Rat hepatic glutaminase: identification of the full coding sequence and characterization of a functional promoter

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Glutamine catabolism in mammalian liver is catalysed by a unique isoenzyme of phosphate-activated glutaminase. The full coding and 5′ untranslated sequence for rat hepatic glutaminase was isolated by screening λ ZAP cDNA libraries and a Charon 4a rat genomic library. The sequence produces a mRNA 2225 nt in length, encoding a polypeptide of 535 amino acid residues with a calculated molecular mass of 59.2 kDa. The deduced amino acid sequence of rat liver glutaminase shows 86% similarity to that of rat kidney glutaminase and 65% similarity to a putative glutaminase from *Caenorhabditis elegans*. A genomic clone to rat liver glutaminase was isolated that contains 3.5 kb of the gene and 7.5 kb of the 5′ flanking region. The 1 kb immediately upstream of the hepatic glutaminase gene (from −1022 to +48) showed functional promoter activity in HepG2 hepatoma cells. This promoter region did not respond to treatment with cAMP, but was highly responsive (10-fold stimulation) to the synthetic glucocorticoid dexamethasone. Subsequent 5′ deletion analysis indicated that the promoter region between −103 and +48 was sufficient for basal promoter activity. This region does not contain an identifiable TATA element, indicating that transcription of the glutaminase gene is driven by a TATA-less promoter. The region responsive to glucocorticoids was mapped to −252 to −103 relative to the transcription start site.

INTRODUCTION

Glutamine is a major substrate for hepatic gluconeogenesis and urea synthesis [1–4]. Within the hepatocyte, the first enzyme of glutamine catabolism is a liver-specific isoenzyme of phosphate-activated glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) [5–7]. Other, non-hepatic, cells that utilize large amounts of glutamine possess a different glutaminase isoenzyme, known as kidney-type. The two isoenzymes show different kinetic properties and are the products of separate genes [5,7].

Liver glutaminase is composed of 58 kDa monomers, is located in the mitochondrial matrix, possibly in loose association with the inner membrane, is characterized by a relatively high $K_{0.5}$ for glutamine, a low $K_{0.5}$ for phosphate, and requires ammonia for activity [2,7,8]. Kidney-type glutaminase, in contrast, is a tetramer of three 66 kDa monomers plus one 68 kDa subunit, all of which are produced from a common primary transcript [9]. This isoenzyme is also found inside the mitochondrial matrix, but shows a relatively low $K_{0.5}$ for glutamine, a high $K_{0.5}$ for phosphate, and is not activated by ammonia but is subject to inhibition by relatively low concentrations of glutamate [3,7]. In the rat, hepatic glutaminase is expressed only after birth [10], and expression is limited to a small periportal population of hepatocytes [11,12].

Hepatic glutaminase is subject to long-term regulation, with the activity being increased 4-fold in uncontrolled streptozotocin diabetes [13], and 2-fold after 48 h of starvation or 10 days of feeding on a high-protein diet [14]. In contrast, the feeding of low-protein diets results in lower hepatic glutaminase activity [14]. These changes are due to changes in the amount of glutaminase protein with no evidence of changes in specific activity [15]. In turn, all changes in hepatic glutaminase activity observed so far are accompanied by changes of equal magnitude in the abundance of glutaminase mRNA and in the rate of transcription of the glutaminase gene in isolated nuclei [10,14,16]. Thus the principal mechanisms responsible for the long-term regulation of hepatic glutaminase are changes in the rate of gene transcription. In contrast, the long-term regulation of renal glutaminase is predominantly via changes in mRNA stability [7,17].

The pattern of expression and long-term regulation of hepatic glutaminase is similar to that observed for the enzymes of the urea cycle and the key gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) [5]. It is interesting to note that the expression of hepatic glutaminase has been observed only in cells expressing the first enzyme of the urea cycle, carbamoyl phosphate synthetase I, and it has been claimed that the amido nitrogen from glutaminase is utilized preferentially by carbamoyl phosphate synthetase I, leading to urea synthesis [18,19]. So far there is little information about the agents responsible for the regulation of hepatic glutaminase gene transcription, but comparison with the regulation of the genes encoding the urea-cycle enzymes and PEPCK allows some predictions. Increased expression would be expected in response to glucagon (acting via cAMP) and glucocorticoids, and the possibility of decreased expression in response to insulin [5].

Previously in this laboratory a 1 kb cDNA to hepatic glutaminase has been isolated and sequenced [10]. Here we report the

Abbreviations used: CAT, chloramphenicol acetyltransferase; CRE, cAMP response element; GRE, glucocorticoid response element; PEPCK, phosphoenolpyruvate carboxykinase; RACE, rapid amplification of cDNA ends; AMV, avian myeloblastosis virus.

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The nucleotide sequence results reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers J05499 (full coding cDNA sequence) and L76175 (promoter sequence).

identification of the full coding sequence together with characterization of the promoter to the hepatic glutaminase gene. The results demonstrate that hepatic glutaminase expression is driven by a promoter lacking TATA that is responsive to regulation by glucocorticoids.

EXPERIMENTAL

Materials

Two rat liver λ ZAP cDNA libraries were screened. One was oligo(dT)-primed and prepared in the laboratory of Dr. Wouter Lamers (University of Amsterdam, Amsterdam, The Netherlands) against liver mRNA from diabetic rats that had received injections of glucocorticoids to increase the abundance of mRNA species to the enzymes of the urea cycle. The second library was an oligo(dT)- and random-primed rat liver λ ZAP cDNA library purchased from Stratagene (La Jolla, CA, U.S.A.). A rat liver genomic Charon 4a library was purchased from Clontech (Palo Alto, CA, U.S.A.). Expression vectors for the protein kinase A catalytic subunit (RSV-PKA) and the glucocorticoid receptor (CDM8-GR) were gifts to U.J. from J. F. Habener and D. D. Moore (Massachusetts General Hospital, Boston, MA, U.S.A.). All other materials were from standard suppliers as previously described [10,14] or as indicated.

