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Cloning and tissue-specific functional characterization of the promoter of the rat diazepam binding inhibitor, a peptide with multiple biological actions

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ABSTRACT **Diazepam binding inhibitor (DBI) is a 10-kDa polypeptide that regulates mitochondrial steroidogenesis, glucose-induced insulin secretion, metabolism of acyl-CoA esters, and the action of γ-aminobutyrate on $GABA_A$ receptors. To investigate the regulation of DBI gene expression, three positive clones were isolated from a rat genomic library. One of them contained a DBI genomic DNA fragment encompassing 4 kb of the 5′ untranslated region, the first two exons, and part of the second intron of the DBI gene. Two other overlapping clones contained a processed DBI pseudogene. Several transcription initiation sites were detected by RNase protection and primer extension assays. Different tissues exhibited clear differences in the efficiencies of transcription startpoint usage. Transient expression experiments using DNA fragments of different length from the 5′ untranslated region of the DBI gene showed that basal promoter activity required 146 bp of the proximal DBI sequence, whereas full activation was achieved with 423 bp of the 5′ untranslated region. DNase I protection experiments with liver nuclear proteins demonstrated three protected regions at nt −387 to −333, −295 to −271, and −176 to −139 relative to the ATG initiation codon; in other tissues the pattern of protection was different. In gel shift assays the most proximal region (−176 to −139) was found to bind several general transcription factors as well as cell type-restricted nuclear proteins which may be related to specific regulatory patterns in different tissues. Thus, the DBI gene possesses some features of a housekeeping gene but also includes a variable regulation which appears to change with the function that it subserves in different cell types.**

Diazepam binding inhibitor (DBI) is a 10-kDa polypeptide that was first purified and partially sequenced from rat brain (1) by monitoring its ability to displace diazepam from brain synaptic membrane recognition sites where this anxiolytic ligand binds with high affinity to type A γ-aminobutyrate receptors and mitochondrial benzodiazepine receptors. The complete amino acid sequence of DBI was deduced from clones derived from rat (2) and human (3) brain cDNA libraries. The amino acid sequences for DBI have been obtained for rat (1, 4), human (3), duck (5), pig (6), and cow (5). cDNAs have been cloned for human (3, 7), rat (2), mouse (8), and cow (7, 9). The DBI cloned from human brain has a longer amino acid sequence (103 aa) than that reported for other species, suggesting that in humans a difference in DBI structure may arise from alternative splicing (3).

More recently DBI has been purified from several peripheral organs (reviewed in ref. 10), and DBI-like immunoreactivity and DBI mRNA were detected in several tissues (11, 12). In rat brain DBI is located in selected neuronal and glial cell populations (such as Bergmann glia) (13). In adrenal gland DBI is expressed in cortical cells, whereas the chromaffin cells are immunonegative (14). The participation of DBI and of its processing product DBI-(33–50) (octadecaneuropeptide, ODN) in the regulation of insulin secretion (6, 15), the role of DBI-(17–50) (triakontatetraneuropeptide, TTN) (16, 17) and des(Gly-Ile)-endozepine or des(Gly-Ile)-DBI in the regulation of steroidogenesis (18), of acyl-CoA ester metabolism (4) and the action of DBI as a negative allosteric modulator of the function of type A γ-aminobutyrate receptors have been established (10).

Chromosomal localization experiments using *in situ* hybridization techniques in human cells (19), as well as Southern blot hybridization of rat (2), mouse (8), and human (3) DNA, have demonstrated that DBI is encoded by a multigene family. The gene (*ACB*) encoding the yeast DBI homolog [DBI/acyl-CoA-binding protein (ACBP)] has been characterized (20), and the rat genomic sequence of the DBI/ACBP gene and four processed pseudogenes has been reported (21). Based on its ubiquity and a marked structural conservation in phylogenesis, DBI was classified as a housekeeping gene (21). In rat this inference was also supported by analysis of the 5′ untranslated region of the DBI gene, which shows features typical for housekeeping genes (lack of TATA and CCAAT boxes) (21). However, a differential, cell type-specific expression (11, 12) and the presence of two different DBI forms in human cells (3, 9) strongly argue in favor of tissue-restricted regulation of DBI transcription. To elucidate the mechanisms of this regulation, we have cloned and characterized the rat DBI promoter and have identified some of the proteins bound to the cis regulatory elements of the rat DBI gene. We also report the cloning of a processed DBI pseudogene that differs from those previously described (21).§

