Cloning of cDNA for DARPP-32, A Dopamine- and Cyclic AMP-Regulated Neuronal Phosphoprotein

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A cDNA clone for the mRNA of bovine DARPP-32 (dopamine- and adenosine 3',5'-monophosphate-regulated phosphoprotein, M_r = 32,000) was isolated from a modified Okayama-Berg plasmid library. Transformed *Escherichia coli* colonies were screened by *in situ* colony hybridization with 2 different oligonucleotide probes corresponding to a region unusually rich in glutamate within the protein. Three positive clones were isolated and shown to encode DARPP-32 by an *in situ* immunoblot assay of their fusion protein products with β-galactosidase. The results of the sequence analysis of the longest cDNA clone, pTKD7 (1771 nucleotides), revealed a 606-nucleotide-long coding region, in exact agreement with the bovine DARPP-32 amino acid sequence (Williams et al., 1986). Southern blot analysis of total bovine genomic DNA showed that there is a single gene coding for DARPP-32. Northern blot analysis of caudate nucleus RNA using antisense RNA derived from the clone pTKD7 demonstrated the existence of 2 abundant mRNA species, corresponding to 1.8 and 1.65 kilobase in length. The high concentration of DARPP-32 mRNAs in the caudate nucleus is in agreement with the known distribution of this protein.

DARPP-32 is a neuron-specific phosphoprotein with an apparent M_r of 32,000. In rat brain, the phosphorylation of DARPP-32 appears to be regulated by dopamine and cAMP (Walaas et al., 1983; Walaas and Greengard, 1984), and the phosphorylated form of the protein is a potent and specific inhibitor of protein phosphatase-1 (Hemmings et al., 1984a, c, d; Williams et al., 1986). In mammalian brain, a variety of biochemical and immunological studies have demonstrated that DARPP-32 is highly enriched in the basal ganglia and may be restricted to the D_1 subclass of dopaminoceptive neurons (Ouimet et al., 1984; Walaas and Greengard, 1984; Hemmings and Greengard, 1986). DARPP-32-like proteins are also present in dopaminoceptive regions of bird and reptile brains but cannot be detected in bony fishes or amphibians (Hemmings and Greengard, 1986). The protein has been purified to homogeneity from bovine caudate nucleus and biochemically characterized (Hemmings et al.,

1984b). The complete amino acid sequence has also been determined (Williams et al., 1986). Knowledge of the structure and organization of the DARPP-32 gene may allow us to elucidate the mechanisms of its neuron-specific expression. In this paper, we describe the isolation and characterization of a cDNA clone for bovine DARPP-32 by use of an *in situ* colony hybridization method with oligonucleotide probes and an *in situ* immunoassay. We have used this clone to study the regional distribution of DARPP-32 mRNA in bovine brain and to carry out a genomic analysis of this protein.

Materials and Methods

Bacterial strains and plasmids. *Escherichia coli* K-12 strains used were WA802 [*metB1, lacy* or *lac3, galK2, galT22,* λ⁻, *supE44, endA, sbcB, hsd3*] (Wood, 1966; Nelson et al., 1981), JM103 [Δ(*lac-pro), thi, strA, supE, endA, sbcB, hsdR⁻, F⁻ traD36, proAB, laciᵠ, zΔM15*] (Messing et al., 1981), and JM83 [*ara, Δlac⁻, pro, strA, thi, φ80d, lacZΔM15*] (Vieira and Messing, 1982). Plasmids included pSV7186 (Pharmacia), pUC18 (Pharmacia), pGEM3 (Promega Biotec), and the newly constructed pTK501 and pTKD7G3. *E. coli* strains harboring these plasmids were grown in LB medium containing 50 μg/ml ampicillin.

Preparation of bovine brain RNA. For preparation of RNA, caudate nuclei were dissected on ice from fresh calf brains, rapidly frozen in liquid nitrogen and stored in it until use. Total RNA (2.8 mg) was isolated from calf caudate nuclei (6 gm) by extraction with guanidine isothiocyanate followed by sedimentation through cesium chloride (Chirgwin et al., 1979). Poly(A)⁺ RNA (200 μg) was enriched by a cycle of oligo(dT)-cellulose Type 3 (Collaborative Research) chromatography (Aviv and Leder, 1972; Zelenka and Piatigorsky, 1974).

Construction of a novel linker plasmid for the Okayama-Berg expression system. In place of the original Okayama-Berg oligo(dG)-tailed linker DNA fragments derived from SV40 DNA (map units 0.71–0.86), *E. coli* lacz containing oligo(dG)-tailed linker DNA (Pharmacia) fragments were used for cyclization of cDNA plasmids (Fig. 1). The new linker fragments were generated from pTK501 constructed from pUC18. Briefly, pUC18 plasmids were digested partially wtih *Pvu*II, which potentially cuts the middle of either *laci* or *lacz* gene. *Hind*III linkers (Pharmacia) were ligated onto the blunt-ended DNAs, and the ends were religated with T4 DNA ligase. Following complete digestion with *Pvu*II, the DNA mixture was diluted 75-fold and self-ligated, a procedure designed to reduce the population of original pUC18 plasmid molecules. *E. coli* JM83 competent cells were transformed with the ligation mixture and plated in the presence of 0.03% 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, obtained from Bachem) and 0.3 mM isopropyl β-D-galactopyranoside (IPTG, from Bachem). Blue colonies containing the *lacz* promoter and operator regions were picked (Messing, 1983), and plasmid DNA was isolated by the alkaline lysis method (Birnboim and Doly, 1979; Maniatis et al., 1982). pTK501 plasmid, which has a *Hind*III site in the *laci* gene instead of the original *Pvu*II site in the pUC18 plasmid, was identified by appropriate restriction enzyme analyses.

