Characterization of a full-length cDNA encoding human liver *S*-adenosylmethionine synthetase: tissue-specific gene expression and mRNA levels in hepatopathies

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The sequence of a full-length cDNA coding for human liver *S*-adenosylmethionine synthetase has been determined. It spans 3217 nucleotides and encodes a protein of 395 amino acid residues, with a calculated molecular mass of 43647 Da. The structural features deduced from the amino acid sequence show a close similarity to those of the rat liver enzyme. The liver-specific *S*-adenosylmethionine synthetase gene appears to be present as a single copy in the genome, as revealed by Southern analysis. The occurrence of a single mRNA species for this enzyme has been determined by primer extension and Northern analysis. Among several human tissues examined, this gene is expressed only in the liver. Similar *S*-adenosylmethionine synthetase mRNA levels have been detected in biopsies from normal human liver and from patients with alcoholic cirrhosis and hepatocellular carcinoma. Based on these results, a possible mechanism of regulation of human liver *S*-adenosylmethionine synthetase is discussed.

INTRODUCTION

In all organisms studied, *S*-adenosyl-L-methionine (AdoMet) acts as the donor of methyl groups for most transmethylation reactions. It is also involved in the biosynthesis of different metabolites such as polyamines, biotin, cysteine and glutathione [1,2]. Besides its central role in cellular metabolism, AdoMet is the subject of an increasing interest due to its pharmacological properties and therapeutic potential in liver damage and affective disorders ([3–5]; reviewed in [6]).

AdoMet is synthesized from ATP and methionine, a reaction catalysed by the enzyme AdoMet synthetase (EC 2.5.1.6) in the presence of Mg^{2+} and K^+. This enzyme has been extensively studied in bacteria, yeast, plants and animals. The enzyme has been purified from several of these organisms [7–14] and cDNAs or genomic clones have been isolated [16–26]. In mammals, different AdoMet synthetase isoenzymes appear to exist. In fact, distinct enzyme forms have been purified from rat liver, rat kidney, bovine brain and human lymphocytes [11–15]. In adult rat liver, two oligomeric forms have been identified and designated high- and low-molecular mass AdoMet synthetase (also called α and β AdoMet synthetase respectively) [14]. The high-molecular-mass form is a tetramer and the low-molecular-mass form is a dimer comprised of the same polypeptide chain [14,27] with a molecular mass of 43.7 kDa, as determined both by SDS/PAGE [25] and from the amino acid sequence deduced from its cloned cDNA [23,25]. Another AdoMet synthetase isoenzyme, named AdoMet synthetase γ, has been characterized in the extrahepatic tissues mentioned. This form has been reported to consist of two or three distinct subunits, with molecular masses ranging between 38 kDa and 53 kDa [13,15].

In human liver, two oligomeric forms of AdoMet synthetase have also been identified, resembling the pattern found in the rat liver [28]. Although human-liver-specific forms are being currently studied due to the involvement of this enzyme in liver diseases [28–30], little is known about its structure and regulation. To gain more insight into these topics, we have isolated cDNA clones coding for human liver AdoMet synthetase. During the course of this work a partial sequence of the enzyme cDNA was reported [26]. Here we describe a full-length cDNA that exhibits some major differences in both the coding and non-coding regions. In addition, the AdoMet synthetase gene and mRNA have been detected. The expression of the enzyme mRNA in normal liver and livers of subjects with alcoholic cirrhosis and hepatocellular carcinoma has been studied. Finally, similarities between rat and human liver enzymes are discussed.

EXPERIMENTAL

Materials

Restriction enzymes were obtained from either Boehringer Mannheim (Mannheim, Germany) or Biotech S.L. (Madrid, Spain). Reverse transcriptase from avian myeloblastosis virus (AMV) was purchased from Promega. Nylon membranes were from Schleicher and Schuell (Keene, NH, U.S.A.). The Sequenase DNA sequencing kit was provided by United States Biochemicals (Cleveland, OH, U.S.A.). [α-^{32}P]dCTP, [α-^{35}S]dATP and [γ-^{32}P]ATP were from Dupont–New England Nuclear (Boston, MA, U.S.A.). Oligo(dT)–cellulose was from Boehringer Mannheim. A λ gt11 cDNA library from human liver was purchased from Clontech (Palo Alto, CA, U.S.A.).