Library screening and subcloning

The cDNA libraries were screened [20] by infection of *Escherichia coli* BB4 cells, and plaque lifts were hybridized with ^{32}P-labelled 1 kb hepatic glutaminase cDNA probes [10]. Phages from positive plaques were rescreened twice; plasmids (pBluescript, Stratagene) were prepared by the λ ZAP *in vivo* excision protocol. Plasmid DNA was isolated with a Magic mini prep kit (Promega, Madison, WI, U.S.A.), followed by digestion with *Eco*RI and 1% (w/v) agarose-gel electrophoresis to determine insert size. The rat liver genomic Charon 4a library was screened with the 5′ end (660 bp) of the 2 kb cDNA (see the Results section). Positive plaques were rescreened and inserts were DNA subcloned into the *Eco*R1 site of the phagemid Bluescript SKII (pBS).

DNA sequencing and analysis

DNA was sequenced by the Sanger dideoxynucleotide chain-termination method [21] with a Sequenase 2.0 sequencing kit (USB, Cleveland, OH, U.S.A.), T3 or T7 universal primers (Stratagene), ^{35}S-labelled dATP (NEN–Dupont), and double-stranded plasmids (deletion set, see below). Problems associated with GC compressions or missing AT bands were resolved by using a ddITP reaction run in parallel with the dGTP mix. DNA was sequenced at least three times in both directions. Unidirectional nested deletion subclones were generated with Exonuclease III and S1 nuclease digestion employing the Erase-a-Base system (Promega). The DNA sequence was analysed with version 7 of the University of Wisconsin Genetics Computer Group software package.

Primer extension analysis

Primer extension analysis was performed to determine the location of the 5′ end of glutaminase mRNA by using a Primer extension analysis kit (Promega). Oligonucleotides (DNA International) were ^{32}P end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Rat liver total RNA (30 μg) was mixed with 1 μl (100 fmol) of ^{32}P-labelled oligonucleotides and 5 μl of avian myeloblastosis virus (AMV) reverse transcriptase 2X buffer to a final volume of 11 μl. The mixture was heated at 58 °C for 20 min and then placed at room temperature to cool for 10 min to anneal the primer and RNA. AMV reverse transcriptase 2X buffer (5 μl), 1.4 μl of 40 mM sodium pyrophosphate, 1.6 μl of water and 1 unit (= 1 nmol of dTTP/10 min at 37 °C) of reverse transcriptase were added to the annealed primer/RNA to start extension. After incubation at 42 °C for 1 h, the reaction was stopped by adding loading dye (98% formamide, 10 mM EDTA, 0.1% Xylene Cyanol and 0.1% Bromophenol Blue). Primer extension products were analysed on denaturing polyacrylamide gels [8% (w/v) gel, 7 M urea].

Construction of chimaeric DNA

Glutaminase promoters were inserted immediately upstream of the chloramphenicol acetyltransferase (CAT) reporter gene in the pCAT-Basic vector (Promega). For cloning the smaller promoter sequences [pCAT(−1022/−225)], the plasmid pBS-1.5 containing 5′ flanking sequences of hepatic glutaminase (see the Results section) was subjected to restriction digestion with *Pst*I and *Acc*I. The 800 bp *Acc*I–*Pst*I fragment from the digestion was ligated with *Pst*I–*Acc*I-digested pCAT-Basic vector. For preparation of a construct containing the larger promoter sequences [pCAT(−1022/+48)], a region ranging from −1022 to +48 of the glutaminase gene was amplified by PCR with the pBS-1.5 plasmid as a template. SK universal primers (5′-TCTA-GAACTAGTGGATC-3′) were used as reverse primers. This primer is complementary to *Xba*I–*Spe*I–*Sma*I recognition sequences of the multicloning site in pBS SKII and flanks the *Pst*I site and the 5′ end of the 1.5 kb glutaminase sequence. Forward primers (5′-GCTCTAGACCTGTCATGGGGTGCATC-3′) were designed to contain the 21 bp sequence complementary to nucleotides from +28 to +48 of the glutaminase gene (see Figure 3) and a *Xba*I site at their 5′ ends. The PCR products (1.1 kb) were subjected to restriction digestion with *Pst*I and *Xba*I and were ligated with *Pst*I/*Xba*I-digested pCAT-Basic plasmids by using T4 DNA ligase. To characterize the basal and glucocorticoid responsiveness of the glutaminase promoter, the pCAT(−1022/+48) construct was used to generate a series of 5′ deletion mutants. Deletion constructs were prepared by the Erase-a-Base system described above and were confirmed by dideoxynucleotide sequencing. Recombinant plasmid DNA for transfection was isolated from overnight cultures of HB101 *E. coli* cells by the alkaline lysis method and purified by twice-performed CsCl/ethidium bromide equilibrium ultracentrifugation.

Cell culture and transfection

Human hepatoblastoma cells (HepG2, ATCC HB8065) were maintained in minimum essential medium (Gibco BRL) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL), 1 × non-essential amino acids (Gibco BRL), 100 mM sodium pyruvate, 50 i.u./ml penicillin G sodium and 50 mg/ml streptomycin. Cells were grown at 37 °C in air/CO_2 (19:1), and the medium was renewed every 2 days. HepG2 cells were plated in 35 mm dishes at a density to reach 50% confluency 24 h after plating. On the day of transfection, 50% confluent cells were refed with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 6 mM glutamine, and transfected 2–4 h later. For the study of basal promoter activity, 15 μg of CAT constructs and 3 μg of β-galactosidase expression vector (pRSV-β-gal) as an internal control were mixed with 50 μl of 2.5 M $CaCl_2$ solution in a volume of 500 μl. For the dexamethasone and cAMP experiments, 6 μg of the glucocorticoid-receptor expression

vector (CDM8-GR) or the protein kinase A catalytic subunit expression vector (RSV-PKA) were included in the DNA/$CaCl_2$ mixture. The DNA/$CaCl_2$ solution (500 μl) was added dropwise into 500 μl of 2 × Hepes-buffered saline and left at room temperature for 20 min. The precipitates (total 1 ml) were distributed to triplicate plates (330 μl to each plate) and incubated for 6 h at 37 °C in an air/CO_2 (19:1) incubator. DNA and media were removed from the plates and the cells were treated with 20 % DMSO in PBS for 90 s to increase transfection efficiency. DMSO solution was removed, and the cells were washed twice with 2 ml of PBS. Fresh medium (3 ml) was added to the cells, after which they were cultured for an additional 48 h. The hormonal response of the promoter was initially studied with the pCAT(−1022/+48) construct. After shock with 20 % DMSO, the cells were refed with starvation medium (Dulbecco's modified Eagle's medium containing 0.1 % BSA, 20 mM Hepes, pH 7.4, 6 mM glutamine, 50 i.u./ml penicillin G sodium and 50 mg/ml streptomycin sulphate). Dexamethasone or 8-bromo-cAMP was added to the medium at a final concentration of 75 or 0.1 mM respectively, and the cells were cultured for a further 48 h.