Construction of a bovine caudate cDNA library. Poly(A)⁺ mRNA from bovine caudate nuclei was used to construct a cDNA library using a modified Okayama-Berg procedure (Okayama and Berg, 1982). Briefly, 6 μg of poly(A)⁺ RNA was used to direct first strand cDNA synthesis with the primer vector fragment and avian myeloblastosis virus reverse transcriptase (Life Sciences). After addition of a (dC)-tail by terminal
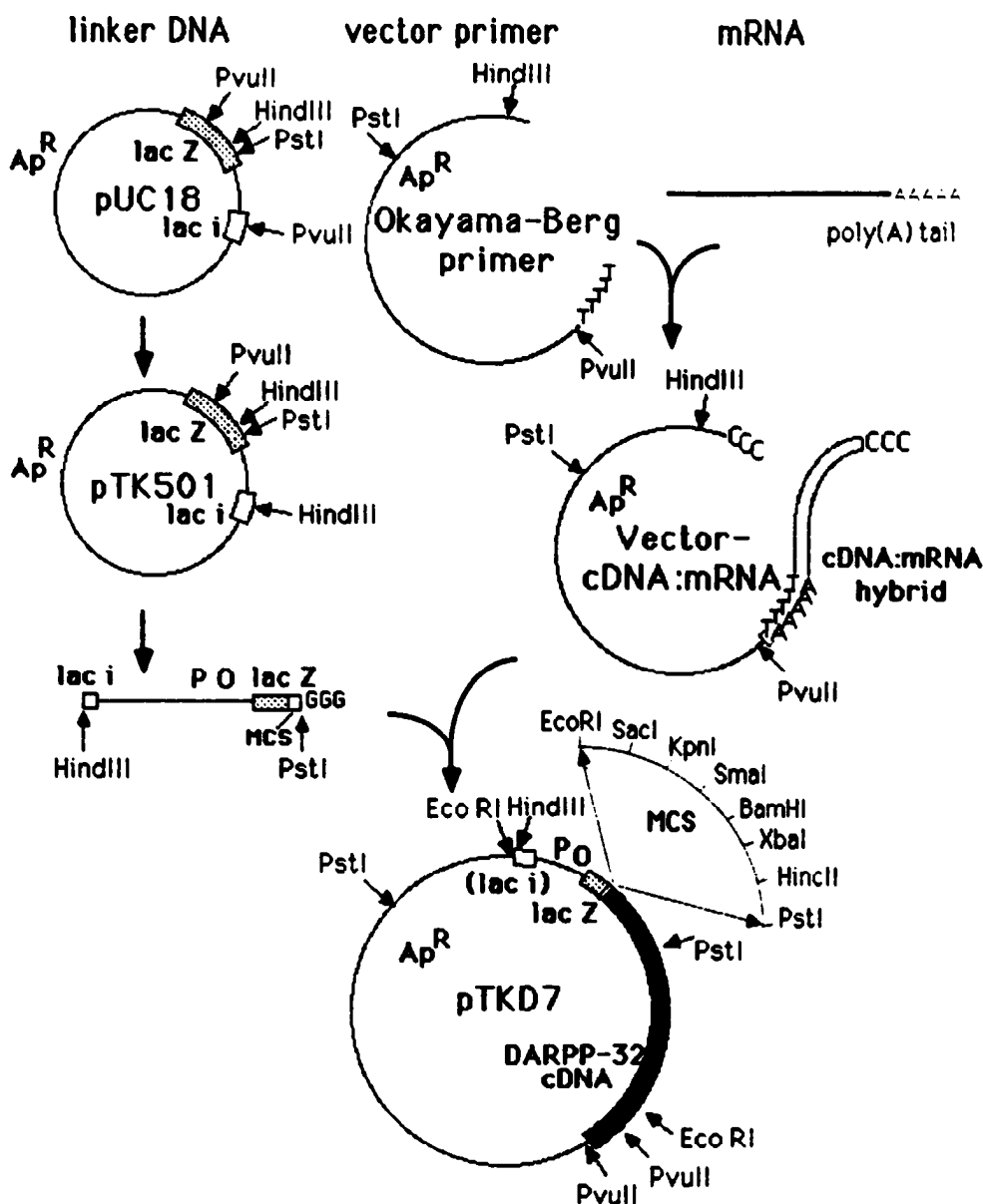
Figure 1. Construction scheme of the recombinant cDNA plasmid pTKD7. Details of the procedures and conditions are described in Materials and Methods and Results. Ap^R, ampicillin-resistant gene; *MCS*, multiple cloning site.

deoxytransferase (Pharmacia), a novel oligo(dG)-tailed *Hind*III linker DNA fragment derived from pTK501 was inserted into the cDNA-RNA hybrid to produce a circular molecule (Fig. 1). Replacement of the RNA strand with DNA was accomplished by *E. coli* ligase (Pharmacia), *E. coli* DNA polymerase I (New England Biolabs), and *E. coli* RNaseH (Pharmacia) as described in the Okayama-Berg procedure. The cDNA/vector products constructed by this method were used to transform the WA802 strain of *E. coli*.

Preparation of oligonucleotide hybridization probes. Two oligonucleotide probes (oligo TK1 and oligo TK2 in Fig. 2) were prepared with the DNA Synthesizer 308A (Applied Biosystems) and purified by Sephadex G-100 (Pharmacia) column chromatography in TEAB buffer (10 mM triethyl amine bicarbonate, pH 8.5). Oligonucleotides were labeled at the 5'-end with [γ-^{32}P]ATP using T4 polynucleotide kinase, followed by purification using Sephadex G-50 (Pharmacia) column chromatography. The specific activities of these probes were >10^8 cpm/μg DNA.

Colony hybridization. Colonies derived from the library were screened at a density of 2500 colonies/82 mm petri dish. The DNA from lysed bacteria was transferred to nitrocellulose filters as described by Maniatis et al. (1982) and Grunstein and Hogness (1975) with the following

modifications. The filters were first prehybridized in 3 × SSC (0.15 M NaCl and 0.015 M sodium citrate) for 30 min at 65°C, then in 3 × SSC, 10 × Denhardt's solution (0.02% Ficoll 400, 0.02% polyvinyl pyrrolidone, 0.02% BSA) for 30 min at 65°C, and finally in 1 M NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.1% SDS, 10 × Denhardt's solution, 10 μg/ml salmon testis DNA for 30 min at 65°C. Hybridization was carried out in 1 M NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.1% SDS, 10 × Denhardt's solution, 100–200 μg/ml salmon testis DNA with ^{32}P end-labeled synthetic oligonucleotides (10^5 cpm/ml, 2 ml/filter) for 15–24 hr at 65°C. Filters were washed at 65°C in 2 × SSC, 0.1% SDS, followed by 1 × SSC, 0.1% SDS, and then twice at 65°C in 0.2 × SSC, 0.1% SDS for 30 min per wash, dried at room temperature, and subjected to autoradiography. Positive colonies were purified by 2 successive screenings.

Southern blot analysis. Southern hybridization (Southern, 1975) was used with the oligonucleotides as probes to analyze the putative positive clones. Cloned cDNAs (1 μg per lane) cut with restriction endonucleases, size-fractionated on 1% agarose mini gels, and blotted onto nitrocellulose filters were hybridized to ^{32}P-labeled probes. In the case of Southern blot analysis of genomic DNA (Fig. 6), 10 μg each of bovine caudate

DNA, following cleavage with individual restriction endonucleases, were hybridized with ^{32}P-labeled antisense RNA (see below) and washed as detailed in the figure legends.

Detection of DARPP-32 cDNA clones with antibodies. Bacterial colonies were grown for 9 hr at 37°C on LB plates supplemented with 50 μg/ml ampicillin. Fusion protein production with β-galactosidase in the bacterial colonies transferred to nitrocellulose filters was induced with 50 μg/ml ampicillin and 0.2 mM IPTG for 9 hr at 37°C. Filters were processed according to the procedure of Helfman et al. (1983) with the following modifications. Each filter was incubated in a separate dish, first with a mixture of mouse anti-DARPP-32 monoclonal antibodies [C24-4D7 and C24-5a (Hemmings and Greengard, 1986) preadsorbed with bacterial lysate] in blocking solution [5 mM Tris-HCl, pH 7.5, 15 mM NaCl, 3% BSA (Sigma fraction V), and 0.1% Triton X100] for 14 hr at room temperature, and then with 1 μCi of goat anti-mouse ^{125}I-Igs (specific activity, 8.8 μCi/μg) in blocking solution at room temperature for 2 hr.

Subcloning and sequencing of cDNA fragments. Restriction fragments of pTKD7 were inserted into the appropriate sites in M13 mp8, mp9, mp10, mp11, and mp18 phages (Pharmacia), and the sequences were determined by the dideoxy method (Sanger et al., 1977; Messing, 1983).

Preparation of RNA probe. DARPP-32 antisense RNA probes were used for Northern and Southern blot hybridizations. The antisense probe was synthesized with SP6-polymerase according to Melton et al. (1984) using the pTKD7G3 plasmid, consisting of a 1667 base pair BamHI/PvuII DNA fragment from the pTKD7 clone subcloned into the BamHI/HincII sites of pGEM3 (Promega Biotec). This template contains the entire cDNA region except a 170 base pair PvuII DNA fragment at the 3'-end containing the poly(A) stretch of the clone. Following linearization of pTKD7G3 with BamHI, the antisense RNA was synthesized according to Melton et al. (1984). The sense RNA (1715 nucleotides) was synthesized by using T7 RNA polymerase with HindIII-linearized pTKD7G3.

