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Putative diazepam binding inhibitor peptide: cDNA clones from rat

(benzodiazepine receptor ligand/hypothalamus, total brain, and liver cDNA libraries/peripheral abundance)

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ABSTRACT **cDNA clones corresponding to the polypeptide that has been shown to be an endogenous diazepam binding inhibitor and may act as a physiological ligand for the benzodiazepine/β-carboline receptor have been isolated from bacteriophage λ recombinant libraries from rat hypothalamus, total brain, and liver. The clones contain an open reading frame corresponding to 87 amino acids. A signal sequence is not present. In addition to high levels of mRNA in various brain regions, RNA blot analysis reveals an abundance of diazepam binding inhibitor mRNA in many peripheral organs (e.g., testes, kidney, liver, and heart) that are known to be rich in peripheral benzodiazepine recognition sites. The size of the mRNA in all tissue examined is approximately 0.7 kilobase. Southern blot analysis of genomic DNA suggests the presence of about six genes in the rat, some of which may be pseudogenes.**

Brain synaptic membranes contain a high affinity recognition site for anxiolytic benzodiazepines and anxiogenic β-carbolines, both of which allosterically modulate the γ-aminobutyric acid $(GABA)_a$ receptor. Given the existence of endogenous ligands for the opioid receptor, there has been similar interest in identifying an endogenous ligand for the benzodiazepine/β-carboline recognition site.

Benzodiazepines facilitate "GABAergic" transmission (1–3) while β-carbolines inhibit GABAergic transmission (4). Hence, the existence of two conformational states of the benzodiazepine's allosteric site has been proposed (5), although it is not known which of the conformational states plays a physiological role. Diazepam binding inhibitor (DBI), an 11-kDa peptide, has been isolated from rat and human brain and has been characterized biochemically and electrophysiologically for its action on the GABA receptor via binding to the benzodiazepine/β-carboline recognition site (6–9). DBI has a specific distribution in various brain nuclei and is highly concentrated in hypothalamus and cerebellum (10). When injected intracerebroventricularly, it causes proconflict action in rats (6, 11). Thus, it has a pharmacological profile of a naturally occurring anxiogenic compound and, accordingly, it preferentially displaces β-[^{3}H]carboline over [^{3}H]diazepam (7).

To obtain more information about this putative neuropeptide, we have isolated its cDNA. The cloning and sequencing of the DBI cDNA should give us the complete structure of the protein functioning as the putative precursor for the endogenous allosteric modulator of GABA transmission. The cDNA may be used as a probe to measure the levels of DBI mRNA in various brain regions and peripheral tissues by RNA gel blot analysis or *in situ* hybridization. This includes examination of mRNA levels at different developmental stages as well as the changes of the mRNA and consequently the peptide's dynamic state (12). In this paper we describe the isolation and characterization of rat DBI cDNA clones from λgt10 and λgt11 libraries using a long synthetic oligonucleotide probe corresponding to the mRNA portion that encodes the octadecapeptide that is a putative active peptide fragment of DBI (13).

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (10–12 weeks old) were obtained from Marland Breeding Farm (Hewitt, CT).

Construction of cDNA Libraries. Cytoplasmic RNA was prepared from rat brain and liver following the procedure of Schibler *et al.* (14). Total RNA was isolated from entire hypothalami (from 9-week-old female Sprague–Dawley rats) by the guanidinium thiocyanate/LiCl procedure of Cathala *et al.* (15). Polyadenylylated RNA was prepared (16) using an oligo(dT) column (PL-Pharmacia, Piscataway, NJ). Eight micrograms of poly(A)$^+$ RNA were converted into double-stranded cDNA essentially following the procedure of Gray *et al.* (17) with the exception that we used regular *Eco*RI linkers (5′ G-G-A-A-T-T-C-C 3′ or 5′ C-C-G-G-A-A-T-T-C-C-G-G 3′ (Pharmacia-PL) after protecting internal restriction sites with *Eco*RI methylase (New England Biolabs). The cDNA was cloned into phage λgt10 (18). Prior to amplification the hypothalamus and liver libraries consisted of about 5×10^6 members, and the entire brain library of about 3.5×10^6 independent members. In addition, a brain cDNA library in λgt11 (18) constructed by V. Aulb and R. Dunn (University of Toronto) was screened. This library, consisting of 3.5×10^6 independent members, was prepared with total polyadenylylated RNA from entire brains of male albino Wistar rats. The RNA was randomly primed, and cDNA was prepared by the Gubler–Hoffman method (19).