Cells were isolated and freeze–thawed three times. Cell lysates were cooled on ice and then microcentrifuged for 5 min at 4 °C. Part of the supernatant was heat-treated at 60 °C for 10 min to inactivate the potential endogenous deacetylase activity. Chloramphenicol acyltransferase activity was determined by the butyrylation of [^{3}H]chloramphenicol (0.025 mCi/ml) (NEN–Dupont). The reaction was terminated by the addition of mixed xylenes (Aldrich) and the CAT reaction products (butyrylated [^{3}H]chloramphenicol) were counted. CAT activity was normalized to β-galactosidase activity determined as described below. β-galactosidase activity was monitored by measuring the hydrolysis of 4-methylumbelliferyl β-D-galactoside to the fluorescent molecule 4-methylumbelliferone. The intensity of fluorescence was read on a TKO 100 mini fluorometer (excitation at 356 nm and emission at 492 nm) calibrated with a standard solution of 0–200 nM of 4-methylumbelliferone.

RESULTS

Hepatic glutaminase coding sequence

Three positive plaques were identified from the library prepared against diabetic-liver mRNA. One clone gave an insert too small for easy detection, whereas the insert of the second clone showed evidence of contamination with unprocessed nuclear RNA. The third cDNA clone was 2 kb in length and on sequencing was found to contain 1568 nt of coding sequence encoding 522 amino acid residues with a predicted molecular mass of 57.6 kDa. Although this clone was close to the size of the full-length mRNA for hepatic glutaminase (2.2 kb), it did not contain a characteristic initiator methionine translation start codon or mitochondrial targeting sequences. Attempts to clone further 5′ sequences by cDNA library screening or the 5′ rapid amplification of cDNA ends (RACE) system were not successful. Finally, a rat genomic library was screened with the 5′ end of the 2.0 kb cDNA, and four identical 11 kb clones were obtained. An additional 42 nt of 5′ coding sequence and 131 nt of 5′ untranslated sequence for the hepatic glutaminase mRNA were identified in the genomic clones (see below).

The newly identified coding sequence, the 660 bp at the 5′ end of the 2 kb cDNA and oligonucleotides (32 nt in length) derived from nucleotides 151 to 182 of the full coding sequence (i.e. derived from the genomic clones) all hybridized with the same 2.2 kb band of RNA from rat liver, but did not hybridize with kidney RNA (results not shown). Together with the sequence data (see below), these results confirm identity with the 1 kb hepatic glutaminase cDNA previously reported [10].

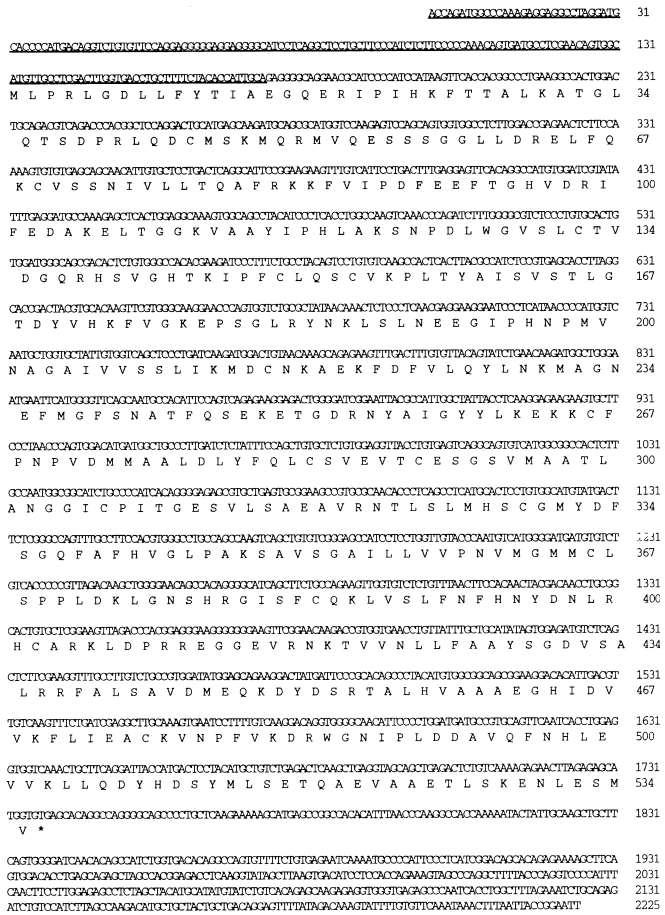

Figure 1 Nucleotide and deduced amino acid sequence of rat hepatic glutaminase cDNA

The nucleotides and amino acid residues (single-letter codes) are numbered on the right. Residue 1 is the initiation methionine and the stop codon is indicated by the asterisk. Sequences derived from the genomic clones are underlined.

Comparison with other glutaminases

The sequence of the 2.0 kb cDNA and the genomic clones is shown as the full-length sequence of hepatic glutaminase mRNA in Figure 1. An open reading frame starts from nt 132 and ends at nt 1739 encoding 535 amino acid residues with a calculated molecular mass of 59 kDa. By comparison with the Kozak consensus sequence [22], an initiator methionine translation start site (ATG) is present in the region derived from the genomic DNA. In addition, the first 16 residues from this putative ATG translation start site are rich in hydrophobic amino acids and show a strong propensity to form an α-helix. When plotted as a helical wheel, these residues form an amphipathic α-helix that closely resembles the helical structure produced by the N-terminal targeting sequences of other mitochondrial proteins.