RNA blot analysis. Poly(A)+ and poly(A)- RNA fractions were stored in 50–75% ethanol and lyophilized immediately before use. RNA blots were done essentially according to Maniatis et al. (1982) and Thomas (1980). The RNAs were transferred onto a nitrocellulose filter with 20 × SSC to prepare the RNA blot. Hybridization procedures were essentially the same as described for the Southern hybridization procedure, except that the incubation was carried out in a mixture containing 50% formamide at 45°C, with 200 μg/ml yeast tRNA, and the filter was washed at 60°C without formamide. Antisense riboprobe (1 × 10^{7} cpm/ml) derived from pTKD7G3 was used for hybridization. Quantitative autoradiography was performed using a Soft Laser Scanning Densitometer, model SL-TRFF (Biomed Instruments, Inc.). The filter was stained with methylene blue (15 min in 5% HOAc, 10 min in 0.04% methylene blue, 0.5 M NaOAc, then H₂O) to visualize the ribosomal RNA bands (Maniatis et al., 1982; Zinn et al., 1983). Calf liver 28S rRNA (4.7 kb) and 18S rRNA (1.9 kb) were used as standards (Pharmacia).

Other enzymes and reagents. E. coli DNA polymerase I (Kenow fragment), RNasin (RNase inhibitor) from human placenta, ribo-, deoxy-, and dideoxy-nucleotide triphosphates, HindIII linker, and DNA size marker, DRIgest III were purchased from Pharmacia. T4 DNA ligase, terminal deoxytransferase, E. coli polynucleotide kinase and restriction endonucleases were purchased from Boehringer Mannheim, Bethesda Research Laboratories, New England Biolabs, or Pharmacia. γ-^{32}P-dATP (3000 Ci/mmol), α-^{32}P-rCTP (800 Ci/mmol), α-^{35}S-dATP (500 Ci/mmol), and ^{125}I-goat anti-mouse Igs (8.8 μCi/μg) were from New England Nuclear. Monoclonal antibodies to bovine DARPP-32 were kindly provided by Dr. Hugh C. Hemmings, Jr. of this laboratory. Other chemicals were obtained from standard commercial suppliers.

Results

Construction of the Okayama-Berg expression system

A novel method was used to clone the cDNA for this protein, which was the development of a modified Okayama-Berg expression cloning vector (Fig. 1). The Okayama-Berg vector is derived from 2 different DNA fragments, a poly(T)-tailed vector-primer DNA and an oligo(dG)-tailed linker DNA, and has the potential capability to be an expression vector (Okayama and Berg, 1982). As a linker DNA fragment, we chose an E. coli lactose gene fragment, which carries a promoter, an operator, the N-terminus of β-galactosidase, and the M13 multiple

cloning sites (MCS) from EcoRI to PstI, immediately followed by oligo(dG). This fragment was constructed from pTK501, which was derived from pUC18 as described in Materials and Methods. The new Okayama-Berg expression vector has several advantages over the standard method, including (1) inducible expression within a host carrying the laci+ or laci^Q gene, (2) the predetermined orientation of the foreign gene in the same direction as the lac gene, since the poly(A) tail of the mRNA is attached to the opposite end of the vector-primer DNA carrying the poly(T) tail, (3) convenient polylinker sites for subcloning, and, finally (4) the ability to sequence the 5'-end of the insert DNA directly by using an M13 reverse sequence primer. Although proper expression of foreign DNA will depend on the reading frame of the DNA insert with respect to that of β-galactosidase, the potential flexibility of the length of the dG tail of the linker DNA fragment and of the dC tail of the 5'-end of the cDNA will increase the likelihood of correct joining. Finally, sequential use of the screening systems should decrease the number of false-positive clones obtained by the use of any single method alone.

Isolation of a cDNA clone for DARPP-32 mRNA

Starting with 0.5 μg of poly(A)+ RNA from bovine caudate nuclei, a cDNA library of 1.0 × 10^{5} clones was constructed using the Okayama-Berg procedure (Fig. 1). The library was screened by the colony hybridization method (Grunstein and Hogness, 1975; Maniatis et al., 1982) using 2 different oligonucleotide probes (oligo TK1 and oligo TK2). These oligonucleotides were designed on the basis of the amino acid sequence of bovine DARPP-32 (Williams et al., 1986), which contains an unusual stretch of acidic residues (amino acid residues 119–134). We chose this Glu-rich region for synthesis of two 47-mer-oligonucleotide hybridization probes. One of them, oligo TK2, contains all possible codons, with a mixture of 2[15] possible species of the nonsense strand. The other probe, oligo TK1, was synthesized on the basis of the following criteria. The mammalian codon usage data (Grantham et al., 1981) indicate that about 60% of the Glu codon is GAG, while the remaining 40% is GAA. In addition, we know that G residues may be bound not only to C residues (by Watson-Crick base pairing) but also to T residues (by the wobble theory proposed by Crick, 1966), and that G:C base pairing is thermodynamically more stable than A:T base pairing. Therefore, we chose the GAG codon or the CTC anticodon as glutamic acid and the sense strand instead of the nonsense strand. The number of species for oligo TK1 is only 2, which is derived from the combination of 2 possible codons for the [128]Asp residue (GAT or GAC). The amino acid sequences and the corresponding oligonucleotide probes are summarized in Figure 2.

We hybridized oligo TK1 and oligo TK2 in parallel against identical replica filters to screen the library (approximately 4.75 × 10^{4} recombinant colonies). Some positive signals were detected only by one of the 2 probes, while others were detected by both probes. Ten colonies (pTKD1–10) detected by both probes were selected. To verify the identity of these 10 clones, restriction enzyme analyses and Southern hybridization with the oligo TK1 were performed in order to look for a similar restriction pattern. Double restriction digests with PstI/PvuII and PstI/EcoRI gave restriction fragments of 1.0 and 0.17 kb (PstI/PvuII) and 0.8 kb (PstI/EcoRI) for 4 clones: pTK1, 3, 7, and 8. In addition, Southern blot analysis showed that both the 1.0 kb fragment and the 0.8 kb fragment hybridized with the oligo TK1 (Fig. 3).

Amino acid No.		119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	
Amino acids	H	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Asp	Glu	Glu	Glu	Glu	Glu	Asp	C
Possible codons (sense strand)	5'	GAG A	GAG A	GAG A	GAG A	GAG A	GAG A	GAG A	GAG A	GAG A	GAT C	GAG A	GAG A	GAG A	GAG A	GAG A	GA	3'
<oligo TK1>	5'	GAG	GAG	GAG	GAG	GAG	GAG	GAG	GAG	GAG	GAT C	GAG	GAG	GAG	GAG	GAG	GA	3'
Actual codons	5'	GAG	GAA*	GAG	GAG	GAG	GAA*	GAG	GAG	GAG	GAT	GAA*	GAG	GAG	GAG	GAG	GA	3'
Possible codons (nonsense strand) <oligo TK2>	3'	CTC T	CTC T	CTC T	CTC T	CTC T	CTC T	CTC T	CTC T	CTC T	CTA G	CTC T	CTC T	CTC T	CTC T	CTC T	CT	5'

Figure 2. Synthetic oligonucleotide DNA probes used for screening of bovine DARPP-32 clones. Possible nucleotide sequences, either sense or nonsense strand, deduced from the amino acid sequence (119–134) of purified DARPP-32 from bovine caudate (Williams et al., 1986) are shown. Oligo TK2 was constructed from a mixture of all nonsense strands with possible codons. Oligo TK1 was made up of 2 species of sense strands. Actual nucleotide sequence is shown as a sense strand, which was derived from the DNA sequence of cDNA clone pTKD7. Three *asterisks* indicate differences in nucleotides between oligo TK1 and actual DNA sequences.

Immunological analysis

Since the Okayama-Berg vector (pSV7186) used in these studies was supplemented with the *E. coli lac* z from the pTK501 (Fig. 1), these 4 clones could be further analyzed via expression of their cDNA fusion proteins. By *in situ* immunoassay with mouse anti-DARPP-32 monoclonal antibodies (mixture of C24-4D7 and C24-5a; Hemmings and Greengard, 1986), only clones pTKD3, 7, and 8 gave positive signals among the 10 putative positive clones (data not shown). Using both *in situ* colony hybridization and *in situ* immunoassay to screen the library, we concluded at this point that clones pTKD3, 7, and 8 were cDNAs coding for DARPP-32 mRNA.