Screening of Libraries. Initially 2.5×10^5 recombinant phage from the unamplified rat hypothalamus library were screened with a long deoxyoligonucleotide probe (for details see Fig. 1) following the procedure of Ullrich *et al.* (20). The other libraries (also using 2.5×10^5 phage) were screened with nick-translated cDNA inserts of DBI clones isolated from previous screenings following the procedures of Maniatis *et al.* (21). Oligodeoxynucleotides were synthesized by B. Goldschmidt (Biochemistry Department, New York University). ^{32}P-labeled radionucleotides were obtained from New England Nuclear.

Subcloning, Sequencing, RNA/DNA Blot Analysis. Preparations of recombinant phage and plasmid DNA, rat liver genomic DNA, insertion into plasmids pUC13, pUC18 (22), or pUC119 (23) as well as Southern and RNA gel blots were done essentially as described (refs. 21, 24, and New England Nuclear manual for GeneScreen*Plus*). DNA sequence analysis was done on both DNA strands using the chemical

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Abbreviations: DBI, diazepam binding inhibitor; GABA, γ-aminobutyric acid.

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method of Maxam and Gilbert (25). Restriction and DNA modifying enzymes were obtained from Pharmacia-PL or New England BioLabs and used according to the supplier's directions.

RESULTS

Isolation and Characterization of DBI Clones. From 2.5×10^5 plaques of the rat hypothalamus library, we identified seven clones using the long oligonucleotide shown in Fig. 1. The smallest *Eco*RI insert of these λgt10 recombinant clones was subcloned into pUC18 (22) yielding pIMh101, and the DNA sequence was determined. The deduced partial amino acid sequence was identical to published peptide fragments of DBI (6, 13). The *Eco*RI insert of pIMh101 (Fig. 2) was then used to identify additional cDNA clones by cross-hybridization to DNA from the remaining six phages. Three more clones were positive at high stringency, and their inserts were transferred into pUC18 yielding plasmids pIMh4, pIMh61, and pIMh102. The remaining three phage clones were either false positives or contain DBI-related sequences. None of the four hypothalamic DBI clones, however, extended to a possible translation start codon at the 5′ end of the mRNA (Fig. 2). To obtain larger clones, we screened a brain cytoplasmic cDNA library with the 5′ *Eco*RI–*Ava* II fragment from clone pIMh102. With this probe (≈100 base pairs long; Fig. 2), we isolated nine recombinant phage and were able to subclone seven inserts into the *Eco*RI site of pUC13. Clones that were likely to extend beyond the previous sequences in the 5′ direction were selected by restriction mapping and then sequenced. Clones pIMc11, pIMc61, pIMc9, and the insert from λc82 (which could not be subcloned and was sequenced directly) are shown in Fig. 2. The latter three clones extend beyond the putative start codon into the 5′-untranslated region. In parallel, the rat liver cDNA library was screened with the insert of pIMh101. Fifteen positive phage clones were identified, and the two largest inserts were subcloned into pUC13 (22), yielding pIMl4 and pIMl5, and subjected to sequence analysis. These inserts are shown in Fig. 2. Finally, a λgt11 cDNA library from rat brain was screened with the 5′ *Eco*RI–*Ava* II fragment from pIMc61. Ten positive phage clones were isolated, and three inserts were subcloned into pUC13 or pUC119 yielding pCG1112, pCG1113, and pCG1114, and their sequence was determined. pCG1114 has the longest insert so far isolated and corresponds to the sequence shown in Fig. 3. The other two inserts from this library extend well beyond the possible ATG start site (Fig. 2).