The 1 kb sequence at the 3′ end of the 2.0 kb cDNA clone is similar to that reported for the original 1 kb clone, with 10 exceptions. The original 1 kb was sequenced commercially as a single piece and not surprisingly the errors locate to the central region of this clone (10). There are four G deletion errors at

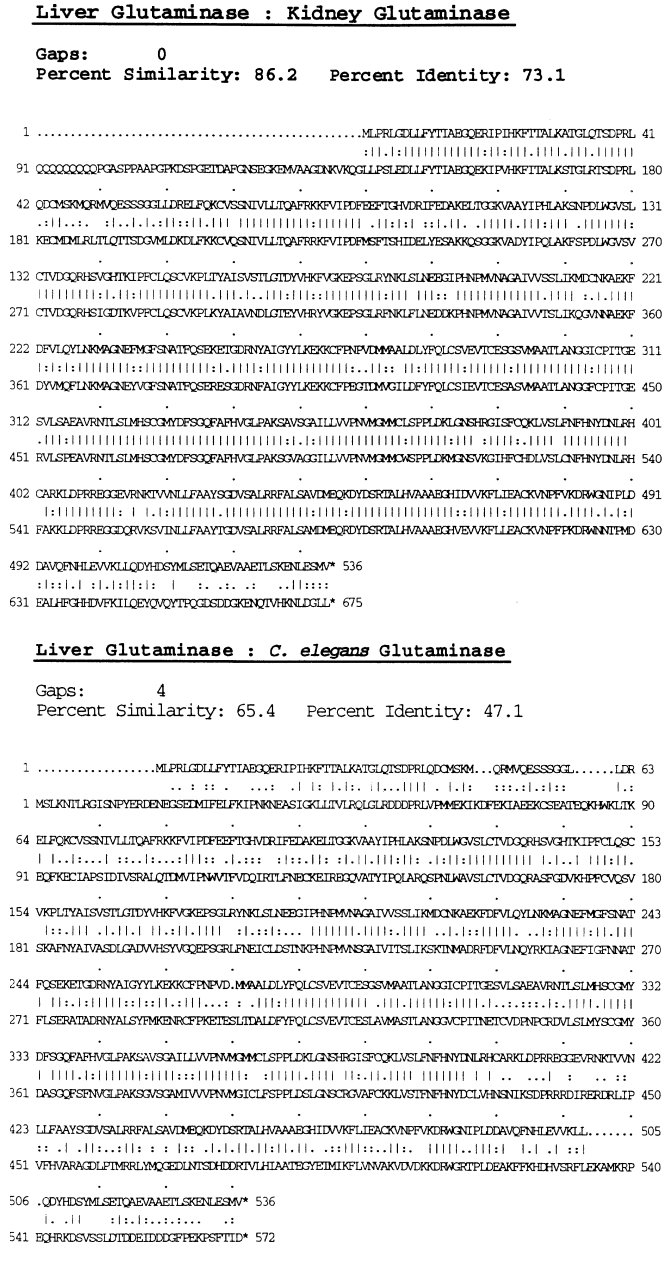

Figure 2 Comparison of the predicted amino acid sequences of rat liver-type and kidney-type glutaminases and the *C. elegans* glutaminase

Upper panel: the sequences for liver glutaminase and kidney-type glutaminase are shown in the upper and lower rows respectively. Lower panel: the sequences for rat hepatic glutaminase and *C. elegans* glutaminase are shown in the upper and lower rows respectively. Identical amino acids are indicated by lines and conservative amino acid substitutions by dots.

positions 1695, 1733, 1750, and 1836, and a C deletion at position 1790. Five substitution errors are also present at locations 1611 (G to C), 1723 (T to G), 1760 (C to T), 1782 (C to G) and 2049 (C to G). The additions of G at positions 1695 and 1733 cause an open reading frame shift, resulting in changes in the encoded amino acid sequence and the position of the stop codon. Therefore the last 14 residues of the newly translated sequences are different from those previously reported; the stop codon is located further 5′ (from nt 1882 to position 1739). Corrections of the nucleotide replacement of C with G at 1611 introduces a valine residue in place of a leucine, but other nucleotide substitutions are located after the stop codon and therefore do not affect the deduced amino acid sequence.

The 2225 nt sequence of hepatic glutaminase was compared with the cDNA sequence of kidney-type glutaminase (4526 nt) [9] with the Bestfit program of the GCG package. The entire coding sequence, with the exception of the last 80 bp of the hepatic glutaminase sequence, showed 67.7% identity to nt 473–2003 of kidney-type glutaminase (results not shown). The deduced amino acid sequence of hepatic glutaminase exhibits a significant similarity to those deduced from the cDNA of kidney type glutaminase and also from a genomic sequence (nt 28531–30944) of *Caenorhabditis elegans* cosmid DH II clone. Kidney-type glutaminase is encoded in 674 residues, encompassing the entire peptide sequence of the 74 kDa primary translation product, which is then processed via a 72 kDa intermediate to generate the 66 and 68 kDa mature proteins. The deduced amino acid sequence of liver glutaminase revealed 86% similarity to residues 140–674 of kidney-type glutaminase (Figure 2, upper panel). The nucleotide sequence of the *C. elegans* cosmid DH II clone were obtained in the Nematode Sequencing Project [23], and coding sequences were predicted by computer analysis, with the program Genefinder. The coding sequence from nt 28531–30944 encodes 572 residues, representing the entire sequence of a putative protein. The deduced amino acid sequence of the liver glutaminase showed 65% similarity to residues 23–572 of that deduced from nt 28531–30944 of the *C. elegans* sequence (Figure 2, lower panel). The *C. elegans* sequence also shows 63% similarity to that of kidney-type glutaminase (results not shown). Thus it is highly likely that, if expressed, this sequence would give rise to a glutaminase activity in *C. elegans*.

