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Frog diazepam-binding inhibitor: Peptide sequence, cDNA cloning, and expression in the brain

(benzodiazepine–receptor ligands/peptide microsequencing/nucleotide sequence/glial cells/evolution)

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ABSTRACT **Three peptides derived from diazepam-binding inhibitor (DBI) were isolated in pure form from the brain of the frog *Rana ridibunda*. The primary structures of these peptides showed that they correspond to mammalian DBI-(1–39), DBI-(58–87), and DBI-(70–87). A set of degenerate primers, whose design was based on the amino acid sequence data, was used to screen a frog brain cDNA library. The cloned cDNA encodes an 87-amino acid polypeptide, which exhibits 68% similarity with porcine and bovine DBI. Frog DBI contains two paired basic amino acids (Lys-Lys) at positions 14-15 and 62-63 and a single cysteine within the biologically active region of the molecule. Northern blot analysis showed that DBI mRNA is expressed at a high level in the brain but is virtually absent in peripheral tissues. The distribution of DBI mRNA and DBI-like immunoreactivity in the frog brain was studied by *in situ* hybridization and immunocytochemistry. Both approaches revealed that the DBI gene is expressed in ependymal cells and circumventricular organs lining the ventricular cavity. Since amphibia diverged from mammals at least 250 million years ago, the data show that evolutionary pressure has acted to conserve the structure of DBI in the vertebrate phylum. The distribution of both DBI mRNA and DBI-like immunoreactivity indicates that DBI is selectively expressed in glial cells.**

Diazepam-binding inhibitor (DBI) is an 86-amino acid polypeptide that was originally identified in mammalian brain from its ability to displace benzodiazepine binding on rat synaptosomal membranes (1, 2). DBI has the potential to generate several biologically active fragments, including the triakontatetraneuropeptide DBI-(17–50) termed TTN (3) and the octadecaneuropeptide DBI-(33–50) termed ODN (4). DBI and its truncated forms TTN and ODN act as inverse agonists on central type benzodiazepine receptors—i.e., they inhibit γ-aminobutyric acid (GABA)-gated chloride channel activity (5, 6) and induce proconflict behavior when injected intracerebroventricularly in rodents (4, 7, 8). As well as its action on the $GABA_A$–benzodiazepine receptor complex, DBI appears to exert various physiological functions including facilitation of cholesterol translocation through mitochondrial membranes (9), inhibition of glucose-induced insulin release (10), and termination of long-chain fatty acid synthesis (11).

The cDNA encoding DBI has been cloned in several mammalian species (12–15), in birds (16, 17), and in yeast (17). Biochemical and immunological studies revealed that DBI-related peptides also occur in the brains of fish (18) and amphibians (19). Concurrently, synthetic mammalian ODN has been shown to modulate the response of frog melanotrope cells to GABA (20), suggesting that the physiological roles of DBI have been preserved during evolution. However, the sequence of DBI has never been determined in any poikilothermic vertebrates.

The present study describes the purification and structural characterization of three large frog DBI fragments, the cloning of the complete frog DBI cDNA, and the distribution of DBI mRNA in the frog brain.§

MATERIALS AND METHODS

Purification of Frog DBI-Derived Peptides. Collection of whole brain (94.5 g) from ≈1200 adult specimens of *Rana ridibunda* and extraction of tissue (boiling for 15 min in 0.5 M acetic acid) have been described (21). The brain extract, after partial purification on Sep-Pak C_{18} cartridges (Waters), was chromatographed on a column of Sephacryl S-100 (2.5 × 100 cm) (Pharmacia LKB) equilibrated with 1 M acetic acid at a flow rate of 2 ml/min. Fractions (10 ml) were collected and absorbance was measured at 280 nm. The fractions with K_{av} between 0.24 and 0.39, containing melanostatin activity (21), were pooled and pumped at a flow rate of 2 ml/min onto a Vydac 218TP510 C_{18} column (25 × 1 cm) (The Separations Group) equilibrated with 0.1% (vol/vol) trifluoroacetic acid/water at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (vol/vol) over 10 min, maintained at this concentration for 20 min, and then raised to 49% (vol/vol) over 60 min. Absorbance was measured at 214 and 280 nm and individual peaks were collected by hand. The prominent peaks designated A, B, and C in Fig. 1*a* (subsequently shown to contain fragments of DBI) were separately rechromatographed on an Ultrapore C-3 column (25 × 1 cm) (Beckman) as described (21). In each case, the peaks denoted by the bars (Fig. 1*b*) contained DBI fragments and were separately rechromatographed on a Vydac 214TP54 (C-4) column (25 × 0.46 cm) as described (21).

