## RESEARCH COMMUNICATION

# The characterization of two diazepam binding inhibitor (DBI) transcripts in humans

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We have investigated the expression of diazepam binding inhibitor (DBI) (also called acyl-CoA-binding protein or endozepine) transcripts in different human tissues and tissue culture cell lines by reverse-transcriptase assisted PCR and RNase protection assay. Two different DBI transcripts capable of encoding polypeptides of 86 and 104 amino acids were detected in all the human tissues and cell lines studied. The transcript coding for the 86 amino acid DBI polypeptide was found to represent the majority of the total DBI transcript pool.

## INTRODUCTION

The diazepam binding inhibitor (DBI) is a 10 kDa highly conserved protein that is widely distributed throughout phylogenesis from yeast to mammals [1–11] and that has been proposed to have multiple biological functions.

Originally, DBI was purified from rat brain on the basis of its ability to displace diazepam from type A $\gamma$-aminobutyrate receptor ($GABA_A$) [12]. Pharmacological and electrophysiological studies, together with intracerebroventricular injection experiments in rats and increased DBI concentrations in cerebrospinal fluid in several mental disorders involving changes in GABAergic transmission, have suggested that DBI might be involved in the endogenous modulation of the $GABA_A$–benzodiazepine receptor complex (see [13] for a review, [14]).

Furthermore, a 10 kDa protein purified from bovine liver and capable of binding and inducing synthesis of acyl-CoA, turned out to be identical with DBI and was named acyl-CoA-binding protein [15]. Structural studies as well as extensive functional studies *in vitro* and *in vivo* have provided strong evidence that DBI acts as a pool former and/or a transporter of acyl-CoA (see [16] for a review). The involvement of DBI in acyl-CoA metabolism has received further support from the observation that in *Drosophila melanogaster*, DBI is only expressed in tissues with a high level of energy consumption, utilizing fatty acids as its primary energy source [10].

DBI has also been purified from bovine adrenal gland on the basis of its stimulatory effect on mitochondrial steroidogenesis [17,18]. The steroidogenic effect of DBI has been demonstrated *in vitro* with mitochondrial preparations from several tissue culture cell lines [19,20] and such effect was proposed to be caused by stimulation of cholesterol delivery to the inner mitochondrial membrane [17]. Also, antisense oligonucleotides to DBI have been shown to inhibit hormone-stimulated steroid production in Leydig cells [21].

DBI purified from porcine intestine has been shown to decrease glucose-stimulated insulin release [4,22,23]. One of the DBI processing products ($DBI_{32-86}$) has been shown to have antibacterial properties [24].

In humans and other mammals the DBI protein consists of 86 amino acids [2,3,4,5,15,25,26]. In addition to the cDNA encoding for DBI of 86 amino acids [25], a human DBI cDNA capable of encoding the protein with 104 amino acids has also been cloned [1]. These different cDNA clones will here be referred to as hDBI-1 [25] and hDBI-2 [1], respectively. So far there is no conclusive evidence of the existence of the hDBI-2 polypeptide or of the expression of the corresponding transcript in human tissues. Nothing is known either about the possible biological function(s) of this putative 104 amino acid DBI polypeptide.

This paper describes the expression of two different DBI transcripts in human tissues and tissue culture cell lines using reverse-transcriptase assisted PCR (RT/PCR) and RNase protection assay.

## EXPERIMENTAL

### RNA preparations and cell lines

The preparation of RNAs from adrenal gland, pancreas, lymphocytes, jejunum, duodenum, heart, kidney and tissue culture cell lines IMR-32 and HS 67 has been described previously [27]. All other RNAs were prepared as described [28]. The following human cell lines were used: Chang (non-malignant liver), IMR-32 (neuroblastoma), Hs 67 (normal thymus), MG-63 (osteosarcoma) and Jurkat (acute T-cell leukaemia).

### cDNA cloning and PCR

cDNA first strand was synthesized in a total volume of 20 $\mu$l, by oligo-dT priming, with M-MuLV reverse transcriptase (Promega), using 2 $\mu$g of the total RNA as a template. Negative controls were carried out with each RNA preparation by omitting reverse transcriptase from the first strand synthesis reaction.

PCR was carried out in a volume of 50 $\mu$l, using 2 $\mu$l of the cDNA first strand synthesis reaction mixture as a template. The reaction mixture was denatured at 96 °C for 4 min and amplified for 30 cycles: 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min. The last polymerization step was extended to 11 min.