MATERIALS AND METHODS

DBI Gene and Pseudogene. Two rat genomic libraries in bacteriophage λ Charon 4A (Clontech) and GEM-11 (Promega) were screened for DBI-specific sequences with α^{32}P-labeled probe generated by PCR using rat DBI cDNA (2) as template and 5′-CGCTCTAGAGCCAGTATGTCTCAGGCTGA-3′ and 5′-GCGAAGCTTAGGCATTATGTCCTCACAGG-3′ as oligodeoxynucleotide primers. Positive clones were further characterized by Southern hybridization. Appropriate restriction fragments were subcloned into pBluescript KS(−) (Stratagene). For determination of the nucleotide sequence, exonuclease III deletion libraries were generated by a nested-deletion kit (Pharmacia). The nucleotide sequence of

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Abbreviations: DBI, diazepam binding inhibitor; ACBP, acyl-CoA-binding protein; GRE, glucocorticoid-responsive element.

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§The sequences reported in this paper have been deposited in the GenBank database [accession nos. Z21846 (functional DBI gene) and X71066 (pseudogene)].

the rat DBI gene was determined by the chain-termination method (22). Sequencing reactions were carried out with an AutoRead sequencing kit (Pharmacia), and the reaction products were analyzed with an A.L.F. automated DNA sequencer (Pharmacia). DNA sequence data were analyzed by the Genetics Computer Group software package (23). Molecular cloning procedures were carried out essentially as described (24). Radioactive compounds were from Amersham.

RNA Isolation and RNase Protection Assay. RNAs from rat tissues were prepared as described (25). For determination of the mRNA start sites, a genomic fragment covering 145 bp of 5′ untranslated region, exon 1, and 110 bp of intron 1 (nt −146 to +119 in Fig. 1) was cloned into pBluescript KS(−). cRNA probes were synthesized using T7 and SP6 RNA polymerases (Promega) and [α-^{32}P]CTP on linearized plasmid templates. RNase protection assays were carried out with the RPA II kit (Ambion). RNA quality was assessed by RNase protection assay using rat cyclophillin cDNA (nt 411–705) cloned into pGEM-1 as a probe (26).

DBI Promoter–Luciferase Constructs, Cell Culture, and Transfection. All DBI promoter fragments were cut by *Kpn* I/*Nhe* I digestion from various clones of the exonuclease III deletion library (the *Kpn* I site is from the pBluescript KS(−) vector and the *Nhe* I site is located at position −84 in Fig. 1) and cloned into *Kpn* I/*Nhe* I-digested pGL-Basic luciferase reporter plasmid (Promega). The DBI promoter–luciferase constructs were pGLbDB122 (containing nt −1061 to −84) pGLbDBI31 (nt −423 to −84), and pGLbDBI41 (nt −146 to −84).

C6 rat glioma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, gentamicin, and 2 mM glutamine. The DNAs used for transfections were purified with the Magic maxipreps system (Promega). DNA transfections were performed with a calcium phosphate mammalian cell transfection kit (5 Prime–3 Prime, Inc.) according to the manufacturer's instructions. Promoter–luciferase construct (10 μg) and the reference plasmid pON260 (2 μg), expressing β-galactosidase (27), were used to transfect C6 cells on 90-mm tissue culture dishes. Sixty hours after transfection, cells were harvested with 10 mM EDTA in phosphate-buffered saline (140 mM NaCl/3 mM KCl/5.4 mM Na_2HPO_4/2 mM KH_2PO_4, pH 7.4) and washed once with phosphate-buffered saline. Half of the cells were used for protein assay (28) and β-galactosidase assay (24). The other half was used for the luciferase assay, which was performed with a kit (Promega) according to the manufacturer's instructions. Luciferase activities were expressed as relative units compared to simian virus 40 early promoter-directed luciferase activity pGL-Control plasmid (Promega)], after normalization to the protein content and β-galactosidase activity.