Human liver samples

Liver biopsies used in this study were obtained from six alcoholic cirrhotic subjects and from a patient with hepatocellular car-

Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; AMV, avian myeloblastosis virus.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession number X69078.

cinoma. The aetiology was established by anatomical pathology studies as previously described [29]. The control group comprised six subjects suffering from cholelithiasis with a totally normal profile of hepatic function.

Library screening and DNA sequence analysis

A human liver λ gt11 cDNA library (Clontech) was screened as described [25] using a 2.3 kb *Eco*RI fragment of rat liver AdoMet synthetase cDNA clone pSSRL as a probe [25]. DNA from positive phage was isolated by the plate lysate method [31]. The DNA was digested with *Eco*RI endonuclease and the inserts subcloned into the *Eco*RI site of PUC 18. Isolated cDNAs were sequenced on both strands by the dideoxy chain termination method [32] using 22 specific oligonucleotides as internal primers. These oligonucleotides were synthesized using the phosphoramidite method with an Applied Biosystem 391 DNA synthesizer. Computer analysis of sequences was performed with Strider 1.0 software (Microsoft).

Primer extension analysis

A 17-base oligonucleotide complementary to nucleotides 94–110 of the human liver AdoMet synthetase cDNA was synthesized and 5′-end-labelled with [γ-^{32}P]ATP (> 5000 Ci/mmol) and T_4 polynucleotide kinase. Then 5 pmol of the end-labelled oligonucleotide was annealed to 3 μg of poly(A)$^+$ RNA from human liver by incubation at 85 °C for 5 min and then at 42 °C for 2 h in 0.1 M Tris/HCl buffer, pH 8.3, containing 0.14 M KCl and 1 mM EDTA. The buffer was then adjusted to 1 mM dNTPs, 15 mM dithiotreitol and 10 mM $MgCl_2$, and 25 units of placental ribonuclease inhibitor and 50 units of AMV reverse transcriptase were added. Reverse transcription was carried out at 42 °C for 90 min. After synthesis, nucleic acids were precipitated and reaction products analysed on 6% polyacrylamide gels containing 7 M urea.

Southern blot analysis

Genomic DNA was isolated from a human liver sample by standard procedures [31]. A 10 μg sample of DNA was digested with appropriate restriction endonucleases, fractionated on 1% agarose gels and blotted to nylon membranes. Prehybridization and hybridization of the membranes were performed as described [33]. The probe used for hybridizations was a 1.4 kb fragment of the human AdoMet synthetase cDNA containing the entire coding region (Figure 1; SHL1 clone). This probe was labelled by nick-translation to a specific radioactivity of 2×10^8 c.p.m./μg.

RNA extraction and Northern analysis

RNA from various human tissues and from liver biopsies was isolated by the guanidinium thiocyanate method [34]. Samples were denatured in formaldehyde, electrophoresed in 1% agarose/formaldehyde gels and transferred to nylon membranes. The probe used for hybridizations was the same as for the Southern analysis.

RESULTS

Isolation of overlapping cDNA clones coding for human AdoMet synthetase

We have previously described the isolation of a cDNA clone (pSSRL) coding for rat liver AdoMet synthetase [25]. A 2.3 kb *Eco*RI fragment of this clone was used to screen a λ gt11 human liver cDNA library. Out of 400000 independent recombinants we obtained six partial clones designated SHL1–6, which contained inserts ranging in size between 0.5 and 1.6 kb (Figure 1). All of them were subcloned into PUC 18 and sequenced on both strands using specific internal primers. The DNA sequence determination showed that clones SHL1 and SHL3 contained the entire coding region for human liver AdoMet synthetase, whereas SHL4 extended to the 3′-untranslated region but did not contain the poly(A) tract. In an attempt to obtain a full-length cDNA, this clone was used to rescreen the library. This screening yielded three additional overlapping clones (SHL11–13). The sequence at the 3′-end of the AdoMet synthetase mRNA, however, was present in a single cDNA clone (SHL13 in Figure 1).