The primers used for PCR were as follows: the 5′ primer for hDBI-1 was GACCTCGAGGTCGGCCAGGATGTCTCAGG

Abbreviations used: DBI, diazepam binding inhibitor; RT/PCR, reverse-transcriptase assisted PCR; $GABA_A$, type A $\gamma$-aminobutyrate receptor.

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[25]; the 5′ primer for hDBI-2 was GACCTCGAGACCGAGCTATGTGGGGCGAC [1]; and the 3′ primer used for both transcripts was GTCGAATTCGGCATCTTCCTTGGAAGTCC [1,25]. The primers included *Xho*I and *Eco*RI restriction sites (5′ primers and 3′ primer, respectively).

For cDNA cloning the 5′ primers were the same as described above but the 3′ primer was TTCCCGAATTCCCACCATCC [1]. Amplified DNA fragments were digested with *Xho*I/*Eco*RI (the *Xho*I site is in the 5′ primers and *Eco*RI site is located in the 3′ untranslated region of the DBI transcript) and cloned into pBluescript II KS-vector (Stratagene). Cloned cDNA sequences were verified by DNA sequencing using the chain-termination method [29] and analysed with an ALF automated DNA sequencer (Pharmacia). The DNA sequence data were analysed by GCG software [30].

### RNase protection analysis

For RNase protection assays *Xho*I/*Sma*I fragments from two different cDNAs were subcloned into pBluescript II KS-vector and designated as P1 and P2 for hDBI-1 and hDBI-2 respectively. *The Xho*I site from the 5′ primers was used for amplifications and cDNA cloning; *Sma*I is located at positions 192 [25] and 205 [1]. cRNA probes were synthesized using T7 RNA polymerase (Promega) and [$\alpha$-$^{32}$P]ATP (Amersham) on linearized plasmid templates. RNase protection assays were carried out with an RPA II Ribonuclease Protection Assay kit (Ambion).

### Transcription-coupled translation *in vitro*

Transcription-coupled translation was carried out with a TNT Coupled Reticulocyte Lysate System (Promega) using 2 $\mu$g of linearized plasmid DNA as a template, with [$^{35}$S]methionine (Amersham) and T3 RNA polymerase (Promega). Proteins were analysed by SDS/PAGE (15–20%) followed by autoradiography.

## RESULTS AND DISCUSSION

To study the expression of DBI transcripts by RT/PCR, two different primers were designed to cover the 5′ ends of the previously published different human DBI cDNAs [1,25]. One common oligonucleotide was used as the 3′ primer (see the Experimental section). Analysis of the total RNA from different tissues revealed the presence of two different amplification products of the expected sizes (237 bp for hDBI-1 and 287 bp for hDBI-2) in all the tissues studied: brain, liver, adrenal gland, pancreas, lymphocytes, jejunum, duodenum, heart and kidney (Figure 1). Likewise, in all cell lines studied (Chang, IMR-32, Hs 67 and MG-63) amplification products corresponding to the two different DBI transcripts were detected (results not shown). To ascertain that amplification products are raised from RNA and not from processed pseudogenes due to the possible contaminating DNA traces in RNA samples [five processed pseudogenes have been cloned from rat [31,32] and one from human genome (accession number L27274)], control reactions were carried out by omitting reverse transcriptase from the cDNA first strand synthesis reaction. No amplification products were detected in negative control reactions (Figure 1).

The RNase protection assay was further used to confirm the results obtained by RT/PCR. First, we cloned both of the DBI cDNAs by RT/PCR from human jejunum RNA. Amplification products were cloned into plasmid vector and nucleotide sequences were verified by DNA sequencing (see the Experimental section). Both cDNA sequences were identical with the respective cDNAs published earlier [1,25].

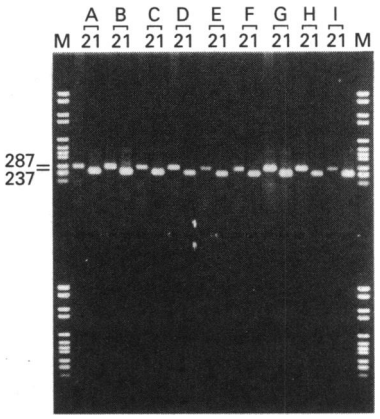


**Figure 1 RT/PCR analysis of total RNA from human tissues**

Amplification was performed using different 5′ oligonucleotide primers corresponding to hDBI-1 cDNA (1) and hDBI-2 cDNA (2) (see the Experimental section). The designation of the lanes is as follows: brain (A), liver (B), adrenal gland (C), pancreas (D), lymphocytes (E), jejunum (F), duodenum (G), heart (H), kidney (I) and molecular mass markers (M). The lower lane is identical to the upper one except that reverse transcriptase was omitted from cDNA first strand synthesis reaction.