Structure of the DBI mRNA and Protein Coding Region. Most clones correspond to the single sequence depicted in Fig. 3. An observed heterogeneity in length (12 or 13 nucleotides) of the poly(T) tract (located in the 3′-untranslated region, positions 447–459; Fig. 3) may be due to slippage of reverse transcriptase during cDNA synthesis or to allelic variation. Another exception is liver clone pIMl5 that starts with 5′ G-G-A-G-C-C-T-C 3′ instead of with 5′ G-T-C-A-C-C-T-C 3′ (corresponding to positions 104–111 in Fig. 3). The length of clone pCG1114 [566 nucleotides without a poly(A) tail] is compatible with a mRNA size of ≈700 nucleotides (see below) and suggests that it is close to full length. The coding region comprises 271 base pairs and predicts a peptide chain of 87 amino acids, or if the N-terminal methionine is removed, of 86 amino acids. The calculated amino acid composition of the predicted DBI peptide (Asn_3, Asp_7, Thr_6, Ser_4, Gln_3, Glu_8, Pro_2, Gly_5, Ala_6 Cys_0, Val_5, $Met_{2/3}$, Ile_2, Leu_7, Tyr_3, Phe_3, His_1, Lys_{15}, Arg_2, Trp_2) is in good agreement with the experimental data obtained on rat DBI (6), except for the high number of serine residues in the amino acid analysis (6). However, serine is known to be a potential contaminant of protein samples. The open reading frame and a TAA stop codon are followed by a 3′-untranslated region of 185 nucleotides. A variant poly(A) addition signal, A-T-T-A-A-A (26–28), is located 17 nucleotides upstream from the poly(A) tail.

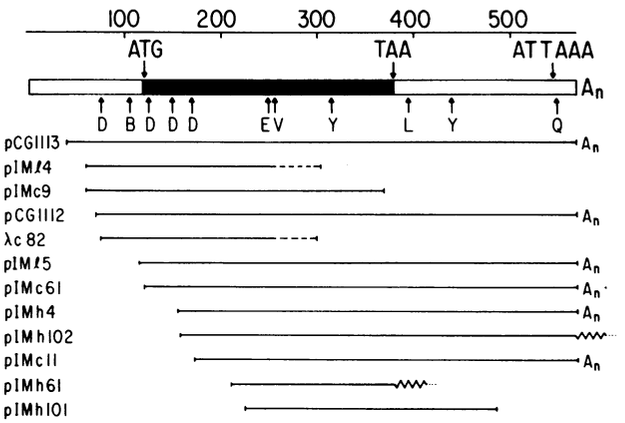

FIG. 2. Schematic map of DBI cDNA clones. The length and landmarks of the largest cDNA insert (in pCG1114) are depicted in the top bar. The ATG translation start codon and the TAA stop codon flanking the filled coding region, as well as the putative poly(A) addition signal (ATTAAA) and the poly(A) tail $(A)_n$ at the 3′ end of the cDNA, are indicated. The locations of selected restriction endonuclease sites are indicated by letters under the top bar (D, *Dra* I; B, *Bst*EII; E, *Eag* I; V, *Ava* II; Y, *Sty* I; L, *Bal* I; Q, *Rsa* I). The remaining cDNA clones are indicated by lines. Broken lines indicate that the 3′ portion of the clones have not been characterized. Zig-zag lines indicate cDNA portions that are probably derived from non-DBI sequences by fusion of two unrelated inserts prior to linker addition. cDNA clones that contain poly(A) tails are marked by A_n. All clones are flanked by *Eco*RI sites contributed by linkers (G-G-A-A-T-T-C-C) [or by (C-C-G-G-A-A-T-T-C-C-G-G) in clones (pIMl4, pIMl5) isolated from the liver library].

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DBI   ...Gln Ala Thr Val Gly Asp Val Asn Thr Asp Arg Pro Gly Leu Leu Asp...

A)  5'  CAG GCC ACA GTG GGA GAT GTG AAC ACA GAC CGG cct ggc ctc ctg ga

B)      gtc cgg tgt cac cct cta cac TTG TGT CTG GCC GGA CCG GAG GAC CT  5'

cDNA    CAA GCT ACT GTG GGC GAT GTA AAC ACA GAT CGG CCG GGG CTG TTG GA
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FIG. 1. Oligonucleotide probe for DBI. The first line shows the part of the DBI protein sequence (6) that has been chosen to design the synthetic oligonucleotide probe. This stretch of 16 amino acids has been "reverse translated" into a hypothetical mRNA (second line) using the anticipated DNA codons (20). Two partially complementary oligonucleotides (A + B, capital letters) were synthesized and labeled with [γ-^{32}P]ATP at their 5′ ends (21). The two labeled probes were then annealed and filled in with [α-^{32}P]dNTPs (lower case letters) using DNA polymerase, large fragment (21). Unincorporated nucleotides were removed by a spin column (21), and the probes were used for screening according to Ullrich *et al.* (20). The last line shows the actual DBI gene sequence of the probe segment. Nucleotides that differ from the probe, which has 78.7% homology to the cDNA, are underlined.