A number of artifacts were also obtained from the library. These sequences appeared fused to cDNA clones SHL 3, 6, 11 and presented a high degree of sequence similarity with rat disulphide isomerase, human 18 S ribosomal RNA and *Bacillus subtilis* spectinomycin resistance gene.

cDNA sequence of pSHL

The resulting sequence of the AdoMet synthetase cDNA (pSHL) has an overall length of 3217 nucleotides followed by the poly(A) tail (Figure 2). The coding region extends from nucleotide 1 to 1185; the 5′- and 3′-untranslated regions are 71 and 1961 nucleotides long respectively. There are major differences between our cDNA sequence (pSHL) and that reported by Horikawa's group (HLSAM) [26]. In the coding region there are eight discrepancies at the nucleotide level (asterisks in Figure 2). The base changes at positions 426, 816, 870, 882, 1089 and 1095 do not alter the amino acid sequence; however, the changes at positions 815 and 818 render two glycines instead of the two alanines reported in HLSAM. In addition, the two sequences differ completely upstream of nucleotide residue −18 in the 5′-untranslated region. These discrepancies are discussed below. Concerning the 3′ non-coding region, pSHL contains an additional fragment of 1677 nucleotides beyond the 3′-end of

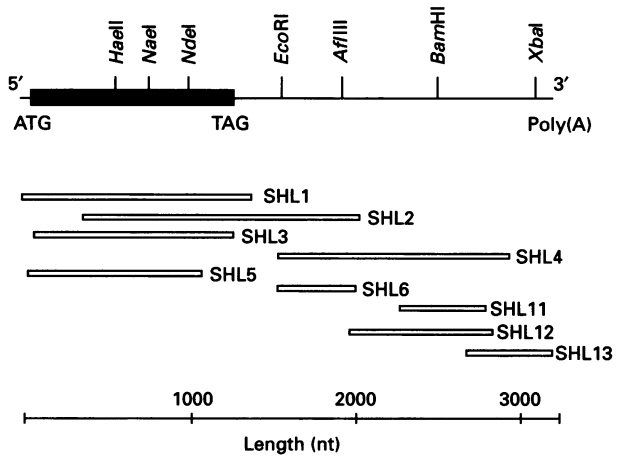

Figure 1 Partial restriction map and pSHL cloning strategy

The closed box represents the protein coding sequence and the thin lines are the 5′- and 3′-non-coding regions. The open bars below the restriction map indicate the sizes and designations for isolated cDNA inserts. The cDNA clones SHL1–6 were identified from a human liver cDNA library with a rat liver probe (see the Experimental section). The remaining clones were obtained by screening the same library with clone SHL4.

Figure 2 Nucleotide and deduced amino acid sequences of the human liver AdoMet synthetase cDNA

The nucleotide sequence is numbered with the A of the ATG initiation codon being designated as +1. In the coding region, discrepancies at the nucleotide or amino acid level compared with a previously reported cDNA, HLSAM1 [26], are marked with asterisks. The ATP-binding site is underlined and cysteine-149 is doubly underlined. The putative polyadenylation signal is underlined with a thick line. The proposed G/T cluster is in bold. The mRNA destabilization consensus sequence ATTTA is indicated by an overbar.

HLSAM. In this extension a putative polyadenylation signal AATAAA (thick underline in Figure 2) is present 16 nucleotides upstream from the poly(A) tail. Also noteworthy is the presence of the trinucleotide TGT repeated in conjunction with oligo(T) stretches (bold letters in Figure 2). This feature, known as a G/T cluster, seems to be implicated in the efficient formation of the 3′-terminus of many mRNAs [35,36]. It is usually located downstream of the AATAAA motif, in contrast with what is observed in the AdoMet synthetase mRNA sequence. Therefore we cannot rule out the possibility of the presence of an alternative polyadenylation site upstream from the one proposed, although another functional AATAAA-like element in this area is not evident. An over-representation of the mRNA destabilization consensus sequence ATTTA [37] is also noted in the 3′-untranslated region (overbar in Figure 2). Although the role of this sequence in AdoMet synthetase mRNA regulation is not clear, it might be of physiological significance.

Primer extension analysis

To determine the length of the 5′-untranslated region of the human liver AdoMet synthetase mRNA, a primer extension analysis was performed. A 17-base oligonucleotide complementary to nucleotides 94–110 of pSHL cDNA was annealed to poly(A)$^+$ RNA isolated from human liver and extended towards the 5′ end of the corresponding mRNA by reverse transcription. As shown in Figure 3, the primer extension reaction yielded a product of approx. 200 nucleotides long, indicating that the

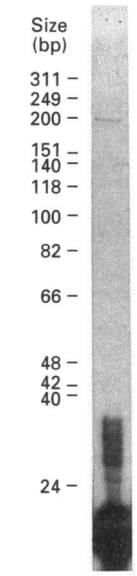

Figure 3 Primer extension analysis of the mRNA corresponding to the pSHL clone

A synthetic primer complementary to nucleotides 94–110 of the pSHL sequence was 5′-end-^{32}P-labelled and annealed with poly(A)$^+$ RNA from human liver. Conditions were as described in the Experimental section. Size markers correspond to ϕx174 digested with *Hin*fI.