The cRNA probes used in the RNase protection assay share 127 nucleotide identity and 17 and 69 nucleotide difference at their 5′ ends respectively (see the Experimental section). If both RNA transcripts are expressed, then both of the probes should yield two protected fragments. A protected fragment of 127 nucleotides, corresponding to identical regions in P1 and P2, should appear with both probes. In addition, P1 should yield a fragment of 144 nucleotides (corresponding to hDBI-1), and P2 should yield a fragment of 196 nucleotides (corresponding to hDBI-2, see also Figure 2). Protected fragments corresponding to the hDBI-1 transcript (144 and 127 nucleotides for P1 and P2 respectively) were detected in all the tissues and cell lines studied (Figure 3). The signal from the protected fragment corresponding to the hDBI-2 transcript (127 for P1 and 196 for P2) is markedly weaker than that of hDBI-1 (Figure 3a, b), indicating that the hDBI-2 represents only a minor fraction of a total pool of the human DBI transcripts.

Our results showing the expression of DBI transcripts in all the human tissues studied are consistent with the findings of an earlier study which revealed expression of DBI-like immuno-

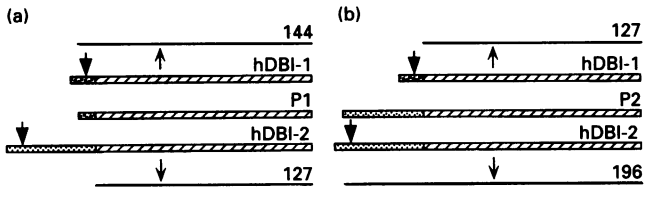


**Figure 2 Schematic representation of RNase protection assay with two different cRNA probes P1 (a) and P2 (b) (see also the Experimental section)**

Striped boxes represent an identical sequence shared by both transcripts and cRNA probes. Boxes with different types of shading represent alternatively spliced first exons of two different transcripts and corresponding sequences in cRNA probes. Lines represent protected fragments with their respective size (in nucleotides) indicated by numbers. Arrows represent initiator ATG codons.

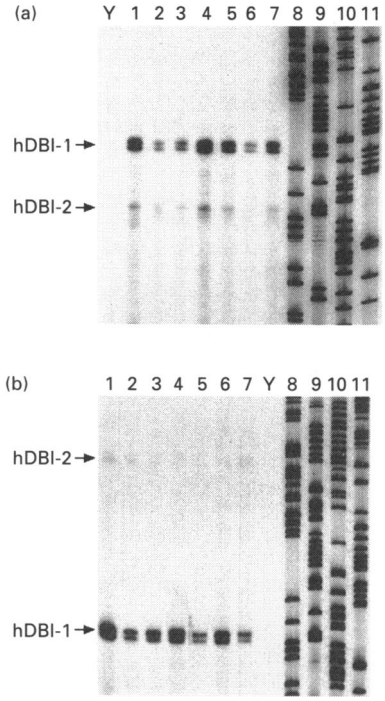


**Figure 3 RNase protection assay with probe P1 or P2**

(**a**) RNase protection assay with probe P1. The lanes are as follows: liver (1), testis (2), cerebral cortex (3), cerebrellum (4), Jurkat (5), Chang (6), Mg-63 (7) and yeast tRNA (Y). The lanes from 8 to 11 are the sequence ladders of unrelated DNA. (**b**) RNase protection assay with probe P2. The lanes are as in (**a**), except that lane 7 represents adrenal gland.

reactivity in human brain and several peripheral tissues [33]. DBI is also expressed in almost all rat tissues studied so far [34,35].