DNase I Footprints. A restriction fragment (200 ng; nt −423 to −84 in Fig. 1) was end-labeled with T4 polynucleotide kinase (Promega) and [τ-^{32}P]ATP and used as a probe in a DNase I protection assay as suggested by the kit manufacturer (GIBCO/BRL), with the following modification. Total nuclear protein extract (5 μg) or 1 footprint unit of recombinant Sp1 protein (Promega) was mixed with 20,000 cpm of the probe and incubated for 30 min at room temperature. DNase I (5 ng) was then added, and 1 min later the reaction was stopped and the reaction products were analyzed by electrophoresis in a denaturing polyacrylamide gel. The same fragment was subjected to a sequencing reaction by the chemical modification method (29) to localize the precise region(s) involved in protein binding.

Nuclear Protein Extract and Electrophoretic Mobility Shift. Nuclear proteins were extracted from various rat tissues (30) and pancreatic islets (31). The buffer and running conditions for the mobility-shift assay were as reported (32). One nanogram of gel-purified double-stranded synthetic oligodeoxynucleotide corresponding to DBI promoter nt −136 to −176 or −293 to −321 was labeled with [τ-^{32}P]ATP and T4 polynucleotide kinase and incubated for 30 min at room temperature with 5 μg of nuclear protein extract. Competition experiments were performed by preincubation of the nuclear extract with a 100-fold molar excess of the unlabeled oligonucleotide for 10 min at room temperature before addition of the labeled oligonucleotide probe. Oligonucleotides (all from Promega), containing consensus sites for known transcription factors were also included in the competition studies: AP1, AP2, AP3, CRE (cAMP-responsive element), GRE (glucocorticoid-responsive element), and Sp1. A synthetic oligonucleotide corresponding to the rat DBI promoter region between −153 and −123 was used as nonspecific competitor.

RESULTS AND DISCUSSION

Isolation and Characterization of DBI Genomic Clones. Two rat genomic libraries were screened for the presence of DBI-specific sequences by using the rat DBI cDNA as a probe. Three different genomic clones were identified and further characterized by Southern hybridization. One of these clones, λ-DBI1, covers about 4 kb of the 5′ untranslated region, exon 1 (coding for the first 3 aa of DBI), intron 1 (968 bp), exon 2 (118 bp), and part of intron 2 (845 bp), as revealed by Southern hybridization analysis and nucleotide sequencing of the 3-kb *Hin*dIII–*Eco*RI restriction fragment of λ-DBI1. The rest of the gene was excluded from the λ-DBI1 clone.

A computer analysis of the 5′ untranslated sequence of the DBI gene revealed no canonical TATA and CCAAT boxes. Consensus sequences for binding of several other known transcription factors, including AP2, Sp1, ETF, Y-box-binding protein, and CTF/NF1 (CAAT-binding transcription factor/nuclear factor 1), as well as two consensus GREs, were detected. Part of the DBI promoter sequence and the positions of selected putative transcription-factor binding sites, some of which are located in regions protected from DNase I digestion, are shown in Fig. 1.

Two overlapping clones, P3 and P28, contained a DBI pseudogene (designated DB1ψ1), including all the features typical for processed pseudogenes: lack of introns, multiple deletions and insertions compared with functional DBI gene, and a short poly(A) tail (data not shown). The DB1ψ1 sequence homology is 94% when compared to the rat DBI

FIG. 1. Partial nucleotide sequence of the 3-kb *Hin*dIII–*Eco*RI genomic fragment, representing an area of the 5′ untranslated region, exon 1, and part of intron 1 of the rat DBI gene. The first nucleotide of the initiation codon is +1. The coding part of exon 1 is shown in uppercase letters. The putative binding sites for the transcription factor ETF are underlined. Consensus GREs and binding sites for various transcription factors are indicated. Putative splicing acceptor sites, able to give rise to alternatively spliced 5′ untranslated regions, are doubly underlined. Arrows indicate transcription startpoints confirmed by two methods: primer extension and RNase protection.

cDNA (2). Comparison of the DB1ψ1 sequence with four DBI/ACBP pseudogenes (accession nos. Z11986–Z11989) described recently (21) revealed homologies of 84%, 90%, 82%, and 84%, respectively, in the aligned region. Thus, DB1ψ1 is a member of the DBI multigene family.