Structural Characterization of DBI-Derived Peptides. Amino acid compositions were determined by precolumn derivatization with phenylisothiocyanate (22) using an Applied Biosystems model 420A derivatizer and a model 130A separation system. Vapor-phase hydrolysis (24 h at 110°C using 5.7 M HCl) of ≈1 nmol of peptide was carried out. The primary structure of the peptides (2–4 nmol) was determined by automated Edman degradation using an Applied Biosystems model 471A sequenator modified for on-line detection of phenylthiohydantoin (PTH)-derivatized amino acids. The

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Abbreviations: DBI, diazepam-binding inhibitor; ODN, octadecaneuropeptide; TTN, triakontatetraneuropeptide; GABA, γ-aminobutyric acid; PTH, phenylthiohydantoin.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U09205).

detection limit for PTH-derivatized amino acids was 0.5 pmol.

Preparation of Frog Brain cDNA Library. Frog brain mRNA was isolated from total RNA using Hybond mAP (Amersham). Oligo(dT)-primed cDNA was prepared by means of a cDNA synthesis kit (Amersham) as described (23). The cDNAs were ligated to λgt10 *Eco*RI arms and packaged according to the protocol (Promega), yielding 10^6 recombinants.

Screening of the Library. One-half of the library was plated directly and the remaining half was amplified and stored. Recombinant plaques were probed in duplicate with a set of degenerate (32-fold) oligonucleotides designed from amino acid residues 22–27 (DDELKE) of frog DBI: 5′-GA(TC)-GA(TC)GA(AG)(TC)TGAA(AG)GA-3′. Hybridization was carried out at 40°C for 48 h in 6× SSC (1× SSC = 150 mM NaCl/25 mM sodium citrate, pH 7.0)/0.01 M sodium phosphate, pH 6.8/1 mM Na_2EDTA/0.5% SDS/100 μg of sonicated salmon sperm DNA per ml/0.1% nonfat dried milk. Filters were washed in 6× SSC and 0.1% SDS for 10 min at room temperature and for 10 min at 40°C. A single clone containing a 426-bp insert was isolated and used as a probe to rescreen the amplified frog brain cDNA library. Numerous phages hybridized with the probe. Inserts were subcloned into the *Eco*RI site of pGEM-3Zf (Promega) and five of them were sequenced. The longest one gave the complete coding sequence. Sequencing was performed on both strands by the dideoxynucleotide chain-termination procedure (24).

Northern Blot Analysis. Total RNA (15 μg) from liver, intestine, testis, lung, heart, kidney, adrenal, and brain was isolated by the guanidinium thiocyanate extraction method (25), electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde, and transferred onto a nylon membrane. A cDNA fragment corresponding to the entire coding region of frog DBI was labeled with ^{32}P by random priming and used as a probe under high-stringency hybridization conditions.