In previous studies the expression of human DBI transcripts has been investigated by Northern blot hybridization. Size heterogeneity in 5′ and 3′ untranslated regions of transcripts (variable length of the poly-A tail and most probably multiple transcriptional starting-points as has been reported for rat DBI [31,32]) may prevent the separation of these different transcripts

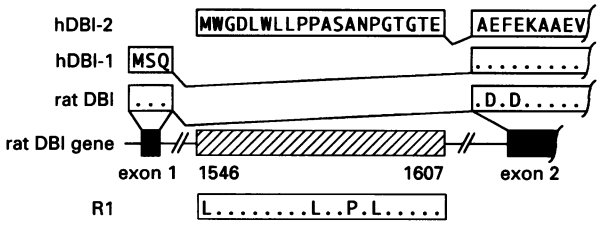


**Figure 4 Alignment of the partial amino acid sequences of the two different human and the rat DBI polypeptides**

Filled boxes represent coding exons 1 and 2 in the rat DBI gene. The striped box indicates the location of a highly homologous nucleotide sequence in the first intron of the rat DBI gene as compared with the cDNA sequence encoding the hDBI-2 N-terminus. The computer translated amino acid sequence of the respective region is designated as R1 and was translated from a nucleotide sequence with accession number Z21846. Deletion of a T nucleotide at position 1585 was introduced into the first intron of the rat DBI gene prior to translation to obtain optimal protein sequence alignment. hDBI-1 and hDBI-2 represent a protein sequence translated from [25] and [1] respectively. Identical amino acids are indicated by dots. The numbers correspond to the numbering of the nucleotide sequence with the accession number Z21846.

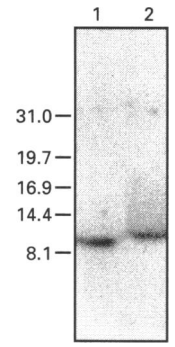


**Figure 5 Transcription coupled translation *in vitro* of hDBI-1 (lane 1) and hDBI-2 (lane 2)**

The numbers indicate the size of the molecular mass markers in kDa.

and cause DBI messages to be detected as a single broad band of 0.6–0.8 kb on Northern blots [1,25]. We have shown here that a DBI transcript capable of encoding a protein of 104 amino acids (corresponding to hDBI-2 cDNA) is expressed in all the human tissues and cell lines studied.

Several studies suggest that these two different DBI transcripts are most likely transcribed from one single gene. First, chromosomal localization experiments of the DBI gene have shown that there is a single functional gene and several pseudogenes in the human genome [36]. Secondly, an extensive nucleotide sequence identity extends throughout most of the coding region and the 3′ untranslated region of DBI transcripts. Thirdly, a highly homologous region exists in the first intron of the rat DBI gene compared with the sequence encoding the N-terminal part of the hDBI-2 cDNA [31; M. Kolmer and H. Alho, unpublished work]. However it has been shown by RNase protection assay that in rat a sequence homologous to the first coding exon of the hDBI-2 is excluded from mature DBI mRNA [31]. Partial protein sequences translated from two different DBI cDNA clones and homologous regions of rat DBI gene are presented in Figure 4.

Different DBI transcripts arise due to alternative usage of the first coding exon. Our data clearly show that the hDBI-1 transcript represents the majority of DBI transcripts. As the human DBI gene remains to be cloned and its promoter(s) to be characterized one can only hypothesize what the mechanisms are that cause such a dramatic difference between two transcript species. One possible explanation is that there are two promoters with different strengths. The other possibility is that the 5′ splice following the alternatively spliced first coding exon of hDBI-1 is favoured over the corresponding hDBI-2 site.

We also studied the translatability of the DBI transcripts *in vitro*. As was revealed by transcription-coupled translation experiments, both DBI transcripts were translated into full-length proteins of the expected sizes and no differences were detected in translation efficiencies from different cDNA clones (Figure 5). However, possible differences in translational efficiencies *in vivo*, due to different 5′ untranslated sequences, could not be excluded.

In conclusion, we have shown that in the human, DBI transcripts are expressed in all the tissues and cell lines studied. We have also established here for the first time the presence of another DBI transcript capable of encoding the 104 amino acid protein (in addition to the DBI transcript encoding an 86 amino acid protein) in humans. Our data clearly show that the hDBI-

1 transcript represents the majority of the DBI transcript pool. It should be noted that all the studies so far attributing different biological functions to the DBI have been carried out with the 86 amino acid form of the protein. The possible functional importance and properties, such as binding of acyl-CoA and/or other functions of the putative 104 amino acid form of DBI, remain to be investigated.

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