Mapping of Transcription Initiation Sites. Multiple transcription startpoints (cap sites) for DBI mRNA were detected in liver, kidney, adrenal gland, and C6 cells by RNase protection as well as primer extension techniques. RNase protection assay revealed multiple protected fragments, with lengths of 72–185 nt (Fig. 2). The protected fragments of length 72, 77, and 78 nt obtained with RNAs from liver, kidney, and C6 cells correspond to cap sites at positions −60, −64, and −65 and are in good agreement with primer extension results (data not shown) and with the data published earlier (21). The efficiency of cap-site usage differed between adrenal gland and other tissues: cap sites at positions −60, −64 and −65 were clearly not favored in adrenal tissue. mRNAs initiating at position −132, detected by primer extension, should yield a protected fragment of 141 nt in the RNase protection assay. However, no protected fragment of this size was detected. Most probably this primer extension product represents a message with an alternatively spliced 5′ untranslated region. Neither the cap sites at −204 (adrenal gland and C6) nor the cap site at −150 (kidney), detected by primer extension, were detected by RNase protection assay, due to their location upstream from the 5′ end of the cRNA probe used, and the origin of those extension products remains to be investigated. In C6 cells there are also two protected fragments which are longer than the longest possible protected fragment (from the 5′ end of the probe to the 3′ end of the exon 1). These fragments either arise from a pre-mRNA population or represent an mRNA population in which the first intron was incompletely spliced. Such a phenomenon has been shown for prostaglandin synthase (33), cytochrome P450IIB6 (34), and eukaryotic translation initiation factor eIF-2a (35). In addition to major cap sites, multiple minor cap sites located between −65 and −118 were detected by RNase protection assay. Multiple transcriptional startpoints are typical for genes with TATA-less promoters (for review see ref. 36). The differences in the efficiency of transcription initiation-site usage might be linked to the various functions that the DBI protein modulates in different tissues (for example, the regulation of steroid synthesis in adrenal gland versus the binding of fatty acid esters of binding to the acyl-CoA in liver). Further, the presence of mRNAs with different 5′ untranslated regions suggests that the expression of the DBI gene may be regulated also at the translational level (37).

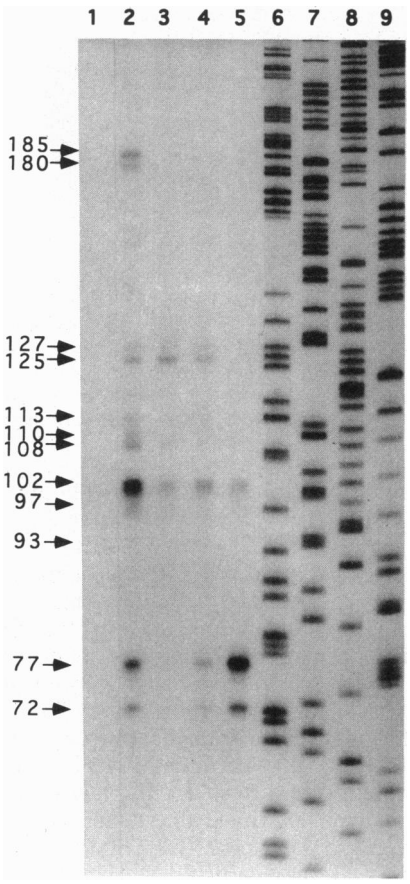

FIG. 2. RNase protection mapping of the 5′ ends of the DBI transcripts. Lane 1, 10 μg of yeast RNA (control); lanes 2–5, 20 μg of total RNA from C6 cell line, adrenal, kidney, and liver, respectively; lanes 6–9, sequence ladders of unrelated DNA. Numbers indicate length (in nucleotides) of the protected fragments.

Promoter Activity. Various fragments located upstream from exon 1 were isolated from the DBI genomic deletion library and fused to the luciferase reporter gene. Luciferase activities were analyzed by transient expression experiments in the C6 glioma cell line. All three constructs promoted luciferase activity (Fig. 3). The construct with 146 bp of upstream sequence conferred 5% activity, whereas the construct with 423 bp of upstream sequence yielded 78% (15-fold compared with the −146 construct). Upstream sequence from −423 to −1061 failed to increase the luciferase activity further (56%). Promoterless pGL-Basic luciferase vector conferred only 0.3% of control activity.