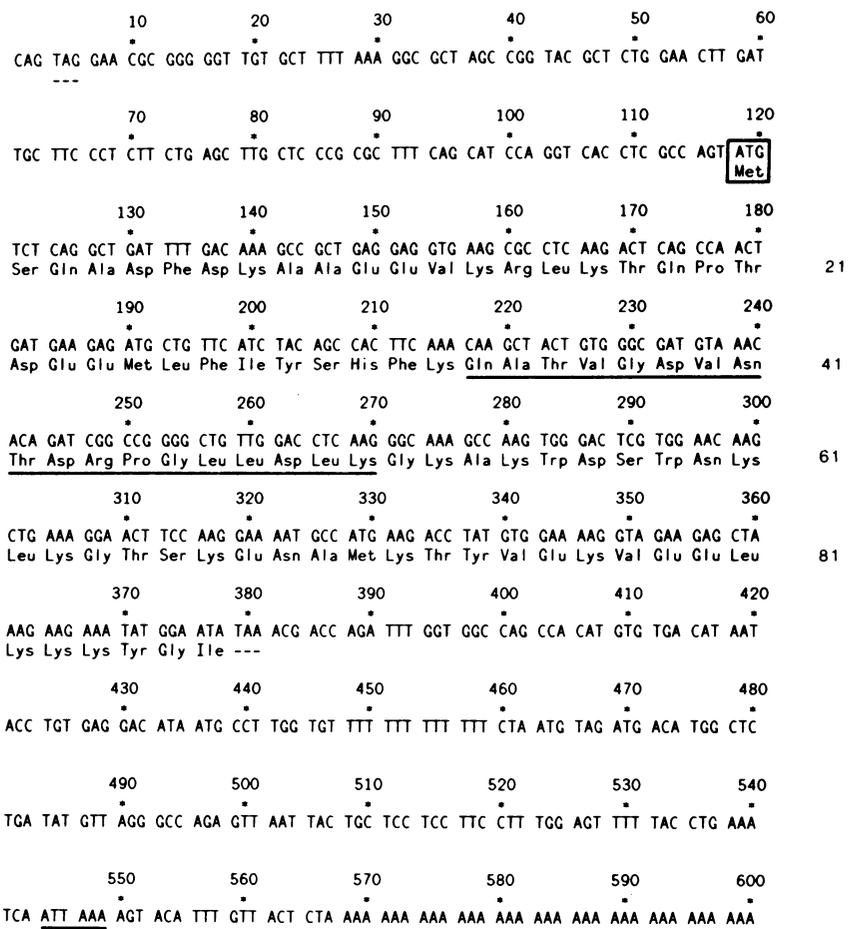

FIG. 3. Nucleotide and derived protein sequence of clone pCG1114. The upper line depicts the nucleotide sequence (without sequences contributed by linkers). The putative poly(A) addition signal (ATTAAA) is underlined. Part of the poly(A) tail is shown at the 3′ end. The ATG start codon and methionine initiation codon (lower line depicting the derived amino acid sequence) are boxed. Numbers at the right margin refer to the amino acid positions of the coding region. The underlined peptide represents the putative active octadecaneuropeptide (6, 13).

RNA Blot Analysis. cDNA probes hybridize to mRNA of ≈700 nucleotides. This mRNA has a relatively high abundance, both in a number of different brain regions, as well as in some peripheral organs (Fig. 4 *a* and *b*). As an internal control, filters were reprobed with the rat β-actin gene from pAc18.1 (29). A shorter exposure (about one-fourth the length) and developing time (about one-third the length) is the reason for the relatively lower intensity of the actin band in the overlay.