Identification of DARPP-32 cDNA by nucleotide sequencing

Clones pTKD3, 7, and 8 had a 0.8 kb *PstI/EcoRI* restriction fragment that hybridized with oligonucleotide probe TK1 (Fig. 3). These 0.8 kb *PstI/EcoRI* fragments were individually subcloned into the *PstI/HincII* sites of M13 phage vectors mp10 and mp11 (Messing, 1983) and sequenced from both ends by the dideoxy chain termination sequencing method (Sanger et al., 1977). The nucleotide sequences of these 3 fragments were identical, and the 130 bases sequenced from the *PstI* site revealed a deduced amino acid sequence that was identical with the experimentally established amino acid sequence of bovine DARPP-32. A restriction map analysis and the sequencing strategy of the longest clone pTKD7 is presented in Figure 3. The complete base sequence and deduced amino acid sequence of clone pTKD7 is shown in Figure 4. The sequence data indicate that pTKD7 is 1771 nucleotides in length. It contains the complete coding sequence corresponding to the bovine DARPP-32 protein, coding for 202 amino acids, including the initiation codon ATG and the termination codon TAG (amber). There are 341 nucleotides of 5' noncoding region and 824 nucleotides of 3' noncoding region. There are 2 potential polyadenylation signals, as the sequence AATAAA (Proudfoot and Brownlee, 1976) is present immediately upstream and 11 nucleotides upstream from the start of the poly(A) tail, which contains 80 nucleotides of A residues. DNA sequence analyses also showed that oligo TK1 contained a sequence almost homologous to DARPP-32 mRNA, i.e., 11 out of 14 Glu codons were GAG (Fig. 2). The codon usage ratio of GAG to GAA for Glu is actually about 80% to 20% in this region. Oligo TK1 gave a

stronger signal than oligo TK2 in the case of Southern blot hybridization with restriction DNA fragments of these clones (data not shown), probably because of the difference of the hybridization stability between the 2 probes and cDNAs.

mRNA analyses by blot hybridization

The cDNA (pTKD7) was subcloned in reverse orientation into the vector pGEM3 downstream of the SP6 promoter to construct a pTKD7G3. The DARPP-32 antisense RNA was synthesized *in vitro* with α-^{32}P-rCTP and the pTKD7G3 as template, and then used as a hybridization probe to poly(A)$^+$ RNA or poly(A)$^-$ RNA from bovine caudate to identify the DARPP-32 mRNA. The antisense RNA hybridized with 2 poly (A)$^+$ RNA species of 1.8 and 1.65 kb (Fig. 5, lane 1). At least 3 faint bands were detected in the poly(A)$^-$ RNA preparation in this
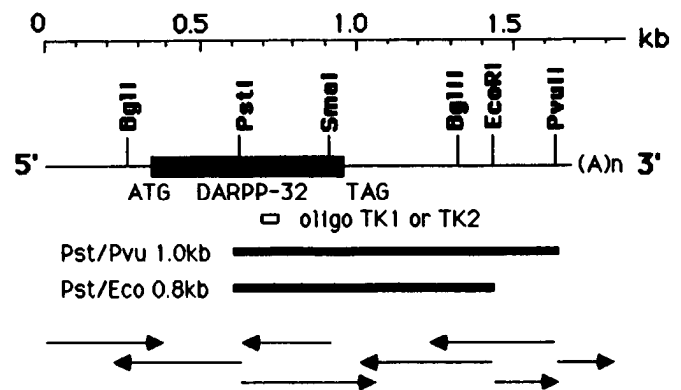

Figure 3. Restriction map and sequencing strategy of the cDNA of clone pTKD7. The scale above the restriction map indicates the nucleotide positions in kilobases (kb) relative to the first nucleotide in the cDNA pTKD7, which is just after the 15 G residues. The *thick black line* depicts the coding region for DARPP-32 protein, while the *thin lines* depict the 5' and 3' untranslated regions of the mRNA. Translation start codon (ATG), translation stop codon (TAG), and poly(A) tail [(A)n] are also shown. Two *black bars* represent the restriction fragments that hybridize with one or both synthetic oligomers, oligo TK1 and oligo TK2, which correspond to the *open box*. The *arrows* indicate the direction and extent of DNA sequence that was determined by the dideoxy chain termination sequencing method (Sanger et al., 1977) using bacteriophage M13 subclones as templates and M13 general sequence primer (Messing, 1983).

AGCGAAACAGAGACAGAGGGGAGACTCGCGGAGAGGGAGCAAGGGAGATAGAGGAAGGTTGGAGAGACACAGAGAGAGCGAGGGAAGGAGG
　　　10　　　　20　　　　30　　　　40　　　　50　　　　60　　　　70　　　　80　　　　90
GCCAGAGACCAGCCCCGCGAGCCCGAGGTGCGGCCCCAGGGGACCGGGCACCCAGGAGCCCCAGAGCCGCGAGCCGGCCGCCCCCCACCC
　　　100　　　110　　　120　　　130　　　140　　　150　　　160　　　170　　　180
CCGCGCGCCCTCCCCTCGCCGGCGGGTATTTTTATCTGTGCGTGAACAGCCCTCCAGCTCCTCTCTGCCACACTCAACCCGCT<u>GCCGCCG</u>
　　　190　　　200　　　210　　　220　　　230　　　240　　　250　　　260　　　270

　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　1
　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　M　D　P　K　D　R
CGGCCCGGAGCAGCGCGGGGGAGCGGAGCGGGATCGCAGCCCGAGACCCCACGACGCGCGCCCCGCCCGCCATGGACCCCAAAGACCGCA
Bg I I　280　　　290　　　300　　　310　　　320　　　330　　　340　　　350　　　360

　　　10　　　　　　　　　　　　　　　　20　　　　　　　　　　　　30
K　K　I　Q　F　S　V　P　A　P　P　S　Q　L　D　P　R　Q　V　E　M　I　R│R　R　R　P　T　P　A│
AGAAGATCCAGTTCTCCGTGCCCGCGCCCCCCAGCCAGCTCGACCCCCGCCAGGTGGAGATGATCCGGCGCAGGAGGACCAACCCCTGCCA
　　　370　　　380　　　390　　　400　　　410　　　420　　　430　　　440　　　450

　　　40　　　　　　　　　　　　　　　　50　　　　　　　　　　　　60
│M　L　F　R│L　S　E　H　S　S　P　E　E　E　A　S　P　H　Q　R　A　S　G　E　G　H　H　L　K　S
TGCTGTTCCGGCTCTCAGAGCACTCCTCACCAGAGGAGGAGGCCTCACCCCACCAGAGAGCCTCAGGAGAGGGGCACCACCTCAAGTCGA
　　　460　　　470　　　480　　　490　　　500　　　510　　　520　　　530　　　540

　　　70　　　　　　　　　　　　　　　　80　　　　　　　　　　　　90
K　R　S　N　P　C　A　Y　T　P　P　S　L　K　A　V　Q　R　I　A　E　S　H　L　Q　S　I　S　N　L
AGAGATCCAATCCTTGTGCCTACACACCCCCCTCGCTGAAAGCCGTCCAGCGCATTGCTGAGTCTCACC<u>TGCAG</u>TCCATCAGCAACCTGG
　　　550　　　560　　　570　　　580　　　590　　　600　　　610 Pst I　620　　　630

　　　100　　　　　　　　　　　　110　　　　　　　　　　　120
G　E　N　Q　A　S　E　E　E　D　E　L　G　E　L　R　E　L　G　Y　P　R　E　E　E　E　E　E　E
GTGAGAACCAGGCCTCGGAGGAGGAGGATGAGCTGGGGGAGCTGCGGGAACTGGGCTACCCAAGAG<u>AGGGGAAGAGGAGGGAGGAAGAGGAGG</u>
　　　640　　　650　　　660　　　670　　　680　　　690　　　700　　　710　　　720