The three glutaminase amino acid sequences were examined to determine whether they contain a highly conserved consensus sequence found in the glutaminase domain of various amidotransferases, especially TrypG-type amidotransferases. This consensus sequence, PVFGICLGHQL [24,25], is absent from all three glutaminase sequences.

Hydropathy analysis of the deduced sequence indicated that hepatic glutaminase lacks a membrane-spanning domain and is not likely to be an integral membrane protein. This is consistent with previous reports that suggest that glutaminase is located in the mitochondrial matrix in loose association with the inner membrane [3].

Genomic sequences

A rat liver genomic Charon 4a library was screened with the 5′ end of the 2 kb cDNA to hepatic glutaminase and four identical clones were isolated. The size of the insert was 11 kb, from which a 4.5 kb *Eco*R1 fragment was identified as overlapping the 5′ end of the cDNA. This 4.5 kb 3′ genomic fragment was subjected to restriction digestion, and the fragments were subcloned and the ends sequenced. Regions complementary to the 2.0 kb cDNA were found in various locations, and the most 5′ (1.5 kb) fragment of the genomic clone contained a region, at its 3′ end, that was complementary to the 5′ end of the 2 kb cDNA. This raised the possibility that this 1.5 kb fragment contained an additional 5′ coding sequence; the fragment was subcloned (pBS-1.5). The first nucleotide of the 2.0 kb cDNA is found at position +174 (nt G) of the 1.3 kb sequence at the 5′ end of pBS-1.5 (Figure 3). The 1.3 kb sequence was compared with the sequence of kidney-type glutaminase: a region of 42 nt immediately upstream from nt +174 showed a high identity (69%) (sequence shown in bold in Figure 3). When these 42 nt were added to the first nucleotide of

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                                                                  -1022  CA
-1020  GGATATCCAG GGCCATACAA AGCAAAAGTT TTCTGGGGTT TGGGGATTTA GCTCAGTTGT
 -960  AGAGCGCTTG CCTAGGAAGC GCAAGGCCCT GGGTTCGGTC CCCAGCTCCG AAAAAAAGAA
 -900  CCAAAAAAAA AAAAAAAAGT TTCCTAAGCC TAATAACATT CACAGAAGTC TTTAGATCGG
 -840  TGGTTCTCCA CCTTCCTGAT GCTGCAATCC TTTCACCCTT CAATGCGGAC TGTGGTGACG
                                                                    CRE
 -780  TCCAATCATG AAATGATCTT CATTGCTGCT TCATAACTTT AGTTTTGCTA CTGTTATTGT
 -720  AATGTGAACA TCTGTGCTTT TCTGATGGTC TTGGGCCACT CCTGCGAAAG GGTCGTTTGA
 -660  CCAAAGGTCA AGGCCTACAG GTTGAGAGCC ATTGCTTTAA GATGCTTCAG GAGTTTTATA
 -600  CCAGTGTGAG AGACCTTTGA GGTTGGTGTG GAGACAGAAG CAGATAGTGG CAGAGAACAG
                                                                    GRE
 -540  ACGGAGTTGG TCATTGGCTG ACTTTTTGTT TTTCTTTTTC TTTTCTTTTT TTTTTTTCA
                  NF-1
 -480  CCGAGCTGGG GACCGAACCC AGGGCCTTGT GCTTGTTAGG CAAGCGCTCT ACCACTGAGC
              CAAT                                C/EBP
 -420  TAAATCCCCA ACCCAATTGG CTGACTTTAT GTCAGGAGGT TTATTTCATA GATAAATAAT
                    NF-1
 -360  ATTAAGTGGG CTACCTAAGG TCTTAGCAAT TCGGAGCCAA GTAGGTCTCT CTTCCCAACA
       TATA
 -300  CAGGGCATCC AGAAGAGGGT GGGTTTGAGA GGAAACACGA AGAATGTGTC CAGTGTTAGA
 -240  CACCAGCTCA CTGTAGACGC AGAGTATGGG AACAGTCAGA CTAAGCCTGT GTTTCCATCC
                            primer 3
 -180  TGTACCAGTC CTCACGGGAA AGACACGAAC CATACCGGAT GCGCTCTGTT TCCAGTGTTT
                                                                    HNF-5
 -120  GCTGGACATC TGGAAGTGGA ATGATTTAAA GGACTGAAAC TTCAGAGTTT CTTTTAAAAG
  -60  AGACTAGAGA GTAAGATGAC AACATCTCGT GCTGGGGTGA AAGTTAGGAT CACAGAATCT
         C/EBP
   +1  ACCAGATGGC CCAAAGAGGA GGCCTAGGAT GCACCCCATG ACAGGTCTGT GTTCCAGGAG
                                        primer 2
  +61  GGGGAGGAGG GGCATCCTCA GGCTCCTGCT TCCCATCTCT TCCCCCAAAC AGTGATGCCT
 +121  CGAACAGTGG CATGTTGCCT CGACTTGGTG ACCTGCTTTT CTACACCATT GCAGAGGGGC
                                                  primer 1
 +181  AGGAACGCAT CCCCATCCAT AAGTTCACCA CGgtaaggaa tcagggttct ctgattcctt
 +241  agagtctttg aaatctggga ggaggatgca ggttgaacag tgcaggtagc taatggctgc
 +301  cactcgcgca tggggaatac t
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Figure 3 Nucleotide sequence of 1.3 kb 5′ upstream of the hepatic glutaminase gene

The transcriptional start site is designated as +1. The 42 nt coding sequence derived from the genomic sequence is shown in bold face. Regions of nucleotide sequence similar to known regulatory elements are boxed and the intron sequence is shown in lower case. The three primers used in primer extension analysis are underlined.