Genomic DNA Blot Analysis. Rat genomic DNA, digested with a number of enzymes, was probed with the insert of clone pIMh101. Five or more bands are identified in each lane (Fig. 5*A*), suggesting that there is more than one DBI gene in the rat. When a similar filter was probed with the *Dde* I fragment from the 5′-untranslated region (corresponding to bases 74–125 in Fig. 3), only one major band was detected (Fig. 5*B*). The bands in the *Bst*EII lane (E) are weak because the hybridizing regions are shorter due to the presence of a *Bst*EII site in the sequence that is covered by the probe.

DISCUSSION

DBI cDNA Clones and Genes. During the isolation of DBI clones, we encountered a relatively large number of scrambled clones. One type contained sections of the cDNA as inverted repeats (pIMl4, pIMc9, λc82), possibly generated by a mechanism suggested by Pletnev *et al.* (30) or by inverted repeat recombination. The other type differs from normal clones by the presence of unrelated sequences. While the former type of clone can be detected easily and "descrambled," the latter type can constitute—if only a single clone is analyzed—a serious source for errors. The two clones of the latter type encountered here (pIMh61 and pIMh102; Fig. 2) do not contain internal *Eco*RI sites, and it is likely that the unrelated segments were fused to DBI sequences by ligation of two cDNA fragments prior to linker addition. Since RNA blot analysis of pIMh61 and pIMh102 gives rise to additional RNA bands not detected with the DBI portions of these inserts (data not shown), it is unlikely that these clones reflect variants of DBI mRNA. None of the sequenced clones isolated from the λgt11 brain library (a gift from R. Dunn), where the second strand of cDNA has been synthesized by the Gubler–Hoffman method (19), were found to be scrambled. The number of clones analyzed is too small, however, to make a valid statement concerning the fidelity of the two cloning methods.

With the exception of the two composite clones discussed above (pIMh61 and pIMh102) and clone pIMl5, which differs only by a stretch of four nucleotides at its 5′ end (possibly another cloning artefact) all clones, including the ones containing inverted repeats, represent only a single sequence. Furthermore, partial sequence analysis of one genomic clone (DBI-G4) reveals 100% homology to six cDNA clones that extend into the 5′-untranslated region. The genomic sequence differs downstream from position 126 of the cDNA (Fig. 3) due to the presence of an intron following the third amino acid of the coding region (unpublished data). A DNA fragment (the pIMh101 insert) covering the 3′ half of the coding region and part of the 3′-untranslated region identifies five or more major DNA bands on a Southern blot (Fig. 5*A*). In contrast, a 5′ *Dde* I fragment (positions 74–125; Fig. 3)