　　　130　　　　　　　　　　　140　　　　　　　　　　　150
E　D　E　E　E　E　D　S　Q　A　E　V　L　K　G　S　R　G　S　A　G　Q　K　T　T　Y　G　Q　G
<u>AGGATGAAGAGGAGGAGGAGGA</u>CAGCCAGGCGGAAGTCCTGAAGGGCAGCAGGGGGTCTGCTGGGCAGAAGACAACTTATGGCCAAGGTC
　　　730　　　740　　　750　　　760　　　770　　　780　　　790　　　800　　　810

　　　160　　　　　　　　　　　170　　　　　　　　　　　180
L　E　G　P　W　E　R　P　P　P　L　D　G　P　Q　R　D　G　S　S　E　D　Q　V　E　D　P　A　L　N
TGGAGGGTCCCTGGGAGCGCCCGCCTCCTCTGGATGGGCCCCAGAGAGACGGAAGCTCTGAGGACCAAGTGGAAGACCCAGCATTGAATG
　　　820　　　830　　　840　　　850　　　860　　　870　　　880　　　890　　　900

　　　190　　　　　　　　　　　200
E　P　G　E　E　P　Q　R　P　A　H　P　E　P　G　T　*
AA<u>CCCGGG</u>GAGGAGCCACAGCGCCCTGCCCACCCTGAGCCTGGCACATAGGCACCCAGCCCTGCATCTCCTGGAAGGAAGTGGGAGGAAG
Sma I　910　　　920　　　930　　　940　　　950　　　960　　　970　　　980　　　990
CATTGATGTTCTCCAGAAACCCATTCTGTTCACACCCTATTTTGTACCCTGCTTCTCACTTGCTAGGGCTGCGGCTTCTGACTTTTAGAA
　　　1000　　　1010　　　1020　　　1030　　　1040　　　1050　　　1060　　　1070　　　1080
GACTAAGGCTGGTCTGTGTTTGCTTGTCTGCCCAACTTCGCTGATCCCAGAGTCCCTGGGCACTTGCTGCCTGATGCCTACCCCTGCCAG
　　　1090　　　1100　　　1110　　　1120　　　1130　　　1140　　　1150　　　1160　　　1170
TCATTCCCCCATACACCCAGCAGGAGGTGGGATGGGAGAGCTTGCATTGGGAAATCCAGTAAATGGGGGACAAAGATTCATCCTTCACAA
　　　1180　　　1190　　　1200　　　1210　　　1220　　　1230　　　1240　　　1250　　　1260
TTCTACTCCCTAGACCCTCTCCCCTGGGCGTAGGAAACCACAGGGCAGGACCC<u>TAAGATCT</u>GGGGAAAAGGGGATACTGAGAACTTGTAAG
　　　1270　　　1280　　　1290　　　1300　　　1310　　　1320 Bg I I I 1330　　　1340　　　1350
TGCCCATAGATCTTTCTCCATCCCCTGGGCAATTCCAAGTCATCACCCCTTCACTGCCTTCTACCAGGGCCC<u>AGAATTC</u>AGGCATCTTTT
　　　1360　　　1370　　　1380　　　1390　　　1400　　　1410　　　1420 EcoR I 1430　　　1440
CCACGGCCTCAGCTTTTGGTAAATCTTCCCCTTATCACCTGCTCCCCAGCCTGGGTGCCTGGAAGATGGACTGGCAGAGACTGCTTTGCT
　　　1450　　　1460　　　1470　　　1480　　　1490　　　1500　　　1510　　　1520　　　1530
GCATTTTATGTGTGCTTTGATGCCAGGAATGCCACCTAGTATAATAAGTCCT<u>TAGGGGGG</u>CACATGGTGGGGGAGCCAAGCTCTCCTTGT
　　　1540　　　1550　　　1560　　　1570　　　1580　　　1590　　　1600　　　1610　　　1620
CCTCC<u>AGCTGC</u>TCTGTCCCCTTCCCCTCTTCCCTGACTCCCGGCCTGAACCTGT<u>AATAAAT</u>CTTTGT<u>AAATAAA</u>AAAAAAAAAAAAAAAAA
　　　1630 Pvu I I 1640　　　1650　　　1660　　　1670　　　1680　　　1690　　　1700　　　1710
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
　　　1720　　　1730　　　1740　　　1750　　　1760　　　1770

Figure 4. Complete nucleotide sequence of pTKD7 and the amino acid sequence of bovine DARPP-32 cDNA encoded therein. The numbering of the nucleotides (below sequence) starts at the first nucleotide after the string of Gs and ends at the 80 adenine residues in the 3′ poly(A) tail. Amino acid residues are numbered (above sequence) by the position of 1 for the Met residue of the predicted amino acid sequence of the protein. The nucleotide sequence (47 residues) used to generate the synthetic oligonucleotide probes (oligo TIK1 and oligo TK2) is indicated by a *single solid underline*. Potential polyadenylation signals in the 3′ untranslated region are indicated by *overlines* and *underlines*. The recognition sites for restriction enzymes shown in Figure 3 are presented with *dotted underlines*. The amino acid sequence around the Thr residue phosphorylatable *in vitro* is enclosed in the *box*.

blot (lane 2). These bands were also detected in the poly(A)$^+$ preparation (lane 1), probably due to contamination by poly(A)$^-$ RNA. Two of these bands appear to correspond to the rRNA bands (28S and 18S), since we observe that the rRNAs themselves tend to cross-hybridize to the DARPP-32 antisense RNA (data not shown). Nevertheless, the predominant bands (1.8 and 1.65 kb bands in lane 1) were considered to be highly specific to the DARPP-32 antisense RNA, since only these 2 bands, but not others, remained under even more stringent washing conditions with RNase A (data not shown).

Southern blot analysis of bovine genomic DARPP-32

In order to investigate the genomic organization, chromosomal DNA from bovine caudate was analyzed using DARPP-32 antisense RNA as a probe followed by washing under stringent conditions [0.1 × SSC, 0.1% SDS for 30 min at 65°C (Fig. 6)]. Single digestion with EcoRI (lane 1), HindIII (lane 3), BamHI (lane 5), PstI (lane 9), or PvuII (lane 11) gave bands of 1.4, 9.5, 21, 2.5, or 0.95 kb, respectively. Double digestion with both EcoRI and either HindIII (lane 2), BamHI (lane 4), XhoI (lane 6), PstI (lane 8), or PvuII (lane 10) gave bands of 0.84, 1.4, 1.4, 1.4, or 0.72 kb, respectively. All combinations gave a single band. Therefore, it is unlikely that the genome coding for DARPP-32 is present in greater than a single copy. Under less stringent conditions (0.2 × SSC, 0.1% SDS for 30 min at 60°C as a final washing), however, EcoRI gave an extra band of 1.6 kb in addition to the band of 1.4 kb, and PstI gave 2 bands of 2.45 and 2.55 kb instead of the single band of about 2.5 kb (data not shown). Since the antisense RNA contains a single restriction site for EcoRI or PstI, it can be concluded that 2 restriction fragments of a single genomic DNA were hybridized to the antisense RNA (Fig. 3). Double digestion studies (Fig. 6) also demonstrated that the 1.4 kb EcoRI fragment was within the BamHI, XhoI, or PstI fragment, and that at least one HindIII and one PvuII site were present in the 1.4 kb EcoRI fragment. The HindIII site in the 1.4 kb EcoRI fragment in the genomic DNA was not detected in the 1424 bp fragment of the cDNA. Furthermore, there is only one PvuII site within the cDNA, which is at the 3′ end of the antisense probe, and the 0.72 kb PvuII fragment of the genomic DNA cannot exist in the antisense RNA region of 1627 nucleotides. Either analysis of the double digestion studies indicates that at least one intron is present in the genome of bovine DARPP-32.