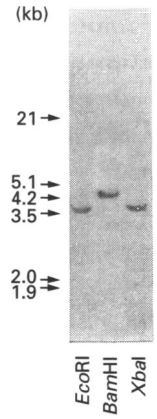

Figure 4 Southern blot analysis of human genomic DNA using an AdoMet synthetase cDNA fragment

High-molecular-mass genomic DNA (10 μg per lane) isolated from human liver was digested with *Eco*RI, *Xba*I or *Bam*HI as indicated, separated on a 1% agarose gel and transferred to a nylon membrane. The radiolabelled probe was SHL1, a 1.4 kb cDNA that comprises the entire coding region of the AdoMet synthetase gene. λ gt11 phage DNA digested with *Eco*RI and *Hin*dIII was used as a size marker; molecular sizes in kb are given on the left.

corresponding mRNA should have a 5′-untranslated region of about 90 bases. This result is in good agreement with the length of the 5′ sequence of pSHL.

Southern analysis of genomic DNA

A Southern blot hybridization of human genomic DNA was performed to determine whether there are multiple AdoMet synthetase genes or other closely related genes. A 1.4 kb cDNA fragment (SHL1), containing the whole protein coding region,

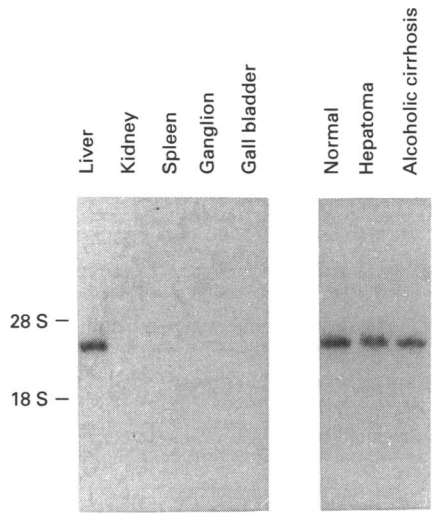

Figure 5 Northern analysis of AdoMet synthetase mRNA in various human tissues, alcoholic cirrhotic liver and hepatocellular carcinoma

Total RNA (15 μg) from the indicated sources was resolved by electrophoresis on agarose gels containing formaldehyde, and transferred to nylon membranes. The blots were probed with ^{32}P-labelled SHL1 clone. The autoradiogram on the right is representative of the results obtained from six biopsies from human normal liver and six from alcoholic cirrhotics. A sample from hepatocellular carcinoma is included. Ribosomal RNAs of 28 S and 18 S were used as size markers.

was used to probe *Eco*RI, *Bam*HI and *Xba*I restriction digests of human genomic DNA. As shown in Figure 4, a single DNA species was detected for each restriction digest. This result suggests that the gene for the liver-specific isoenzyme is present in the human genome as a single copy.

Northern analysis

RNA from various human tissues was analysed by Northern hybridization using the SHL1 cDNA as a probe. A hybridization signal corresponding to an mRNA species of about 3.3 kb was detected only in liver (Figure 5), indicating a liver-specific expression of the gene. A similarly sized mRNA was obtained when human liver RNA was probed with the rat liver cDNA pSSRL [25]. A hybridization band was also detected when total RNA isolated from biopsies of patients with alcoholic cirrhosis or hepatocellular carcinoma was analysed (Figure 5). Thus liver-specific isoenzyme mRNA is being expressed in human hepatopathies, in contrast with the data reported in rats, where the hepatic AdoMet synthetase is replaced by the extrahepatic isoenzyme in carcinogenesis [38]. On the other hand, no significant differences in AdoMet synthetase mRNA levels were observed when samples obtained from six alcoholic cirrhotic subjects were compared with the corresponding controls.