***In Situ* Hybridization.** Frog brains were removed and quickly frozen in isopentane at −30°C. Coronal sections (10 μm thick) were cut in a cryomicrotome (Leica, Nussloch, Germany) and collected on 0.5% gelatin/0.05% chrome alum/0.01% polylysine-coated slices. Sections were pretreated with 2× SSC buffer containing 0.2% Triton X-100 for 5 min and prehybridized at room temperature for 1 h in 50% formamide, 5× SSPE (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA)/0.1% SDS/5× Denhardt's solution (1× Denhardt's solution = 0.02% each Ficoll, polyvinylpyrrolidone, and bovine serum albumin)/200 μg of yeast tRNA per ml/20 μg of poly(A) per ml/200 μg of denatured salmon sperm DNA per ml. Hybridization was carried out in the same buffer supplemented with the ^{35}S-labeled frog DBI cDNA probe at a concentration of 10^7 cpm/ml and 4% dextran sulfate. Successive washings were performed at room temperature in 2× SSC for 2 h, 1× SSC for 2 h, 0.5× SSC for 1 h, and then at 37°C in 0.5× SSC for 1 h. After hybridization, the sections were dehydrated and apposed onto Hyperfilm-βmax (Amersham) for 72 h. Then the tissue sections were dipped into Kodak NTB-2 liquid emulsion at 40°C. After 15 days of exposure, the sections were stained with hematoxylin and eosin. Control sections were treated with pancreatic RNase A (40 μg/ml) at room temperature for 30 min before the prehybridization step.

Immunocytochemical Study. The distribution of DBI-like immunoreactivity in the frog brain was investigated by the indirect immunofluorescence method using an antiserum against rat ODN as described (19). The sections were incubated with the ODN antiserum (26) at a final dilution of 1:250. The tissue sections were then incubated with fluorescein isothiocyanate-conjugated anti-rabbit γ globulin (GAR/FITC; Nordic, Tilburg, The Netherlands) diluted 1:60. Sections were examined on a Leitz Orthoplan microscope equipped with a Vario-Orthomat photographic system.

RESULTS

Amino Acid Sequence Analysis. The elution profile on a semipreparative Vydac C_{18} column of the extract of frog brain is shown in Fig. 1*a*. The prominent peak denoted by M contained melanostatin (21). All the major peaks in the chromatogram were rechromatographed on a semipreparative Ultrapore C-3 column. The elution profiles of peaks A,