Distribution of DARPP-32 mRNA in bovine CNS and rat tissues

The distribution of DARPP-32 mRNA was studied in 6 separate regions of bovine CNS by using the DARPP-32 antisense RNA (Fig. 7, lanes 1–6). Forty micrograms of total RNA were analyzed by Northern blot hybridization as described in Materials and Methods and the legend to Figure 5. DARPP-32 mRNA was found to be highly enriched in the caudate nucleus, which is in agreement with the enrichment of the protein in this region (Ouimet et al., 1984; Walaas and Greengard, 1984; Hemmings and Greengard, 1986). Lower, but still significant, levels were demonstrated in the hippocampus, cerebellum, and medulla white matter. These levels were approximately 1–2% of that of caudate nucleus. Cerebral cortex contained the mRNA at slightly higher concentrations than these areas, i.e., at about 3% of that of caudate nucleus. In the substantia nigra the level of DARPP-32 protein, as measured by immunoprecipitation and Western blot analyses (J.-A. Girault, personal communication),
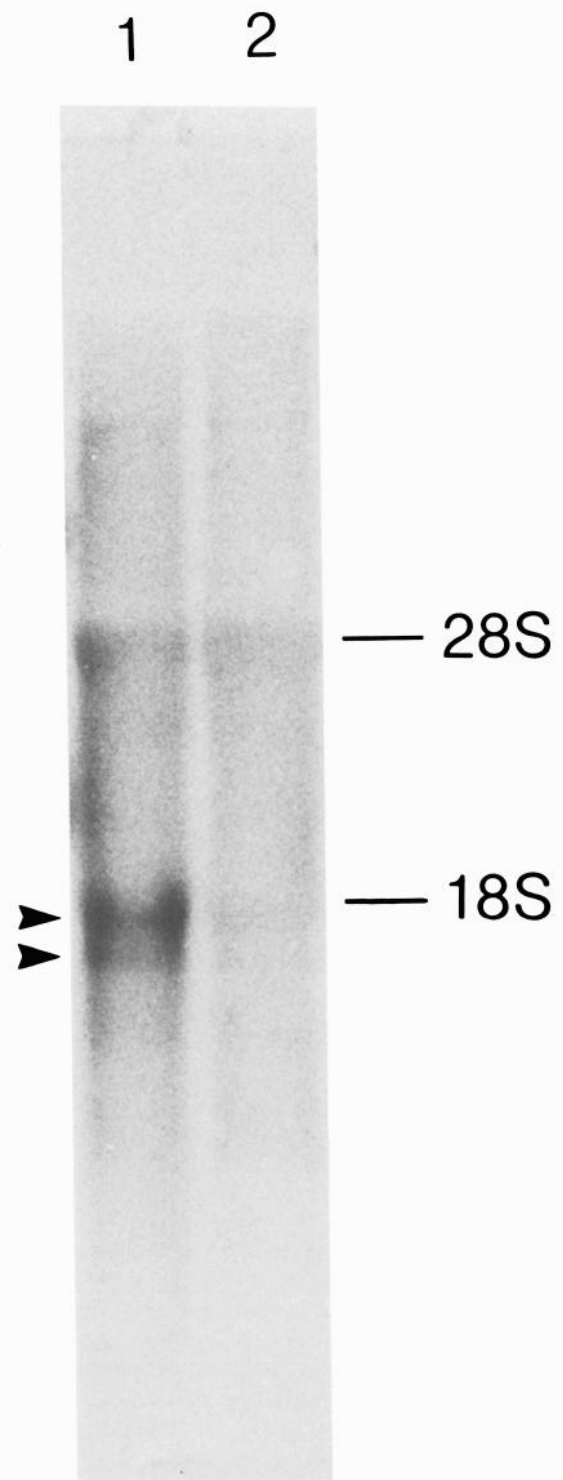

Figure 5. Northern blot analysis of poly(A)$^+$ RNA from bovine caudate nucleus. Twenty micrograms of poly(A)$^+$ RNA (*lane 1*) and poly(A)$^-$ RNA (*lane 2*) were separated in a 1.2% agarose gel containing 2.2 M formaldehyde. After transferring to nitrocellulose, they were hybridized to a ^{32}P-labeled antisense RNA probe derived from pTKD7G3 as template. Sizes of the bands (1.8 and 1.65 kb) marked by *arrowheads* in lane 1 were determined by rRNA markers.

is about half that of the caudate nucleus. The DARPP-32 mRNA level in the same region, however, was only 3% of that of caudate nucleus. The levels of DARPP-32 mRNA were also measured in the caudate nucleus and the liver from the rat (Fig. 7, lanes
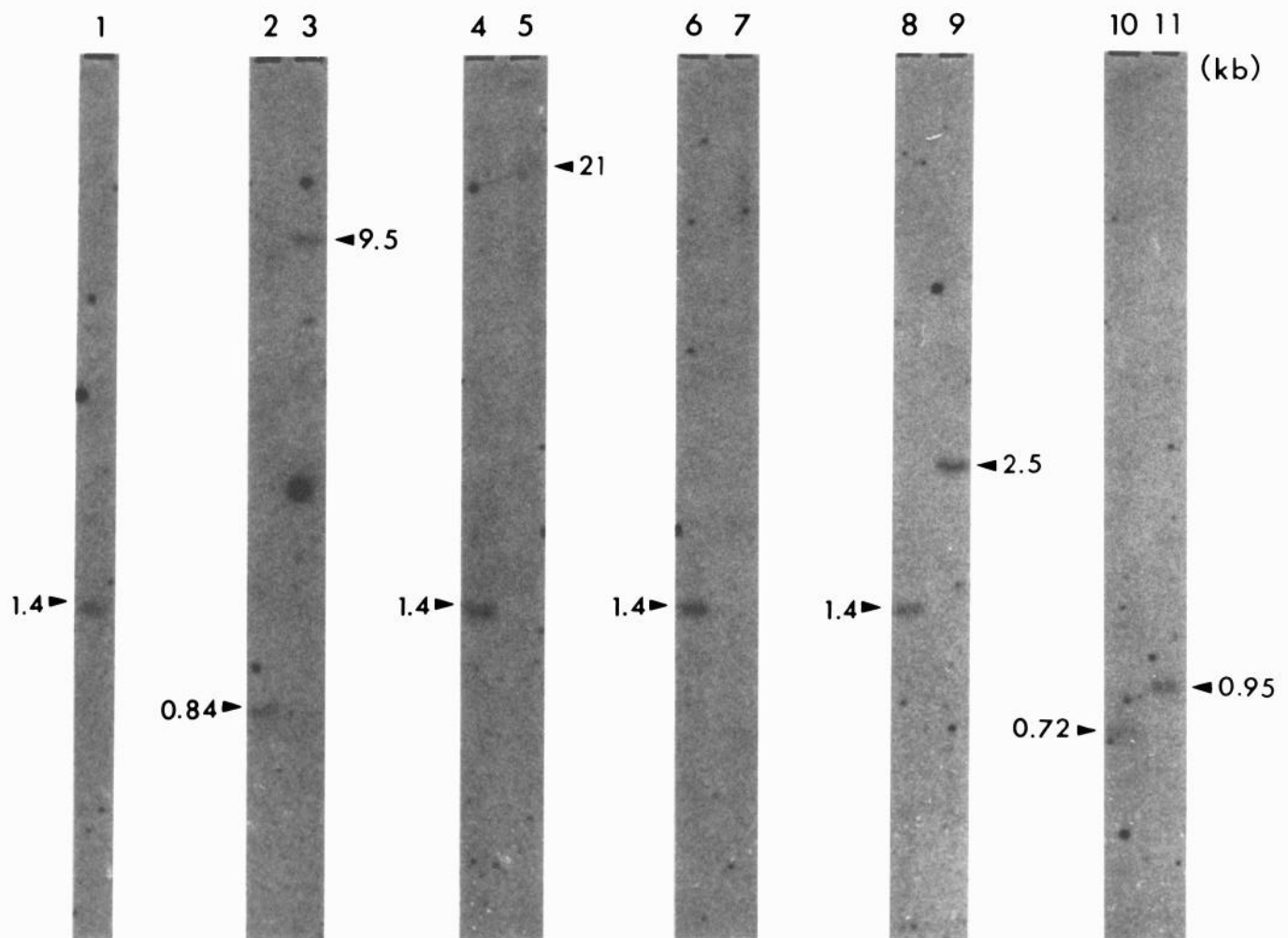
Figure 6. Southern blot analysis of genomic DNA. Ten micrograms of bovine caudate DNA were digested with restriction endonucleases, electrophoresed through a 0.9% agarose gel, transferred to a nitrocellulose filter, and hybridized with ³²P-labeled DARPP-32 antisense RNA as described in Materials and Methods. Stringent conditions were used for final washing (0.1 × SSC, 0.1% SDS for 30 min at 65°C). *Lane 1, EcoRI; 2, EcoRI/HindIII; 3, HindIII; 4, EcoRI/BamHI; 5, BamHI; 6, EcoRI/XhoI; 7, XhoI; 8, EcoRI/PstI; 9, PstI; 10, EcoRI/PvuII; 11, PvuII.* RNase was used for reduction of the background (1 μg/ml RNaseA in 2 × SSC for 15 min at room temperature). Sizes were calculated from co-electrophoresis of DRIgest III (Pharmacia; mixture of both phage λcI/⁸⁵⁷ Sam7 DNA digested with HindIII and φX174DNA digested with HaeIII), and are indicated in kilobases.