DISCUSSION

The sequence of the human liver AdoMet synthetase mRNA has been determined from nine overlapping cDNA clones (Figure 1). The resulting cDNA sequence (pSHL) differs significantly from the one previously reported (HLSAM) [26]. In the coding region there are eight discrepancies at the nucleotide level. Six of the sequence differences from the HLSAM sequence are silent at the amino acid level, but those located at positions 815 and 818 result in two glycines instead of the two alanines previously reported. Two arguments support our sequence determinations:

(1) the same sequence has been found in four independent cDNA clones (SHL1, 2, 3 and 5 in Figure 1); and (2) rat liver and kidney enzymes, which display sequence similarities of 95% and 84% respectively with the human liver enzyme, also present two glycines at these positions. Nevertheless, the reason for these discrepancies could be due to the source of the respective cDNA libraries. Regarding the 5′-untranslated region, pSHL contains 71 nucleotide residues, whereas HLSAM has 268. In addition to their length, both sequences differ completely upstream from nucleotide −18. A search of the GenBank nucleic acids sequence database revealed that the 5′-non-coding region of pSHL is highly similar to the same region in the rat liver cDNA [25]. On the contrary, the 5′ sequence of the HLSAM showed 100% sequence identity with a fragment of human mitochondrial DNA. Furthermore, primer extension analysis showed that the 5′-non-coding region of AdoMet synthetase mRNA is about 90 nucleotides long (Figure 3). Therefore it seems that the sequence of HLSAM upstream from nucleotide −18 is an artifact from the cDNA library. pSHL and HLSMA cDNAs also differ in the length of the 3′-untranslated region, the former being 1677 nucleotides longer than the latter. In this extension, the poly(A) tail has been identified, as well as other regulatory features (Figure 2).

Previous studies have reported evidence of a close similarity between AdoMet synthetase detected in human liver and that purified from rat liver. The human liver enzyme shows, on gel filtration chromatography, two peaks of activity that coincide with the elution positions for the rat liver high- (200 kDa) and low- (110 kDa) molecular-mass forms [28]. It has been shown that the rat liver forms correspond to two different oligomeric states of the same subunit, which possesses a molecular mass of 43697 Da and is encoded by a single mRNA species of 3.4 kb [23,25]. Here we have shown, by primer extension and Northern analysis, the existence of only one mRNA for the human liver enzyme, which encodes a protein of 43647 Da. It can therefore be expected that the two forms of the human liver AdoMet synthetase detected by gel filtration correspond to oligomeric forms similar to those observed in rat liver.

The tissue distribution of the human liver-specific AdoMet synthetase mRNA is also similar to that found in the rat. An AdoMet synthetase cDNA isolated from rat liver hybridized to an mRNA species in liver, but not in kidney, spleen, heart, brain or testis [24,25]. Conversely, when total RNA from these tissues was analysed using a cDNA clone specific for the rat kidney AdoMet synthetase (γ form), two mRNA species were identified, but no hybridization signal was observed in the liver sample [24]. Thus the mRNAs for the liver- and kidney-type isoenzymes appear to be expressed in the rat in a tissue-specific manner, consistent with the tissue distribution of the different AdoMet synthetase isoenzymes identified. The cDNA isolated from human liver detects a specific mRNA in liver, but not in kidney, spleen, gall bladder or ganglion (Figure 5), suggesting tissue-specific AdoMet synthetase mRNA expression similar to that found in rat.

Consistent with these data is the finding that the liver-specific AdoMet synthetase gene appears to be present as a single copy in the human genome (Figure 4). This result suggests the existence of at least two different AdoMet synthetase genes to explain the distinct isoenzymes found. In this context, two structural genes for AdoMet synthetase have also been characterized in *Saccharomyces cerevisiae* [17,18] and *Arabidopsis thaliana* [19,20]. Therefore, in addition to its primary structure, the genetic pattern of AdoMet synthetase is exceptionally well conserved through evolution.

Intriguing questions arise from the detection of normal levels of human liver AdoMet synthetase mRNA in liver biopsies from subjects with alcoholic cirrhosis and hepatocellular carcinoma (Figure 5). It has been reported that in adult rats treated with the hepatocarcinogen N-2 fluorenylacetamide, the liver-specific AdoMet synthetase is replaced by the extrahepatic isoenzyme according to the progress of the hepatocarcinoma [38]. Our results, however, indicate that the mRNA for the human liver enzyme is being expressed in hepatocellular carcinoma. However, it cannot be excluded that the extrahepatic form is also present, since, as mentioned above, the mRNA corresponding to this isoenzyme is not recognized by the liver-specific cDNA.

