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Analysis of the 5′ non-coding region of rat liver *S*-adenosylmethionine synthetase mRNA and comparison of the M_r deduced from the cDNA sequence and the purified enzyme*

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A 3 kb cDNA coding for rat liver *S*-adenosylmethionine (AdoMet) synthetase has been isolated. The M_r of the protein has been unequivocally determined by cDNA sequencing and enzyme purification on a thiopropyl-Sepharose column. The length of the mRNA 5′ non-coding region has been defined by primer-extension analysis. The rat liver cloned cDNA has been also used to detect *S*-adenosylmethionine synthetase mRNA in human liver.

S-Adenosylmethionine synthetase; cDNA sequence; Sulfhydryl groups; Messenger RNA; Rat liver; Human liver

1. INTRODUCTION

S-Adenosylmethionine (AdoMet) synthetase (ATP:L-methionine *S*-adenosyltransferase, EC 2.5.1.6) is the enzyme responsible for the synthesis of AdoMet using methionine and ATP as substrates [1,2]. In rat liver, two distinct oligomeric forms of the enzyme have been identified and designated as high- and low-M_r AdoMet synthetases. It has been shown that both forms are constituted by the same polypeptide chain, which has an apparent molecular mass of 48.5 kDa on polyacrylamide gel electrophoresis [3,4]. Recently, Horikawa et al. [5] have isolated a 1.8 kb cDNA coding for rat liver AdoMet synthetase. The deduced amino acid sequence corresponds to a protein with an M_r of 43.7 kDa instead of the 48.5 kDa described for the purified enzyme subunit. However, as indicated by the authors and since they have only sequenced 72 nucleotides upstream the proposed ATG initiation codon, the possibility of the presence of another translational start site upstream from the nucleotide residue −72, that results in a larger protein, could not be excluded.

In an attempt to elucidate the full sequence of the protein, we have isolated a 3.0 kb cDNA encoding rat liver AdoMet synthetase from a phage lambda gt 11 library. The sequence of the cDNA completes that previously reported and unequivocally establishes the exact M_r of the protein. Moreover, the M_r deduced from the cDNA sequence and that obtained with the enzyme purified on a thiopropyl-sepharose column are compared.

*The nucleotide sequence data reported will appear in the EMBL, Genebank and DDBJ Nucleotide sequence Databases under the accession number X60822

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2. MATERIALS AND METHODS

Restriction enzymes were obtained either from Boehringer Mannheim or Biotech S.L. Reverse transcriptase from avian myeloblastosis virus was purchased from Seikagagu America. Nylon membranes were from Schleicher and Schuell. The sequenase DNA sequencing kit was purchased from United States Biochemicals (Cleveland OH). [α-^{32}P]dCTP, [α-^{35}S]dATP and [τ-^{32}P]ATP were from Du Pont-New England Nuclear. Synthetic oligonucleotides were provided by Bio-synthesis S.L. Oligo(dT)-cellulose was from Boehringer Mannheim. Thiopropyl-sepharose 6B was a product from Pharmacia, and L-cysteine was purchased from Aldrich. Lambda gt11 cDNA library was kindly provided by Dr Frank González (National Cancer Institute).

2.1. *Library screening and DNA sequence analysis*

Recombinant plaques were screened at a density of 10 000 plaques/100 mm plate using a synthetic oligonucleotide probe of 18 bases corresponding to AdoMet synthetase cDNA nucleotide residues +61/+78 [5]. The probe was end-labelled at the 5′ end using standard procedures [6]. DNA from positive phage was isolated by the plate lysate method [6]. The DNA was digested with *Eco*RI endonuclease and the inserts subcloned into the *Eco*RI site of PUC 18. The insert was digested with *Hae*III, *Alu*I, *Sac*I, *Eco*RI and *Cla*I according to the restriction map already described [5], and that shown in Fig. 1. Sequencing of both strands was performed by the dideoxy chain termination method [7].