These results suggest that the region including 146 bp (upstream from the ATG initiation codon) may regulate basal promoter activity. Extension of the untranslated part to −423 enhanced luciferase activity about 15-fold, suggesting that most of the binding sites for the important transcription factors are located between nt −146 and −423. The functional responses of the DBI promoter to different treatments and in unrelated cell lines and primary cell cultures have been investigated. In C6-2B glioma, treatment with 1 μM isoproterenol stimulated the DBI promoter activity by about 3-fold, whereas this effect was not seen in the HITT-15 pancreatic beta-cell line, which also expresses β-adrenergic receptor (L.P. and E.C. unpublished data). These data suggest that the regulation of the DBI promoter differs among the cells that use DBI to mediate diverse physiological responses.

DNase I Footprint and Electrophoretic Mobility-Shift Assays. To localize the DBI promoter consensus sequences for binding of transcription-regulatory proteins, the initial analysis was restricted to tissues where DBI functions have been well documented and where the DBI/ACBP expression

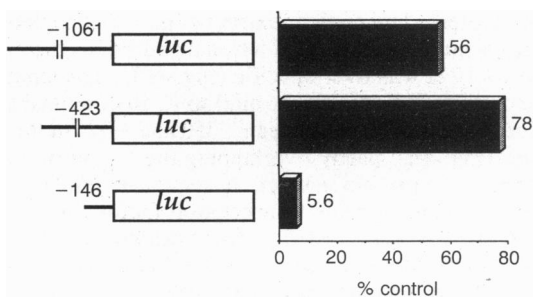

FIG. 3. Functional analysis of the rat DBI promoter by transient transfection in the C6 cell line. Genomic DNA fragments upstream from the translation initiation codon were fused to luciferase reporter gene (*luc*). Results are expressed as % of luciferase activities directed by a simian virus 40 promoter-driven control plasmid (pGL-Control). Numbers represent mean values of three experiments performed in triplicate.