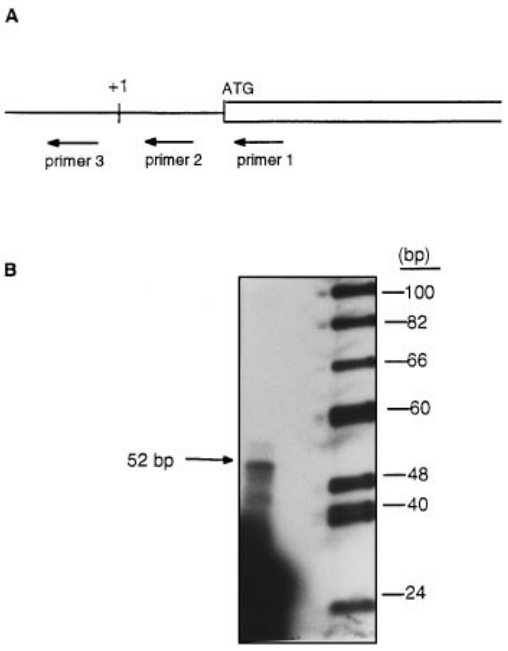

Figure 4 Transcription initiation site of hepatic glutaminase

(**A**) Schematic representation of the hepatic glutaminase gene promoter and the position of primers used for primer extension analysis. (**B**) Primer extension of rat liver RNA: 30 μg of total RNA from rat liver was hybridized with the 5′ end-labelled synthetic oligonucleotide primer 2 (5′-ACACAGACCTGTCATGGGGTGCATC-3′) followed by extension with reverse transcriptase. The size of the extension produced was determined by comparison with molecular size markers (ϕX174 *Hin*fI DNA markers, Promega) shown at the right.

the 2.0 kb cDNA sequence, one open reading frame was found with the characteristic ATG initiator codon at the 3′ end (see above and Figure 1).

Primer extension analysis

To identify definitively the end region of the coding sequence, primer extension analysis was performed with three primers to the genomic sequences. Primer 1, complementary to nt +151 to +182, which corresponds to residues 8–16 of the protein (Figure 3), produced many bands on the autoradiogram, and it was not possible to identify unequivocally the true 5′ end of the mRNA. This problem probably arose from the high GC content of the region causing reverse transcriptase to pause or terminate, and is similar to the problems encountered with cDNA cloning and 5′ RACE (see above). To eliminate this problem a second primer was prepared beyond the high-GC region, complementary to the region +28 to +52 (Figure 3). As shown in Figure 4, a single product of 52 bp in length was detected. A third primer complementary to sequence −199 to −228 (Figure 3), i.e. outside the predicted coding sequence, gave no detectable products by primer extension. These results indicate that the transcription start site is located 131 nt from the translation start site and that the full length of hepatic glutaminase mRNA is 2.2 kb. This result is in good agreement with the size determined by Northern blotting and indicates that the pBS-1.5 subclone and the 11 kb genomic clones contain 1 and 7.5 kb respectively of 5′ upstream sequence to the glutaminase gene.

Promoter sequence

The 1.3 kb sequence from hepatic glutaminase genomic DNA represented in Figure 3 contains 1 kb of 5′ flanking region, exon 1 (+1 to +212) and part of intron 1 (+213 to +331). The transcription initiation site is designated +1. Computer analysis of the 1 kb 5′ upstream of the gene revealed putative binding sites for a number of transcription factors. A putative HNF-5-binding site is contained between nt −118 and −125, and two HNF-1 sequences are found at −528 to −522 and −405 to −397. Two putative C/EBP-binding sites are present, at −52 bp to −43 bp and −445 to −437; cAMP response element (CRE) and glucocorticoid response element (GRE) consensus sequences are present at nt −785 to −780 and −547 to −540 respectively. However, an identifiable TATA box is not found in the region −25 to −30, where this sequence is usually located in TATA-containing eukaryotic promoters. Instead a putative TATA box is found at nt −361 to −357 (TATTAA), with a putative CAAT box at −407 to −404.

Promoter activity and hormonal responsiveness

Two constructs were prepared in the promoterless pCAT-Basic vector to study the promoter activity of the 1 kb 5′ region to the

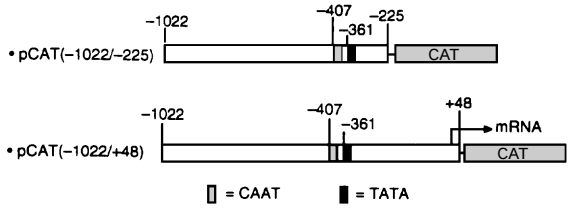

Figure 5 Hepatic glutaminase promoter–CAT constructs

Schematic diagram of the constructs containing the CAT coding sequence (shaded) with regions of the hepatic glutaminase promoter (5′ of position +48). Putative CAAT and TATA boxes in the promoter are indicated by shading.

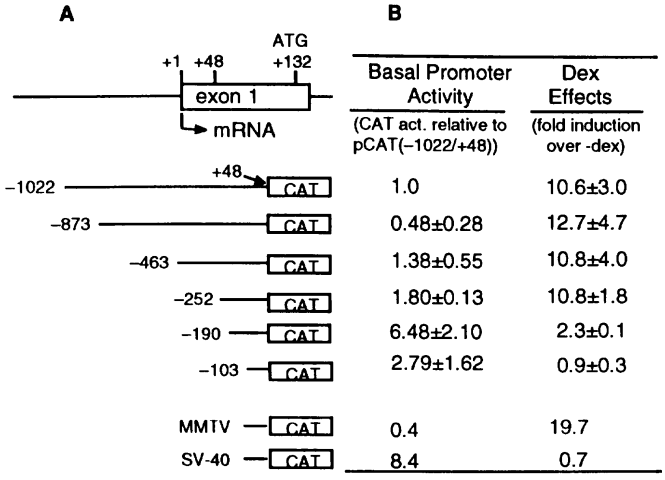

Figure 6 Structure and activity of the glutaminase promoter–CAT deletion constructs