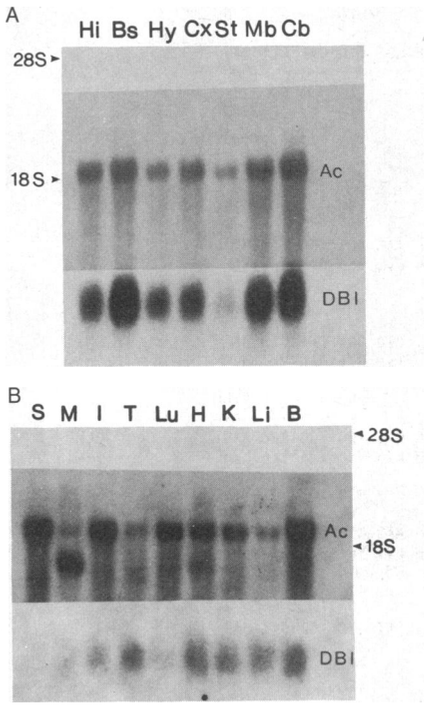

FIG. 4. RNA blot analysis of various rat brain regions (*A*) and peripheral tissue (*B*). (*A*) Polyadenylylated RNA from hippocampus (lane Hi, 8 μg), brain stem (lane Bs, 14 μg), hypothalamus (lane Hy, 6 μg), cortex (lane Cx, 10 μg), striatum (lane St, 4 μg), midbrain (lane Mb, 13 μg), and cerebellum (lane Cb, 16 μg) were electrophoresed on a 1.1% agarose/formaldehyde gel and transferred to nitrocellulose (21). The filter was probed with nick-translated cDNA insert from pIMh101 and hybridized in 50% (vol/vol) formamide/0.75 M NaCl/25 mM Pipes, pH 6.8/25 mM EDTA/0.2% $NaDodSO_4$/1× Denhardt's solution/denatured salmon sperm DNA at 100 μg/ml at 42°C overnight. The filter was washed twice for 1 hr in 2× SSC/0.2% $NaDodSO_4$ at 42°C and for 15 min in 0.1% SSC/0.2% $NaDodSO_4$ at 67°C. (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0.) The filter was autoradiographed for 24 hr. The filter was then reprobed with nick-translated pAc18.1 (ref. 29; kindly provided by U. Nudel) containing the rat β-actin gene under the same conditions, except that autoradiography was for 8 hr, and the developing time was reduced. The reprobed blot is an overlay with the actin bands exactly on top of their position on the DBI blot. The bands corresponding to actin mRNA are shown in the overlay (Ac). Positions of 28S and 18S rRNA, as well as DBI mRNA are indicated. (*B*) Polyadenylylated RNA from rat spleen (lane S, 7 μg), muscle (lane M, 9 μg), intestine (lane I, 8 μg), testes (lane T, 10 μg), lung (lane Lu, 8 μg), heart (lane H, 11 μg), kidney (lane K, 10 μg), liver (lane Li, 10 μg), and total brain (lane B, 10 μg) was probed, reprobed, and exposed as in *A*. Data are presented as in *A*.

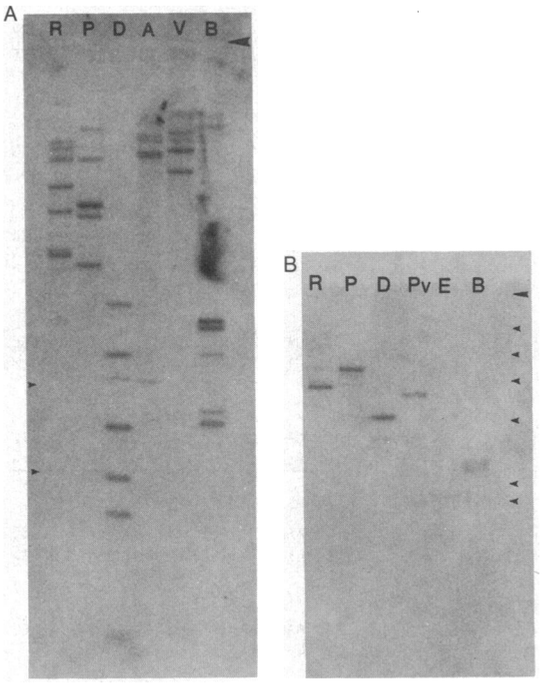

FIG. 5. Southern analysis of rat genomic DNA with DBI cDNA. (*A*) Coding region and 3′-untranslated region as probe. Ten micrograms of rat liver genomic DNA were digested with *Eco*RI (lane R), *Pst* I (lane P), *Dra* I (lane D), *Apa* I (lane A), *Eco*RV (lane V), and *Bam*HI (lane B) and electrophoresed on 1% agarose. After transfer to nylon membrane, the filter was hybridized [in the presence of 50% (vol/vol) formamide] with the insert of pIMh101 (Fig. 2) and washed under the stringent conditions described in the GeneScreen *Plus* manual (New England Nuclear). The filter was exposed to Kodak XAR-5 film with intensifying screen for 7 days. The larger arrowhead near the top depicts the origin of the gel, while the smaller arrowheads on the left side depict 2.4- and 1.35-kilobase size markers. (*B*) 5′-Untranslated region as probe. Ten micrograms of DNA were digested with *Eco*RI (lane R), *Pst* I (lane P), *Dra* I (lane D), *Pvu* II (lane Pv), *Bst*EII (lane E), and *Bam*HI (lane B), electrophoresed, and hybridized as described in *A* with the exception that the *Dde* I fragment from pIMc9 (positions 74–125; Fig. 3) was used as a probe. The small arrowheads depict size markers of 8.5, 7.2, 6.4, 5.7, 4.8, and 4.3 kilobases.