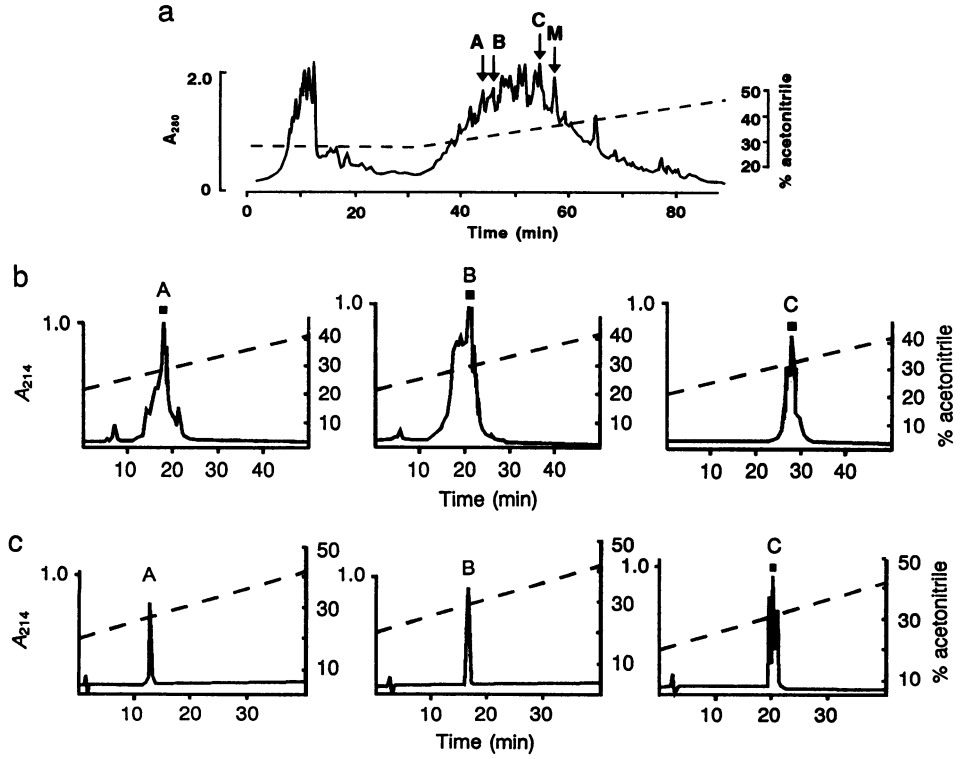

FIG. 1. (*a*) Reversed-phase HPLC on a semipreparative Vydac C_{18} column of frog DBI fragments after partial purification by gel-permeation chromatography. Arrows show peaks containing DBI fragments A, B, and C. The peak containing melanostatin is denoted by M. (*b*) Purification of peak peptides A, B, and C on a semipreparative Ultrapore C-3 column. In each chromatogram, the peak containing the collected material is denoted by a bar. (*c*) Purification of peak peptides A, B, and C on an analytical Vydac C-4 column. In the right chromatogram, the peak containing the selected material is denoted by a bar. Dashed lines show concentration of acetonitrile in the eluting solvent.

Table 1. Automated Edman degradation of purified peptide fragments of frog DBI

	Fragment A (residues 1–39)		Fragment B (residues 70–87)		Fragment C (residues 58–87)	
Cycle	Residue	Yield	Residue	Yield	Residue	Yield
1	Ser	187	Ala	747	Ala	2348
2	Pro	830	Met	717	Trp	1674
3	Gln	636	Ser	54	Asn	1289
4	Ala	932	Ala	571	Leu	2419
5	Asp	659	Tyr	425	Lys	1871
6	Phe	614	Val	518	Lys	2137
7	Asp	672	Ser	32	Gly	1362
8	Lys	977	Lys	361	Leu	1723
9	Ala	705	Ala	379	Ser	175
10	Ala	735	His	142	Lys	1536
11	Gly	542	Glu	130	Glu	706
12	Asp	575	Leu	302	Asp	404
13	Val	568	Ile	218	Ala	1234
14	Lys	863	Glu	118	Met	1205
15	Lys	898	Lys	285	Ser	117
16	Leu	772	Tyr	212	Ala	940
17	Lys	664	Gly	140	Tyr	779
18	Thr	79	Leu	84	Val	879
19	Lys	550			Ser	71
20	Pro	191			Lys	605
21	Thr	55			Ala	682
22	Asp	234			His	513
23	Asp	242			Glu	188
24	Glu	51			Leu	514
25	Leu	213			Ile	474
26	Lys	180			Glu	180
27	Glu	41			Lys	470
28	Leu	194			Tyr	375
29	Tyr	108			Gly	173
30	Gly	113			Leu	100
31	Leu	189				
32	Tyr	100				
33	Lys	122				
34	Gln	40				
35	Ser	14				
36	Thr	23				
37	Val	84				
38	Gly	59				
39	Asp	18				

Yields of PTH-derivatized amino acids are shown in pmol.

B, and C, subsequently shown by Edman degradation to represent fragments of DBI, are shown in Fig. 1*b* (A–C). The DBI fragments were purified to near homogeneity by chromatography on an analytical Vydac C-4 column (Fig. 1*c*). The final yields of pure peptides were as follows: fragment A (residues 1–39), 8 nmol; fragment B (residues 70–87), 3 nmol; fragment C (residues 58–87), 11 nmol.

The results of automated Edman degradation of the purified DBI fragments are shown in Table 1. It was possible to assign without ambiguity PTH-derivatized amino acids during 39 cycles of operation of the sequencer for fragment A, 18 cycles for fragment B, and 30 cycles for fragment C. The amino acid compositions of the peptides were consistent with their proposed structures and demonstrated that their full amino acid sequences had been obtained. The peptides were identified as fragments of DBI using the FASTA protein sequence data base (27).

FIG. 2. Nucleotide sequence and deduced amino acid sequence of *R. ridibunda* DBI cDNA. Numbers on right correspond to the last nucleotide of the line. Amino acid residues are numbered on the left from the starting methionine (−1). Pairs of basic amino acids are shaded. Bioactive domain (ODN) is double underlined with arrows. Polyadenylylation signal is underlined. Substitution at position 326 is denoted by an asterisk.