(**A**) Schematic diagram of the promoter–CAT constructs generated by 5′ deletion. The top diagram shows the genomic organization of the 5′ untranslated region and the first exon of the glutaminase gene. The transcription start site is indicated by an arrow. The lengths (in bp) of the promoter fragments fused to the CAT reporter gene are shown. Abbreviation: MMTV, mouse mammary tumour virus. (**B**) The basal promoter activities and the effects of dexamethasone (Dex effects) on the promoter activity of the constructs shown in (**A**). CAT activity was normalized for transfection efficiency by using co-transfection with pRSV-β-gal. Basal promoter activity is expressed relative to the CAT activity by using the pCAT(−1022/+48) construct. Dexamethasone (75 nM plus co-transfection with the glucocorticoid receptor expression vector CDM8-GR) effects are expressed as fold induction over control (no hormone) activity for each construct. Results are means ± S.D. for three separate experiments.

glutaminase coding sequence and the functionality of the putative TATA box (Figure 5). The construct, pCAT(−1022/−225) contained a region upstream of the glutaminase coding sequence spanning from nt −1022 to −225. This construct contains the TATA-like element and CAAT-box consensus sequence, but lacks the region immediately 5′ of the coding sequence. When transfected into HepG2 hepatoma cells this construct did not result in CAT activity (less than 200 c.p.m. per assay). A second construct, pCAT(−1022/+48), containing the entire 1 kb upstream sequence plus 48 bp downstream to the transcription start site did drive CAT expression (2900 ± 800 c.p.m. per assay, mean ± S.D. for three experiments). In subsequent experiments deletion analysis of the promoter region showed that a construct spanning −103 to +48 was sufficient to drive CAT expression (see Figure 6). These results indicate that the hepatic glutaminase promoter lacks an identifiable TATA element, but that the region −103 to +48 contains elements critical for basal promoter activity.

To investigate the effects of cAMP and glucocorticoids on the glutaminase promoter activity, Hep G2 cells were transfected with the pCAT(−1022/+48) construct and either the glucocorticoid receptor expression vector (for glucocorticoid effects) or the catalytic subunit of protein kinase A expression vector (for cAMP effects). The transfectants were then treated with dexamethasone or 8-bromo-cAMP in serum-free medium for 48 h. Although a putative CRE was identified in the 5′ flanking region (around −781 bp) to the glutaminase gene, cAMP treatment was without effect on CAT expression in Hep G2 cells (results not shown). Treatment with dexamethasone significantly increased (10-fold) the level of CAT activity (Figure 6). In the absence of co-transfection with the glucocorticoid-receptor expression plasmid, there was no response to dexamethasone treatment (results not shown). The Hep G2 cell line does not express hepatic glutaminase and this was not affected by the addition of cAMP or dexamethasone.

A 5′ deletion series was prepared for the pCAT (−1022/+48) construct. The resulting constructs (−1022 CAT, −873 CAT, −463 CAT, −252 CAT, −190 CAT and −103 CAT), all containing the 48 bp 3′ to the transcription start site plus the length of 5′ sequence indicated, were able to drive CAT expression (Figure 6). With CAT activity for the original construct (pCAT −1022/+48) set to 1, the −190 CAT construct showed a 6-fold increase, whereas that with −873 CAT showed a decrease in expression of approx. 50%. All other constructs showed increased activity relative to the original. To locate the 5′ boundary of the GREs, the 5′ deletion series constructs were transfected with the glucocorticoid receptor expression plasmid. Plasmids containing the glutaminase promoter from −1022 to −252 all showed a 10–13-fold increase in CAT expression on treatment with glucocorticoids. A marked decrease in the glucocorticoid response occurred with construct −190 (from a 10-fold to a 2-fold induction) and the remaining 2-fold induction was lost with the removal of the region −190 to −103.

DISCUSSION

Glutaminase coding sequences

Rat liver glutaminase is encoded by a single species of mRNA 2225 bp in length, consisting of 131 bp of 5′ untranslated sequence, 1608 bp of open reading frame encoding a polypeptide of 535 residues with a molecular mass of 59.2 kDa, and 386 bp of 3′ non-translated sequence. Shapiro et al. [9] cloned and sequenced the full-length cDNA for rat kidney-type glutaminase, which results in two mRNA species: a more abundant 5.0 kb and a less abundant 3.4 kb mRNA. The full-length cDNA for the 5 kb mRNA contains 60 bp of 5′ untranslated sequence, 2022 bp of open reading frame (encoding 674 residues, molecular mass 74 kDa) and 2445 bp of 3′ untranslated sequence [7]. The long 3′ untranslated region of kidney-type glutaminase mRNA contains a region (939 bp) of that has been shown to play an important role in the regulation of mRNA stability, the primary mechanism for the regulation of renal glutaminase expression [7,17]. The relatively short 3′ untranslated region of hepatic glutaminase mRNA is unlikely to have a role in the regulation of hepatic glutaminase expression, where transcriptional control is the major regulatory mechanism.

Comparison of the hepatic glutaminase DNA sequence with that of kidney-type glutaminase [9] shows 67.7% identity throughout the coding region, with the exception of the last 80 nt. In addition, the deduced amino acid sequences show 86% similarity. A region between residues 317 and 370 of hepatic

glutaminase and between residues 456 and 519 of kidney-type glutaminase is most highly conserved and most hydrophobic, suggesting an important role for this region, such as a core element during protein folding or a mitochondrial inner membrane interaction site. However, a region of approx. 27 amino acids from the putative C-terminus shows less similarity and might be responsible for the kinetic differences observed between the two isoenzymes. The deduced hepatic and kidney-type glutaminase sequences also show high similarity (65% and 63% respectively) to a protein sequence deduced from the genomic sequence (nt 28531–30944) of the *C. elegans* cosmid DH II clone [23]. The high degree of similarity of the *C. elegans* sequence to the rat glutaminases suggests that, if expressed, it would result in glutaminase activity. The lack of the conserved glutamine-binding site of the TrypG amidotransferases [24,25] in any of the three glutaminase sequences examined here suggests that they did not evolve from the common ancestor of the amidotransferases.