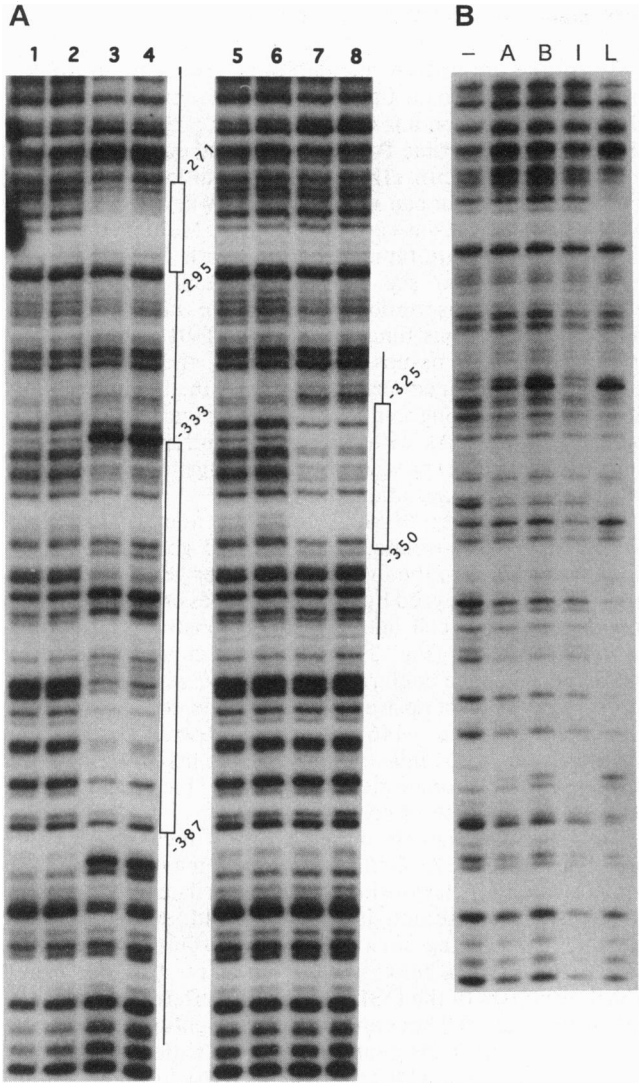

FIG. 4. DNase I footprint analysis of the DBI promoter region from nt −255 to nt −413. (*A*) Lanes: 1, 2, 5, and 6, naked control digested DNA; 3 and 4, samples incubated with liver nuclear protein extract; 7 and 8, samples incubated with pure recombinant Sp1. Boxes indicate protected regions and numbers correspond to nucleotide position as depicted in Fig. 1. (*B*) Lanes: −, no extract (control); A, adrenal; B, brain; I, pancreatic islets; L, liver.

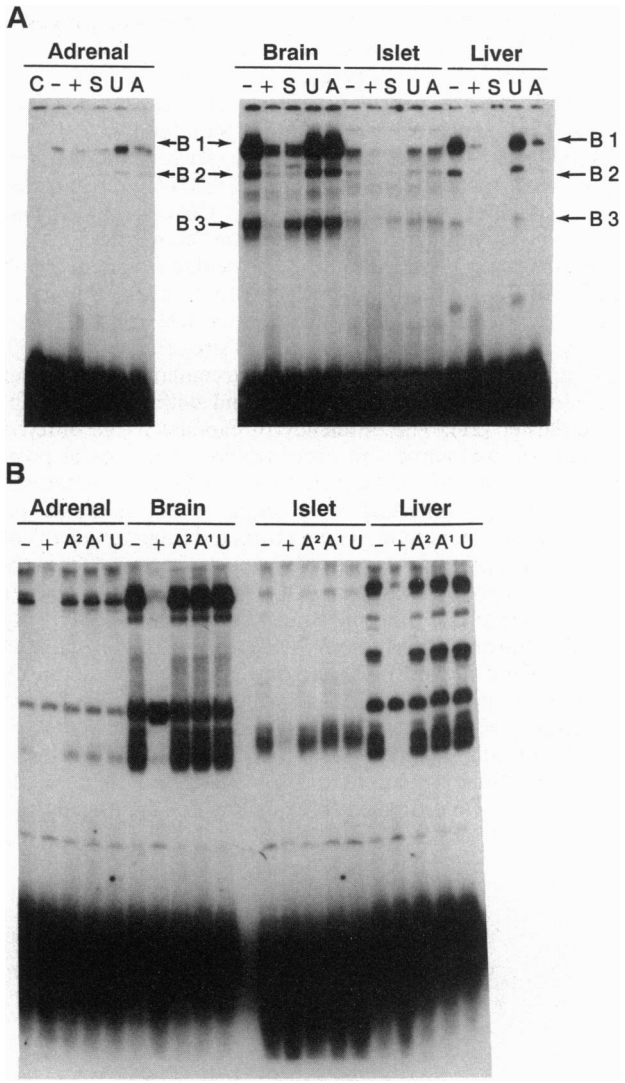

FIG. 5. Mobility-shift analysis of the DBI promoter region between nt −139 to −176 (*A*) or −293 and −321 (*B*) with nuclear extract from adrenal gland, brain, pancreatic islets, and liver. Labels indicate no competition (−) or 100-fold molar competition (+) by oligonucleotide identical with the probe or by Sp1 (S), AP1 (A or A^1), AP2 (A^2), or unrelated (U) oligonucleotide. C, no nuclear extract (control lane). Arrows B1, B2 and B3 indicate the three complexes discussed in the text.