On the other hand, previous reports have shown that AdoMet synthetase activity is greatly reduced in human alcoholic cirrhosis [28,29]. The results presented here indicate that the human cirrhotic liver presents normal levels of AdoMet synthetase mRNA compared with the controls. Thus the reduced AdoMet synthetase activity observed seems to be the consequence of a post-translational event. In this regard, it has been observed that cirrhosis induced in rats by carbon tetrachloride caused a marked reduction of AdoMet synthetase activity without producing significant changes in AdoMet synthetase mRNA levels [39].

The mechanism of regulation of AdoMet synthetase in different organisms is so far unknown, despite recent efforts focused on the wheat embryo, *Saccharomyces cerevisiae* and rat liver enzymes [9,40,41]. The importance of the thiol groups of the molecule in maintaining its structure and activity is well established. Thus the modification of specific cysteine residues of *Escherichia coli* and rat liver AdoMet synthetases leads to enzyme inactivation [42–44]. These cysteines correspond to residues 90 and 240 of the *E. coli* sequence and to residue 150 of the rat liver sequence. Furthermore, related to the role of the thiol groups in AdoMet synthetase activity, it has been shown that the rat liver enzyme activity is modulated by glutathione [41,45].

Based on these data and on the presence of some putative phosphorylation sites in the rat liver AdoMet synthetase sequence, a regulatory mechanism combining phosphorylation and oxidoreduction of thiol groups has recently been proposed for this enzyme [46].

Some structural features deduced from the human liver AdoMet synthetase amino acid sequence suggest that his enzyme may undergo a similar regulation to that proposed for the rat liver enzyme. An analysis of the deduced human AdoMet synthetase sequence indicates the existence of several putative phosphorylation sites: six for casein kinase II, five for protein kinase C, two for Ca^{2+}/calmodulin-dependent protein kinase and one for cyclic AMP-dependent protein kinase (Figure 6). Some of these sites, e.g. for protein kinase C or casein kinase II, are located on the most hydrophilic area of the molecule, as revealed by a comparison of their locations with the hydrophobicity profile of the protein (Figure 6). Therefore it could be expected that human liver AdoMet synthetase is phosphorylated by some of these kinases. In fact, the rat liver enzyme, which exhibits most of the potential phosphorylation sites at the same positions as those observed in the human liver enzyme [45], appears to be phosphorylated *in vitro* by protein kinase C (M. A. Pajares, C. Durán and J. M. Mato, unpublished work).

On the other hand, the cysteine residue involved in maintaining rat liver AdoMet synthetase activity (Cys-150) is conserved in the sequence of the human liver enzyme (Cys-149; doubly underlined in Figure 2). Interestingly, in both the rat and human liver enzymes this cysteine is located close to the ATP binding site (Figure 2).

In spite of the evidence obtained so far, further work is necessary to fully determine the underlying mechanisms of the regulation of human liver AdoMet synthetase. The cDNA

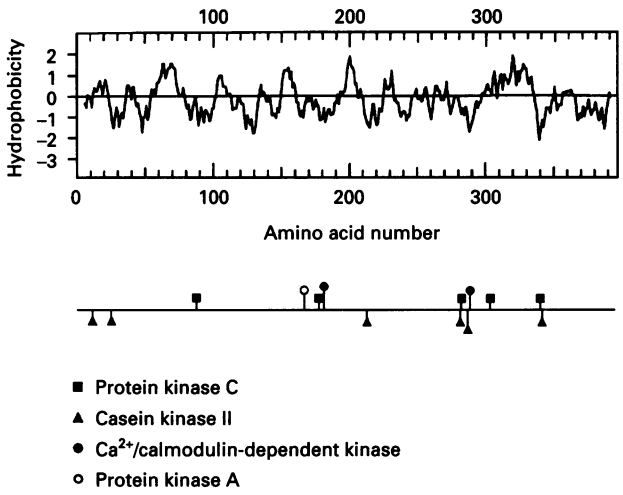

Figure 6 Structural features of human liver AdoMet synthetase

The hydrophobicity of the deduced sequence of AdoMet synthetase was analysed by the Kyte–Doolittle algorithm. The relative locations of putative phosphorylation sites are shown.

described in this paper will provide a tool for expression studies to address this question.

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