini, and D. R. Colman (1986) Cloning and characterization of myelin CNP cDNAs. Trans. Am. Soc. Neurochem. *17:* 106.

Bolton, A., and W. Hunter (1973) The labelling of proteins to high specific activities by conjugation to a ^{125}I-containing acylation agent. Biochem. J. *133:* 529–539.

Bradbury, J. M., and R. J. Thompson (1984) Photoaffinity labelling of central nervous system myelin. Biochem. J. *221:* 361–368.

Bradbury, J. M., R. S. Campbell, and R. J. Thompson (1984) Endogenous cyclic AMP-stimulated phosphorylation of a Wolfgram protein component in rabbit central nervous system myelin. Biochem. J. *221:* 351–359.

Coudrier, E., H. Reggio, and D. Louvard (1981) Immunolocalization of the 110,000 molecular weight cytoskeletal protein of intestinal microvilli. J. Mol. Biol. *152:* 49–66.

Dale, R. M. K., B. A. McClure, and J. P. Houchins (1985) A rapid single-stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: Application to sequencing the corn mitochondrial 18S rRNA. Plasmid *13:* 31–41.

de Ferra, F., H. Engh, L. Hudson, J. Kamholz, C. Puckett, S. Molineaux, and R. A. Lazzarini (1985) Alternative splicing accounts for the four forms of myelin basic protein. Cell *43:* 721–727.

Dietzschold, B., M. Lafon, H. Wang, L. Otvos, E. Celis, W. H. Wunner, and H. Koprowski (1987) Localization and immunological characterization of antigenic domains of the rabies virus internal N and NS proteins. Virus Res. (in press).

Drummond, R. J., and G. Dean (1980) Comparison of 2′,3′-cyclic nucleotide 3′-phosphodiesterase and the major component of Wolfgram protein WI. Neurochemistry *35:* 1155–1165.

Folch-Pi, J., and M. Lees (1951) Proteolipids, a new type of tissue lipoproteins. J. Biol. Chem. *191:* 807–817.

Frischauf, A. M., H. Lehrach, A. Poustkau, and N. Murray (1983) Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. *170:* 827–842.

Goldman, B. M., and G. Blobel (1978) Biogenesis of peroxisomes: Intracellular site of synthesis of catalase and uricase. Proc. Natl. Acad. Sci. USA *75:* 5066–5070.

Gubler, U., and B. J. Hoffman (1983) A simple and very efficient method for generating cDNA libraries. Gene *25:* 263–269.

Jahnsen, T., L. Hedin, V. J. Kidd, W. G. Beattie, S. M. Lohmann, U. Walter, J. Durica, T. Z. Schulz, E. Schiltz, M. Browner, C. B. Lawrence, D. Goldman, S. L. Ratoosh, and J. S. Richards (1986) Molecular cloning, cDNA structure and regulation of the regulatory subunit of type II cAMP dependent protein kinase from rat ovarian granulos cells. J. Biol. Chem. *261:* 12352–12361.

Karin, N. J., and T. V. Waehneldt (1985) Biosynthesis and insertion of Wolfgram protein into optic nerve membranes. Neurochem. Res. *10:* 897–907.

Kaufman, D. L., J. F. McGinnis, N. R. Krieger, and A. J. Tobin (1986) Brain glutamate decarboxylase cloned in gt11: Fusion protein produces aminobutyric acid. Science *232:* 1138–1140.

Keller, S. E., S. J. Schleifer, and F. P. McKegney (1982) A simplified method for assessing PHA-induced stimulation of rat peripheral blood lymphocytes. J. Immunol. Methods *51:* 287–291.

Kurihara, T., and Y. Tsukada (1968) 2′3′-Cyclic nucleotide 3′-phosphohydrolase developing chick brain and spinal cord. J. Neurochem. *15:* 827–832.

Kurihara, T., J. L. Nussbaum, and P. Mandel (1970) 2′3′-Cyclic nucleotide 3′-phosphohydrolase in brains of mutant mice with deficient myelination. J. Neurochem. *17:* 993–997.

Kurihara, T., A. V. Fowler, and Y. Takahashi (1987) cDNA cloning and amino acid sequence of bovine brain 2′,3′-cyclic-nucleotide 3′-phosphodiesterase. J. Biol. Chem. *262:* 3256–3261.

Lewis, S. A., and N. J. Cowan (1985) Genetics, evolution, and expression of the 68,000-mol-wt neurofilament protein: Isolation of a cloned cDNA probe. J. Cell Biol. *100:* 843–850.

Lewis, S. A., J. Balcarek, V. Krek, M. Shelanski, and N. J. Cowan (1984) Sequence of a cDNA clone encoding mouse glial fibrillary acidic protein: Structural conservation of intermediate filaments. Proc. Natl. Acad. Sci. USA *81:* 2743–2746.

Lipman, D. J., and W. R. Pearson (1985) Rapid and sensitive protein similarity searches. Science *227:* 1435–1441.

Liu, C. P., D. Slate, R. Gravel, and F. H. Ruddle (1979) Biological detection of specific mRNA molecules by microinjection. Proc. Natl. Acad. Sci. USA *76:* 4503–4506.

Mentaberry, A., M. Adesnik, M. Atchison, E. M. Norgard, F. Alvarez, D. D. Sabatini, and D. R. Colman (1986) Small basic proteins of myelin from central and peripheral nervous systems are encoded by the same gene. Proc. Natl. Acad. Sci. USA *83:* 1111–1114.

Milner, R. J., C. Lai, K. A. Nave, D. Lenoir, J. Ogata, and J. G. Sutcliffe (1985) Nucleotide sequences of two mRNAs for rat brain myelin proteolipid protein. Cell *42:* 931–939.