levels are relatively high (4). Footprint experiments with liver nuclear protein extracts revealed two protected regions, nt −333 to −387 (Fig. 4*A*) and −295 to −271; whereas the first was also protected by nuclear extract of pancreatic islets and partially by nuclear extracts of adrenal and brain, the region at −295 to −271 was liver-specific (Fig. 4*B*), suggesting that a tissue-restricted factor might bind to it. Recombinant Sp1 also protected the region between −350 and −325 in the DBI promoter (Fig. 4*A*), partly overlapping the region protected by liver nuclear protein extract. A sequence similar to the consensus binding site for transcription factor Sp1 was located at nt −339 and −333 (upper strand, CACGCCC); however, the fact that Sp1 can protect some DNA sequences not protected by the liver nuclear extract argues that in liver, Sp1 could be bound to this particular region. A region further downstream, at −177 to −147, was also protected by recombinant Sp1 protein (data not shown). The latter corresponded to an area containing consensus sites for Sp1 and CAAT-binding factors as revealed by a computer-assisted analysis (Fig. 1). To investigate whether or not the promoter for the rat DBI gene could recruit divergent protein complexes in different tissues, a region between nt −136 and −176 was challenged with nuclear proteins extracted from adrenals, brain, pancreatic islets of Langerhans, and liver. In each of the above tissues a dissimilar yet very specific function has been extensively described (10). Mobility-shift assay showed that every extract included complexes with a different migration profile. Although the total amounts of protein were comparable and control experiments showed no signs of protein degradation (data not shown), the relative abundance of the shifted bands differed dramatically (e.g., islets and liver in Fig. 5*A*). The gel shift experiments revealed three possible regulatory protein complexes (B1, B2, and B3 in Fig. 5*A*). Competition studies confirmed that unlabeled Sp1 oligonucleotide was able to complex with B1 and B2. Formation of labeled complex B3 was clearly blocked by the unlabeled Sp1 oligonucleotide only in liver nuclear extract, suggesting that the Sp1 protein could be a major component of the proteins binding at −177 to −136. The Sp1 consensus site in the DBI promoter partially overlaps with that for ETF, which is believed to be a specific transactivator for TATA-less genes

(38). Another level of such cell-restricted regulation is shown by the AP1 consensus (39), which was able to compete with the same Sp1-bound complexes only in liver nuclear extract (Fig. 5*A*). Finally, in brain nuclear extract, only the oligonucleotide with the same sequence as the probe competed with the probe for formation of the complex B3, indicating that in some tissues DBI gene could also be regulated by other specific nuclear factors. These findings allow us to infer that two orders of control regulate the DBI gene, one probably common to all tissues and related to its housekeeping structure and another, which presumably expresses a transcriptional control related to each of the multiple biological actions of DBI and therefore might be regulated by cell-specific signals.

Since the transient expression experiments showed that the DBI promoter activity was greatly reduced when sequence upstream from nt −146 was removed (Fig. 3), and since a cluster of putative AP2 sites was present between −321 and −295, an oligonucleotide spanning that region was synthesized. Once again the pattern of bound complexes differed significantly among the tissues tested, confirming the tissue-restricted regulation of the DBI promoter (Fig. 5*B*).

Conclusions. We have cloned and characterized part of the rat DBI gene covering 4 kb of the 5′ untranslated region, the first two exons, intron 1, and part of intron 2. The putative promoter region of the DBI gene was functionally characterized by transient expression studies in the C6 rat glioma cell line. It was shown that the 423-bp sequence upstream from the translation initiator codon was responsible for the complete activity of the DBI promoter in C6 cells. Mapping of the 5′ ends of DBI mRNA revealed multiple transcriptional startpoints in all tissues studied. Remarkable differences were found in the transcriptional startpoint usage among different tissues. DNase I protection assay using a nuclear protein extract from rat liver with the −423 promoter fragment of the DBI gene revealed several protected regions. Two of these regions were also protected with recombinant Sp1 protein. Different efficiencies in mRNA cap-site usage by different tissues, as well as detection of tissue-specific DNA–protein complexes (footprints and gel shift assay with several other tissues and cell lines), substantiate the tissue segregation of different protein complexes. These findings strongly suggest the possibility of complicated regulatory patterns of DBI gene expression in different cell types according to the specific functional tasks. Further, the existence of DBI mRNAs with different 5′ untranslated regions suggests that the DBI gene might be also regulated at the translational level. Accordingly, preliminary evidence of alternative splicing observed in the rat DBI mRNA 5′ untranslated region (M.K. and H.A., unpublished data) supports the view that the DBI gene, thought to be a housekeeping gene (21), is subject to additional regulatory mechanisms probably related to functional tissue specificities subserving the multiple biological activities of this peptide present in different tissues (4, 10).

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