7 and 8). No detectable band was observed in the rat liver, but a strongly positive band was detected in the rat caudate nucleus, although only 5% of that in bovine caudate nucleus. Although the efficiency of cross-hybridization between the rat and bovine species is unknown, only a single band was detectable, at least under these experimental conditions. The molecular size of the rat mRNA was slightly larger than that of the bovine mRNA. Using immunoprecipitation and Western blot analyses, rat DARPP-32 protein is immunoreactive with anti-bovine DARPP-32 monoclonal antibodies, but the molecular size of rat DARPP-32 protein is slightly larger than that of the bovine protein (Ouimet et al., 1984; Hemmings and Greengard, 1986).

Discussion

In this paper, we describe the cloning of a cDNA for bovine DARPP-32, a neuron-specific phosphoprotein. Our sequence data (Fig. 4) indicate that the longest clone, pTKD7, has 341 nucleotides before the Met codon, which corresponds to the amino terminal end of the DARPP-32 protein. Since this clone has 15 nucleotides of G residues between the *Pst*I restriction site

and the cDNA insert, there are 414 nucleotides, corresponding to 138 amino acids, before the structural gene. There is also one termination codon (TAG) at the 41st position from the β-galactosidase translation start codon in the same reading frame. The bacterial host harboring this plasmid is WA802 carrying *supE44*, which suppresses the TAG termination codon. This could potentially overcome the translation termination signal, or the translation could be reinitiated after the TAG codon. The second longest clones, pTKD3 and pTKD8, which also gave positive signals with *in situ* immunoassay, contained 118 nucleotides in the 5' noncoding region and 10 nucleotides of G residues after the *Pst*I site. These clones have 62 amino acids before the structural gene of the DARPP-32. Although we did not sequence the coding region of the clone pTKD1, the restriction map indicated that it was very similar to that of the clones pTKD3 and pTKD8. Clone pTKD1, however, gave no positive signal in the *in situ* immunoassay, perhaps because it was out of phase with β-galactosidase.

The open reading frame sequence of 606 nucleotides starting with the Met codon at position 342 encodes a protein of 22,614
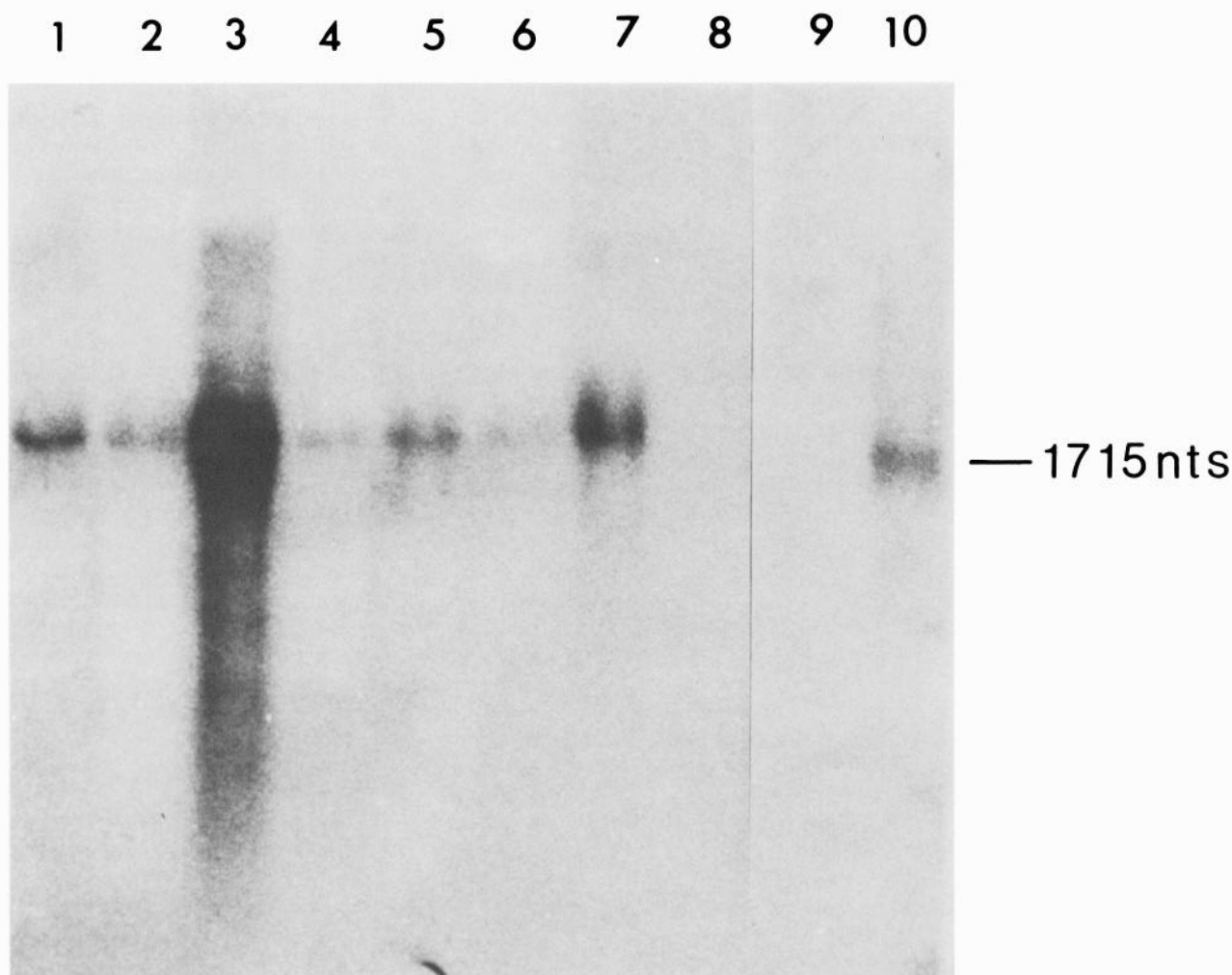
1 2 3 4 5 6 7 8 9 10

—1715nts

Figure 7. Regional distribution of DARPP-32 mRNA in bovine CNS and rat tissues by Northern blot analysis. Forty micrograms of total RNA were separated in a 1.0% agarose gel containing 2.2 M formaldehyde and analyzed by Northern blot hybridization as described in the legend to Figure 5. *Lanes 1–6* contained bovine cerebral cortex, hippocampus, caudate nucleus, cerebellum, substantia nigra, and medulla white matter; *lanes 7* and *8,* rat caudate nucleus and liver. *Lanes 9* and *10* contained about 2 and 20 pg of *in vitro* synthesized bovine DARPP-32 mRNA [1715 nucleotides (nts)], which were prepared by using T7 RNA polymerase and *Hind*III-linearized pTKD7G3 as template.