Mutzel, R., M.-L. Lacombe, M.-N. Simon, J. deGunzburg, and M. Veron (1987) cAMP-dependent protein kinase from *Dictyostelium discoideum*: Cloning and cDNA sequence of the regulatory subunit. Proc. Natl. Acad. Sci. USA *84:* 6–10.

Nabeshima, Y., Y. Fujii-Kuriyama, M. Muramatsu, and K. Ogata

(1984) Alternative transcription and two modes of splicing result in two myosin light chains from one gene. Nature *308:* 333–338.

Nishizawa, Y., T. Kurihara, T. Masuda, and Y. Takahashi (1985) Immunohistochemical localization of 2',3'-cyclic nucleotide 3'-phosphodiesterase in adult bovine cerebrum and cerebellum. Neurochem. Res. *10:* 1107–1118.

Norton, W. T., and S. E. Poduslo (1973) Myelination in rat brain: Method of myelin isolation. J. Neurochem. *21:* 749–757.

Olafson, R. W., G. I. Drummon, and J. F. Lee (1969) Studies on 2'3'-cyclic nucleotide 3'-phosphohydrolase from brain. Can. J. Biochem. *47:* 961–966.

Roach, A., K. Boylan, S. Horvath, S. B. Prusiner, and L. E. Hood (1983) Characterization of cloned cDNA representing rat myelin basic protein: Absence of expression in brain of shiverer mutant mice. Cell *34:* 799–806.

Robert, B., P. Daubas, M.-A. Akimenko, A. Cohen, I. Garner, J.-L. Guenet, and M. Buckingham (1984) A single locus in the mouse encodes both myosin light chains 1 and 3, a second locus corresponds to a related pseudo-gene. Cell *39:* 577–586.

Roman, R., J. D. Brooker, S. N. Seal, and A. Marcus (1978) Inhibition of the transition of a 40 S ribosome met-t-RNA complex to an 80 S ribosome met-C-RNA complex by 7 methylguanosine 5'-phosphate. Nature *260:* 359–360.

Roussel, G., J. P. Delaunoy, P. Mandel, and J. L. Nussbaum (1978) Ultrastructural localization study of two Wolfgram proteins in rat brain tissue. J. Neurocytol. *7:* 155–163.

Roussel, G., M. Sensenbrenner, G. Labourdette, E. Wittendorp-Rechenmann, B. Pettmann, and J. L. Nussbaum (1983) An immunohistochemical study of two myelin specific proteins in enriched oligodendroglial cell cultures combined with an autoradiographic investigation using [^{3}H]-thymidine. Dev. Brain Res. *8:* 193–204.

Sanger, F., S. Nicklen, and A. R. Coulson (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA *74:* 5463–5467.

Sheedlo, H. J., J. E. Doran, and T. J. Sprinkle (1984) An investigation of 2',3'-cyclic nucleotide 3'-phosphodiesterase in peripheral blood elements and CNS myelin. Life Sci. *34:* 1731–1737.

Sikela, J. M., and W. E. Hahn (1985) Identification of genes using ligand probes: Calmodulin binding proteins in gt11 expression libraries. Soc. Neurosci. Abstr. *11:* 353.

Sims, N. R., and P. R. Carnegie (1978) 2',3'-Cyclic nucleotide 3'-phosphodiesterase. In *Advances in Neurochemistry,* vol. 3, B. W. Agranoff and M. H. Aprison, eds., pp. 1–41, Plenum, New York.

Sprinkle, T. J., M. R. Wells, F. A. Garver, and D. B. Smith (1980) Studies on the Wolfgram high molecular weight CNS myelin proteins: Relationship to 2',3'-cyclic nucleotide 3'-phosphodiesterase. J. Neurochem. *35:* 1200–1208.

Sprinkle, T. J., F. A. McMorris, J. Yoshino, and G. H. deVries (1985) Differential expression of 2',3'-cyclic nucleotide 3'-phosphodiesterase in cultured central, peripheral, and extraneural cells. Neurochem. Res. *10:* 919–931.

Suda, H., and Y. Tsukada (1980) Improved method for purification of 2'3'cyclic nucleotide 3'-phosphohydrolase from bovine cerebral white matter. J. Neurochem. *34:* 941–949.

Takio, K., S. B. Smith, E. G. Krebs, K. A. Walsh, and K. Titani (1984a) Amino acid sequence of the regulatory subunit of bovine type II adenosine cyclic 3',5'-phosphate dependent protein kinase. Biochemistry *23:* 4200–4206.

Takio, K., R. D. Wade, S. B. Smith, E. G. Krebs, K. A. Walsh, and K. Titani (1984b) Guanosine cyclic 3',5'-phosphate dependent protein kinase, a chimeric protein homologous with two separate protein families. Biochemistry *23:* 4207–4218.

Titani, K., T. Sasgawa, L. H. Ericsson, S. Kumar, S. B. Smith, E. G. Krebs, and K. A. Walsh (1984) Amino acid sequence of the regulatory subunit of bovine type I adenosine cyclic 3',5'-phosphate dependent protein kinase. Biochemistry *23:* 4193–4199.

Towbin, H., T. Staehelin, and J. Gordon (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA *76:* 4350–4354.

Traktman, P., P. Sridhar, R. C. Condit, and B. E. Roberts (1984) Transcriptional mapping of the DNA polymerase gene of vaccinia virus. J. Virol. *49:* 125–131.

Weissbarth, S., I. R. Maker, T. S. Brannan, E. P. Lapin, and G. M. Lehren (1981) The activity of 2',3'-cyclic nucleotide 3'-phosphodiesterase in rat tissues. J. Neurochem. *37:* 677–680.

Young, R. A., and R. W. Davis (1983) Efficient isolation of genes by using antibody probes. Proc. Natl. Acad. Sci. USA *80:* 1194–1198.