Isolation and Sequence of DBI cDNA. The longest cDNA sequenced (470 nt) comprised the whole coding region (267 nt) flanked by 12 nt at the 5′ end and 191 nt at the 3′ end (Fig. 2). The five clones differed only in the extent of the 5′ end and in a single substitution (G/A) at position 326 in the 3′ untranslated region. At the 3′ end, a variant consensus polyadenylylation signal (ATTAAA), 10 nt before the terminal poly(A) sequence, was found. The structure of frog DBI mRNA predicts an 87-amino acid protein without any obvious N-terminal signal peptide (Fig. 2). Frog DBI possesses an extra amino acid (proline at position 2) as compared to mammalian DBI and a single cysteine at position 44 within the ODN region (Fig. 3).

Tissue Distribution of DBI mRNA. The distribution of DBI mRNA was examined by Northern blot analysis. A single band (≈690 nt long) was detected in the brain extract but no expression was apparent in liver, intestine, testis, lung, heart, adrenal, and kidney (Fig. 4*a*), although equivalent amounts of total RNA were loaded in the wells as shown by ethidium bromide staining of the gel before blotting (Fig. 4*b*).

In situ hybridization with the DBI cDNA probe produced a strong autoradiographic signal in ependymal cells and circumventricular organs lining the entire ventricular system (Fig. 5 *a* and *b*). Other brain regions showed only a background signal similar to that observed in control sections

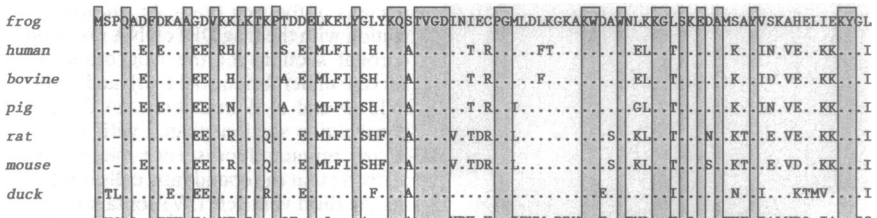

FIG. 3. Comparison of amino acid sequences of DBI from eight different species. Points represent amino acids that are identical with the frog sequence. Regions with 100% interspecies homology are boxed. Pig, human, bovine, rat, mouse, duck, and yeast sequences have been taken from refs. 10, 12–15, 16 and 17, and 17, respectively.

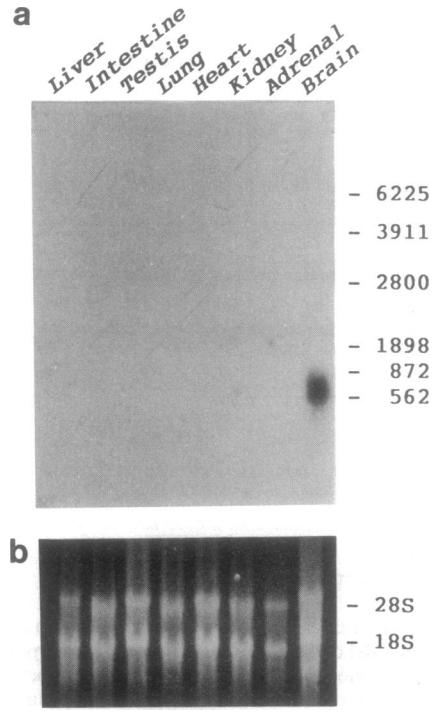

FIG. 4. (*a*) Northern blot analysis of frog RNA probed with the random-primed frog DBI cDNA. Fifteen micrograms of RNA from each tissue sample was electrophoresed on an ethidium bromide-stained 1% agarose/formaldehyde gel. Size markers in bases are indicated on the right. (*b*) Photograph of the gel stained with ethidium bromide before blotting.

treated with RNase A (Fig. 5*c*). The periventricular glial cells that were labeled by the DBI cDNA probe also exhibited ODN-like immunoreactivity (Fig. 5*d*).