Da, with the exact amino acid sequence of the bovine DARPP-32 protein. Although the amino terminal residue of this protein is blocked, and could not be identified by amino acid sequence analysis (Williams et al., 1986), we can conclude that the Met codon at position 342 corresponds to the first N-terminal amino acid of DARPP-32 for the following reasons: (1) the codon at position 342 is the first Met codon in the open-reading frame sequence; (2) a typical consensus sequence ($CC^A/_GCCATGG$) around a translation initiation codon (Kozak, 1986) is present around the Met codon at position 342 (CCGCCATGG).

The amino acid sequence derived from the cDNA matched that of the protein. There are 37 Glu, 13 Gln, 10 Asp, and 4 Asn in the DARPP-32 protein, as well as in those derived from the corresponding codons, indicating that there is no amino acid change after the translation of this protein. The Thr codon, which is phosphorylated by cyclic AMP-dependent protein kinase *in vitro,* is present at position 441.

RNA blot analysis of the bovine caudate demonstrated 2 homologous mRNAs of 1.8 and 1.65 kb. The probe was the antisense RNA derived from the *PstI/PvuII* 1642 nucleotide sequence of the cDNA clone pTKD7, which does not contain

the *PvuII* restriction fragment of 170 nucleotides at the 3'-end with the poly(A) tail. The clone pTKD7 may actually be derived from the 1.8 kb mRNA, as judged by the similarity of its size to that of the DARPP-32 mRNA visualized in Northern blots (Figs. 5 and 7). The fact that RNA blot hybridization revealed the presence of 2 DARPP-32 mRNA species suggests several possibilities. First, the 2 mRNAs may be derived from 2 different genes. Second, the 2 mRNAs may be derived from a single gene, with 2 different messages being produced by posttranscriptional modification. In the latter case, one would expect a different splicing pattern, a different polyadenylation site, such as is seen in dihydrofolate reductase (Setzer et al., 1980) and CaM mRNA species (Lagacé et al., 1983), or degradation of the 5'-end and/or 3'-end. Third, it is possible that the 1.8 kb mRNA may be a nuclear precursor for the 1.65 kb mRNA. The larger mRNA is almost certainly not heterogeneous nuclear RNA, since it was as abundant as the smaller mRNA. Fourth, it is possible that one of the 2 mRNAs is the actual DARPP-32 mRNA and that the other is an unrelated, but homologous, mRNA. Since the antisense RNA used as a probe for Northern hybridization contained not only the structural gene but also

the 5' and 3' noncoding regions, this homology may exist either in the regulatory region or in the structural region. Phosphatase inhibitor-1 is a candidate for the latter possibility, since there is amino acid sequence homology and immunological cross-reactivity between them (Hemmings and Greengard, 1986; Williams et al., 1986). Further experiments are needed to distinguish among these possibilities.

Southern blot analysis of bovine caudate genomic DNA revealed that there were a small number of restriction fragments that hybridized to DARPP-32 antisense RNA (Fig. 6). Under stringent washing conditions, a single band was observed using several restriction enzymes. Figure 6 also shows the double digestion pattern using both EcoRI and HindIII, BamHI, XhoI, PstI, or PvuII. All combinations gave a single band. Therefore, it can be concluded that the DARPP-32 gene is present in a single copy, ruling out the possibility that the 2 mRNA species are derived from different genes. Furthermore, the analyses of the double digestion studies revealed that the structure of the DARPP-32 mRNA was different from that of its genome, indicating that the 2 mRNAs may be derived by alternate splicing of a single precursor mRNA.

The regional distribution of DARPP-32 mRNA in bovine CNS was examined by Northern blot hybridization with the antisense RNA (Fig. 7). DARPP-32 mRNA was highly concentrated in the caudate nucleus. This region in the bovine brain corresponds to the caudatoputamen in the rat brain, in which the rat DARPP-32 protein is highly enriched, as shown by in vitro back-phosphorylation (Walaas and Greengard, 1984), immunocytochemistry (Ouimet et al., 1984), and radioimmunoassay (Hemmings and Greengard, 1986). Similar results were obtained in the bovine brain by distribution analysis with immunoprecipitation, back-phosphorylation, and Western blot analysis (J.-A. Girault, personal communication). The caudate nucleus in the bovine brain and the caudatoputamen in the rat brain are known to receive a strong dopaminergic input, and both the cell bodies and dendrites of dopaminoceptive cells are present in these regions. The enrichment of DARPP-32 mRNA in this region correlates with the enrichment of the protein.

Distribution studies of DARPP-32 in the rat brain demonstrated that the substantia nigra is another region highly enriched in the protein (Hemmings and Greengard, 1986; Walaas and Greengard, 1984). It should be noted that only the axons and the nerve terminals of the dopaminoceptive cells are present in the substantia nigra. Northern blot hybridization analysis demonstrated that the level of DARPP-32 mRNA in the substantia nigra was low (3%) compared with the caudate nucleus. These results indicate that the DARPP-32 gene is expressed in the caudate nucleus and that most of the DARPP-32 mRNA is present in neuronal cell bodies. The physiological significance, if any, of the small amount of mRNA present in the substantia nigra is not clear at present. Cerebellum, hippocampus, and medulla white matter also contained low concentrations of DARPP-32 mRNA (<2% of that of caudate nucleus). Slightly higher concentrations (approximately 3% of that of caudate nucleus) were detected in cerebral cortex. The upper band (1.8 kb) was detected in all regions, while the lower band was not detectable in these regions, probably because of the low concentrations (Fig. 7, lanes 1, 2, 4–6). Although it is not yet clear whether the 1.65 kb band represents DARPP-32 mRNA or another cross-hybridizable mRNA species, the 1.8 kb band in these regions most likely represents DARPP-32 mRNA, since the cDNA clone pTKD7 encoding the DARPP-32 protein was

derived from the 1771 nucleotide mRNA. Biochemical studies (Walaas and Greengard, 1984) and radioimmunoassays (Hemmings and Greengard, 1986) on the regional distribution of the protein indicate that many neurons contain low concentrations of DARPP-32. Walaas and Greengard (1984) showed that the lower brain stem, hypothalamus, and hippocampus of the rat do contain small amounts of the DARPP-32 protein. Immunocytochemical studies have also shown that the neurons in layers III and VI throughout most of the cerebral cortex, the Purkinje cells of the cerebellum, and certain glial cells are also weakly immunoreactive for the protein (Ouimet et al., 1984). In situ hybridization experiments using the antisense RNA probe derived from pTKD7G3 are in progress to determine the precise cellular localization of DARPP-32 mRNA in the mammalian brain.

In conclusion, we have isolated a cDNA clone that encodes a neuron-specific phosphoprotein, DARPP-32. We have found 2 species of mRNA for DARPP-32, which are probably derived from a single copy of the gene via alternative splicing. The mRNAs were found to be highly enriched in the caudate nucleus compared with other regions of the brain. Further study of this gene should provide a basis for a detailed investigation of the regulatory mechanisms of neuron-specific expression and developmental expression within the brain. Furthermore, the physiological role of DARPP-32 can now be investigated using an isolated gene-expression system.

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