Promoter sequence

Sequence analysis of the 1.3 kb 5′ region of the 4.5 kb hepatic glutaminase genomic fragment indicates that this region contains approx. 1 kb of 5′ upstream sequence, the entire 5′ untranslated sequence (131 bp), 81 bp of coding sequence and part (119 bp) of the first intron sequence. The 1 kb 5′ upstream sequence to the hepatic glutaminase gene contains several putative regulatory elements. Two putative NF-1 binding sites are identified by computer analysis. NF-1 is a ubiquitous nuclear protein that is known to bind to the promoters of liver-specific genes such as those coding for PEPCK [26] and albumin [27]. Quinn et al. [28] demonstrated that the 3′ region of the NF-1 site in the PEPCK promoter was required for the normal level of basal transcription from the PEPCK promoter. However, Christ et al. [29] suggested that NF-1 is involved in the cAMP responsiveness of the PEPCK promoter by interacting with protein(s) binding to the CRE of the PEPCK promoter. In addition, NF-1 has been shown to participate in the glucocorticoid regulation of mouse mammary tumour virus gene transcription. Putative binding sites for two liver-enriched transcription factors, C/EBP and HNF-5 [30], are also found in the 5′ flanking sequence to the liver glutaminase gene. Such sites have been identified in the promoters of a number of liver-specific genes [31] and because hepatic glutaminase is expressed only in the liver, the putative C/EBP- and HNF-5-binding sites might have important roles in liver-specific glutaminase expression.

The best-characterized core promoter element, TATA-box, and the distal promoter element, CAAT box, are found −361 and −407 bp respectively from the transcription start site of hepatic glutaminase. The canonical TATA and CAAT boxes are usually located approx. 25–30 bp and −80 bp upstream of the transcription initiation site. Thus the TATA sequence 5′ to the glutaminase gene was predicted to be too far away from the identified transcription initiation site to function as a core promoter element. Functional analysis of these sequences indicated that a construct including a promoter region between bases −190 and +48, lacking an identifiable TATA element, was sufficient for the expression of a CAT reporter gene. Therefore the expression of hepatic glutaminase is controlled by a TATA-less promoter. Interestingly, the promoter of kidney glutaminase also lacks an identifiable TATA box [7], indicating that the transcription of both glutaminase isoenzymes is driven by TATA-less promoters. The glutaminase promoter–CAT constructs including sequences between bp −252 and +48 have lower promoter activity than the construct containing −190 to +48 bp. This suggests the presence of inhibitory elements in the region between −252 and −190 bp. Similarly, regions upstream of −190 bp also resulted in lower expression than the −190 to +48 bp construct. Thus the region from −252 to −103 might play an important role the basal expression of hepatic glutaminase.

Hormonal regulation

Putative hormonal response elements (CRE and GRE) are found in the 5′ upstream region to the hepatic glutaminase gene. Hepatic glutaminase expression shows similar regulation patterns to those of the genes encoding urea-cycle and gluconeogenesis enzymes and might therefore share regulatory mechanisms. Glucagon, acting via cAMP, and glucocorticoids are major hormones involved in the increased transcription of genes encoding gluconeogenic enzymes such as PEPCK [32–36] and the urea-cycle enzymes [37,38]. In accord with this hypothesis, increased levels of hepatic glutaminase mRNA are found in rats after injection of dibutyryl cAMP) [39] and in H35 cells treated with dibutyryl cAMP or dexamethasone [40]. In addition, McGivan et al. [41] have shown that hepatic glutaminase activity increases in primary rat hepatocytes after treatment with glucagon for 24 h.

The widely accepted mechanism [42] for cAMP-induced transcription involves the cAMP activation of protein kinase A, which in turn phosphorylates a transcription factor, CRE-binding protein (CREB). There is a large family of CREB proteins [43], but other proteins have also been implicated as mediators of cAMP effects. Moreover, for several promoters, cAMP responsiveness involves multiple *cis*-elements [44,45]. Although a putative CRE (−785 to −780) was identified in the 5′ flanking region to the glutaminase gene, the activity of the glutaminase promoter was not changed in response to 8-bromo-cAMP treatment. Thus the hepatic glutaminase promoter might require multiple *cis*-elements, including CRE(s) that might not be present in the region from −1022 to +48 of the glutaminase promoter. Alternatively, the HepG2 cell might not be appropriate for the induction of the hepatic glutaminase promoter by cAMP, although it has been successfully used for other cAMP-regulated genes [30,44,46]. Thirdly, the putative CRE in the glutaminase promoter might simply not be functional. Further analysis with different cell lines or 5′ flanking sequence beyond −1022 bp of the glutaminase promoter will resolve the cAMP responsiveness of the hepatic glutaminase promoter.

Glucocorticoids are known to regulate gene transcription by activation of the receptor. On binding hormone, the cytosolic glucocorticoid receptor protein translocates to the nucleus and binds in a site-specific manner to the GRE consensus sequence (T/G)GTACANNNTGTTCT [47]. However, some functional GREs identified in glucocorticoid-responsive promoters such as PEPCK [48] and proliferin [49] lack this consensus sequence. Such GREs are complex and require additional transcription factors for glucocorticoid induction [50,51]. The chimaeric construct containing the glutaminase promoter region from base −1022 to +48 was highly responsive to glucocorticoid treatment. Deletion mutants of the promoter indicate that a functional GRE in the glutaminase promoter is located between −252 and −103 bp, a region that lacks the consensus GRE sequence. Deletion analysis also demonstrated that the putative GRE consensus sequence at −547 to −540 was not required for the glucocorticoid induction of expression. Thus the functional GRE in the region between −252 and −103 bp of the glutaminase promoter might have weak affinity for the glucocorticoid receptor, and other factors might be necessary to achieve the

binding specificity and affinity required for glucocorticoid *trans*-activation, as seen with the GREs in PEPCK and proliferin promoters.

Concluding remarks

Rat hepatic glutaminase shows striking similarities in nucleotide and amino acid sequences to kidney-type glutaminase and to a putative glutaminase identified from the *C. elegans* genome. These glutaminases are clearly the products of related but separate genes, but they do not share a close evolutionary background to the glutamine amidotransferases. Rat liver glutaminase gene expression is driven by a TATA-less promoter that is responsive to glucocorticoids. The availability of the full coding sequence and 5′ upstream genomic sequences to the hepatic glutaminase gene will be valuable for further investigation of the liver-specific, hormonal and developmental regulation of expression of this gene.

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