DISCUSSION

This study presents a structural characterization of DBI from a poikilotherm vertebrate and contributes to our understanding of the molecular evolution of the DBI family of polypeptides. Two large peptide fragments were isolated from an acetic acid extract of 1200 frog brains and the structure of these peptides appeared to correspond to DBI-(1–39) and DBI-(58–87). A third peptide exhibiting the sequence of DBI-(70–87) was also characterized. Alignment of the amino acid sequences of these peptides (Fig. 2) shows that they arise from cleavages of peptide bonds at the C-terminal side of an aspartic acid residue. It is uncertain whether such cleavages are the result of the action of a specific proteolytic enzyme or are artifactual hydrolyses occurring during the extraction procedure. It has been established that peptide bonds incorporating aspartyl residues are cleaved in acidic solution at a rate that is at least 100 times greater than other peptide bonds (28). The extraction procedure used in this study involved heating the brain tissue at 95–100°C for 15 min in dilute acid. It is possible, therefore, that appreciable hydrolysis of aspartic acid-containing bonds would occur under these conditions. However, the possibility that a proteolytic enzyme that preferentially hydrolyzes peptide bonds at the C-terminal side of aspartyl residues may be present in frog brain warrants further study. Despite an intensive search involving sequence analysis of >70 peptides from the brain extract, the peptide fragment corresponding to DBI-(40–57) was not identified. Nucleotide sequence analysis of the cloned cDNA (Fig. 2) indicates that this fragment contains a single cysteine residue, also present in duck DBI (16), and it is possible that the fragment may have oligomerized or be bound covalently to protein.

A series of observations suggest that frog DBI may serve as a precursor for smaller neuropeptides. (*i*) Several molecular forms of DBI-derived peptides have previously been detected in the frog brain by combining HPLC analysis with RIA detection using an antiserum directed against the central region of rat ODN—i.e., DBI-(46–49) (19). (*ii*) Several molecular forms of DBI have also been found in the brains of different mammalian species using various extraction procedures (3, 4). (*iii*) The 18-residue peptide DBI-(40–57), which would result from the processing at the Gly^{38}-Asp^{39} and Trp^{56}-Asp^{57} sites of DBI, contains the C-terminal sequence of ODN that includes the biologically active determinant of DBI (6, 20).

The amino acid sequence of frog DBI deduced from the corresponding cDNA sequence exhibits 80% similarity with duck DBI (16, 17), whereas the similarity with various mammalian DBI ranged between 61% and 68% (10, 12–15). Evolutionary pressure has particularly acted to conserve the ODN domain, which interacts with both central and peripheral type benzodiazepine receptors (6, 20, 29) and which binds acyl-CoA esters (17, 30). Several aromatic residues