identifies only one major band on genomic DNA (Fig. 5*B*) that also corresponds to the respective band generated by digests of genomic clone DBI-G4. Furthermore, of the six distinct genomic DBI clones isolated on bacteriophage λ clones (unpublished data) only one hybridizes to the *Dde* I fragment under stringent conditions. The 5′ probe gives rise to the same broad DBI mRNA band on an RNA gel blot (data not shown). The above data suggest that probably all cDNAs are derived from one mRNA template that is transcribed from only one gene (unless the structures of mRNAs derived from the other genes would interfere with their ability to be converted into cDNA or with the stability of the λ clones). This, in turn, would suggest that some of the other identified genomic sequences might be transcribed at much lower levels or might represent inactive pseudogenes.

DBI Peptide. An in-frame translational stop codon is located at nucleotide position 4–6 (Fig. 3). Consequently, the first possible ATG start codon is located at position 118–120. Since the next ATG codon (positions 190–192) is located in tryptic peptide T7 (corresponding to positions 169–216; Fig. 3) of DBI (13), there is only one possible initiation codon. This predicts that DBI is 87 or, if the N-terminal methionine is posttranslationally removed, 86 amino acids long. The calculated molecular weight of 10,026.4 of the 87-amino acid unmodified peptide chain is in good agreement with an experimental value of 11,000 from $NaDodSO_4$/polyacrylamide electrophoresis (6), and the calculated amino acid composition agrees with the experimental data (6). Furthermore, the deduced amino acid sequence is in excellent agreement with the peptide sequences of CNBr fragments 1 and 2 (with the exception of the two C-terminal amino acids in fragment 1; ref. 6) as well as with tryptic fragments T4, T5, and T7 (13).

The sequence contains a large number of charged residues (15 negative and 18 positive residues for 38%) and no extended stretches of hydrophobic amino acids. In particular, DBI lacks an apparent N-terminal signal sequence for transmembrane passage.

Expression of DBI. There are relatively high levels of DBI mRNA in most brain regions (Fig. 4*A*), and the distribution of mRNA in different brain areas correlates with the distribution of DBI protein content (M. Miyata and I. M., unpublished data). DBI mRNA is also found in peripheral tissue (Fig. 4*B*); while there is little DBI mRNA in spleen, muscle,

lung, or intestine, higher levels are detected in liver, testes, kidney, and heart. Peripheral benzodiazepine-binding sites have been located in platelets (31), mast cells (32), thymocytes (33), heart (34, 35), kidney (35, 36), testes (37, 38), liver (39), and adrenal gland (37, 38, 40, 41), where they were found to be associated with the mitochondrial outer membrane (42). It could be speculated that DBI synthesized and stored in peripheral organs may interact with these binding sites even though the presence of a receptor in a given tissue does not imply that that tissue is the site of neurotransmitter synthesis.

Conclusion. DBI, the putative endogenous ligand to the benzodiazepine receptors, has been cloned as cDNA. The clones encode a hydrophilic peptide of 86 or 87 amino acids. The sequence does not include an N-terminal signal sequence as one would expect for a secreted neuropeptide. On the other hand, secreted proteins are known that lack a leader peptide (43). The high levels of DBI mRNA in most brain regions and many peripheral tissues would suggest that DBI is a structural or housekeeping protein. Interestingly, though, *in situ* hybridization experiments (R. Fremeau, H. Alho, C. Wilcox, I.M., J.B., E. Costa, and J. Roberts, unpublished data) reveal that DBI mRNA is not equally distributed but that there are distinct cell clusters and areas in the brain where the mRNA is highly enriched. Either DBI is not the endogenous ligand for the benzodiazepine receptor and its real function remains to be established, or DBI is an exceptional neuropeptide, lacking a signal sequence and being abundantly expressed in nonneural tissue. Data from Southern blot analysis suggest that DBI might be encoded by as many as six different genes. It is likely, however, that most of the hybridizing sequences are pseudogenes, since probably all transcripts are derived from only one active gene. Nevertheless, it cannot be ruled out yet that more than one true gene exists. The product of one of these genes or even a peptide from a more distantly related gene might exhibit the expected characteristics of a neuropeptide. More work is necessary to clarify these questions.

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