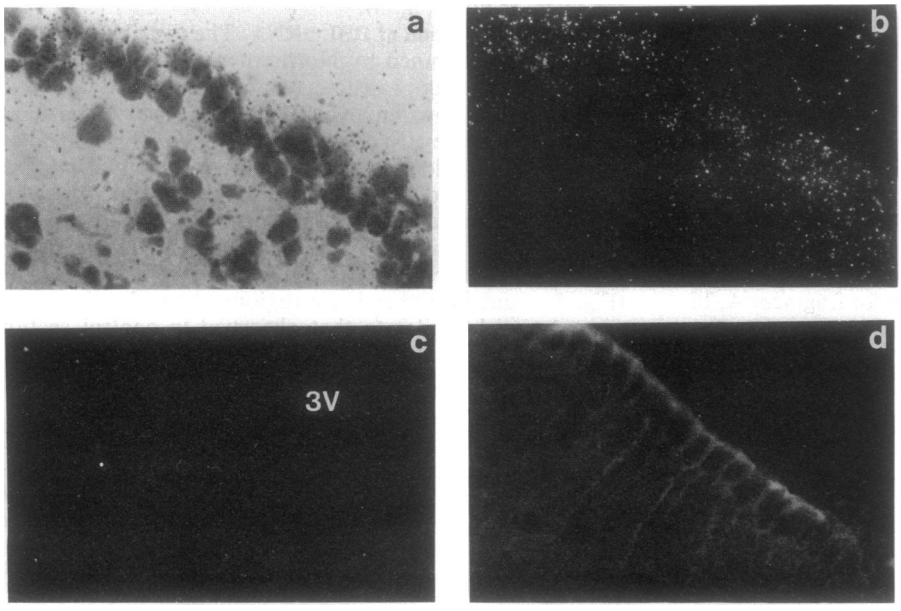

FIG. 5. Cellular distribution of frog DBI mRNA and DBI-related peptides in the periventricular region of the preoptic nucleus of the frog brain. *In situ* hybridization with the frog DBI cDNA probe on coronal sections of the frog brain observed under bright-field (*a*) and dark-field (*b* and *c*) illumination. (*c*) Control consecutive section treated with RNase A. (*d*) Immunofluorescence micrograph showing that the ependymal cells bordering the third ventricle (3V) contain ODN-like immunoreactivity. (×650.)

were also remarkably conserved—e.g., Phe[6], Tyr[29], Trp[56], Trp[59], Tyr[74], and Tyr[85]. The presence of paired basic residues, that constitute potential proteolytic cleavage sites, was found in frog DBI at positions 14–15 and 62–63. These dibasic sequences do not occur in several other species such as human, bovine, pig, and yeast. In contrast, the three lysines flanking ODN and TTN have been highly preserved during evolution (Fig. 3). Inasmuch as the polypeptide DBI-(1–39) isolated from the frog brain extract corresponds to the 5′ end of the open reading frame in the frog DBI cDNA, the present data demonstrate that, in amphibians as in all other species investigated so far (31), the sequence of DBI does not possess a signal peptide.

Mandrup *et al.* (32) have recently reported the existence of a DBI gene family in the rat, comprising one expressed gene and four pseudogenes. In the present study, five cDNA clones have been characterized and all of them exhibited the same sequence, suggesting that only one DBI gene is expressed in the frog brain. In two of the five frog cDNA clones, a single nucleotide mutation (G/A) was observed at position 326, suggesting that the DBI gene exists in several allelic forms. The occurrence of such alleles is consistent with the fact that the cDNA library was constructed using a pool of pituitaries from animals captured in the wild.

Northern blot analysis revealed that the DBI gene is highly expressed in the frog brain. Concurrently, *in situ* hybridization and immunocytochemical studies showed that the expression of the DBI gene is restricted to a discrete layer of glial cells lining the ventricular system. Taken together, these observations indicate that the rate of expression of the DBI gene in the circumventricular organs must be very great. Similarly, the high concentrations of DBI-derived peptides found in frog brain extracts indicate that the DBI mRNA is actively translated. The physiological role of DBI in ependymal cells bordering the ventricular cavity is currently a matter of speculation. Recent studies have shown that, in frog, ODN modulates the action of GABA on the $GABA_A$–benzodiazepine receptor complex (6, 20). The fact that glial cells possess $GABA_A$ receptors (33) suggests that DBI may regulate GABA neurotransmission through a paracrine and/or autocrine mechanism. In support of this hypothesis, we have recently observed that (*i*) DBI is released by rat glial cells and (*ii*) ODN modifies cytosolic calcium concentrations in cultured rat astrocytes (unpublished data).

In mammals, a number of studies have demonstrated that DBI-related peptides and mRNA are widely distributed not only in the brain (26, 34–36) but also in various peripheral organs including pituitary (37), liver, intestine, kidney, testis, and adrenal gland (38–40). Surprisingly, we did not detect DBI transcripts in frog peripheral organs by Northern blot analysis with our brain cDNA probe. It is interesting to note that the frog DBI exhibits a substitution (serine for alanine at position 35) in the DBI-(1–39) region, which is highly conserved from yeast to human (ref. 3; see also Fig. 3). Since this domain coincides with the putative hydrophobic binding site for acyl-CoA (17, 30), the frog DBI identified here may not act as an acyl-CoA binding protein. Alternatively, it is conceivable that, in frog, several DBI genes could exist and that these genes could be differentially expressed in the brain and in peripheral organs. Screening of a frog genomic library will be performed to search for the existence of other putative DBI genes.

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