2.2. *RNA extraction and Northern analysis*

Total RNA was isolated by the guanidinium thiocyanate method [8]. For primer extension analysis, poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose column chromatography [9]. Aliquots of 25 μg of

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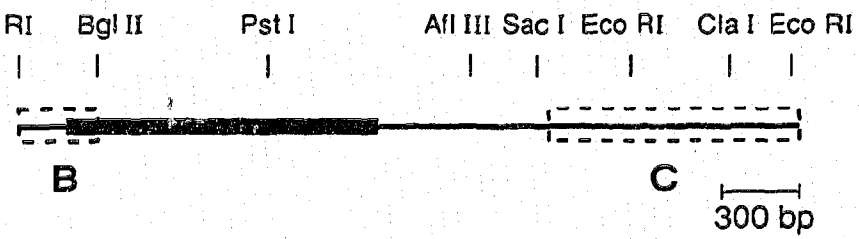
A



B

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-210                                                    TCCCTCTTTGCCCATCCTCAATCT
-186 GTTCTCATAAGCTCATAGGCACTTAGGCAGAAGTCATCTCCTTGTGATTCTGCCCAAGATCT
-124 GCCCGTTGGGTTTGTAGGGGAGGAAGAAAAAAAAAAAGAGAGAGACAGAGAAAAGAAACTA
 -62 CAGGCAAACTTTCAGCTTTGAAGTTGTCACCTTGGAGAAGTGAAGTCGAGAAGTGTGACACC
   1 ATGAATGGACCTGTGGATGGCTTGTGTGACCATTCTCTAAGTGAAGAGGGAGCCTTCATGTT
  63 CACATCTGAATCGGTAGGAGAAGGGCATCCAGATAAGATCTGTGACCAGATTAGTGATGCAG
 125 TGCTGGATGCCCAT
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C

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1748                                                    TGAGGCTCGGAGAGTTATGGAGCTC
1773 TGACAGGTTCCTATAGCTGCGTGCACCAGAGTCAAGAAACAGATCTTGACTCTAGGAATTTT
1835 GACTCTAACCCTCACACCTGCATCCCAAACAAGATGTTGACCAGAGTGACGACACATGGATT
1897 GACAAGAGGTTATGACCTCTTATTTAAGGCCTGGGTTTCAGGAGAATTAGATGAACCTAATG
1959 GCTCTTGGTGAGCCCTGGACAGTGTTCCCACAAAATCAAGATGGAAACATGTAAGTGTCTCA
2021 TTTGTTCTGGAAGAGGGCTTAAGGAAAGCTGAGCATCTTGGTCTATAAAGCTGGACTGGGGT
2083 CTGTAATTAAGAGGAAGCTTCATGGTCCTAGCGCCCAACACTCAAAGACAGGTGTGTGAATT
2145 CTTTGAGCATGGGATCTAATCATGGACAGCCCCATGTGCTTGCTTCCTCACAGAGGAGAGGC
2207 AGTTCGTTGTTCCAGCACAGACAACCAGCCTCTATAGGGGACCCTTCAAGGCTTTCTTGTGC
2269 CCTGGACCCTCACACTTATGCAGTCTGAATGCTGCTAGGGAAGTGGGTGATCCAGGTGAGGA
2331 GCCAGCGTCCAAGCAGTTGACAAGTTGCCCAAGATCTCACGAGAACTGACTTTTACCAGGTA
2393 GCCTGGGTGTGTCTGTCTAGCAGAGTCCTGAGAACATGTCTCTAGGTGTCCCATCATGATCA
2455 GCATCACACACCCAGCCTGTCCCTCGTTGGGGCTTCCCCACAGCATACTGCCCTCATAGCTC
2517 TCAAACACGTGGCTTCAGGAAGGGACCTGGCTCATATCGATGTTCTGATTAGAGAGACTGAG
2579 CAGCTTCATCAAGGAGCAGACACCTTGCATCTGCCTTAACTGGACAGAGACCCCAGCTTTCC
2641 CCATTCCACCAGGGCGAGGAAACAGCAGAGAGAAAAGCCCCAGGAGCCAACAGATCCTGAAG
2703 CTCTATGTGATGTAGAAGCTTGGGTCTTTCCATGGGGTCAGGGCTGCCTGGGAGCCAAGTTC
2765 CTTACAGCAGAGGGAAGGATGGGTGTGCAGAAGGCTG
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Fig. 1. Nucleotide sequence of the 5′ and 3′ regions of pSSRL cDNA. (A) Partial restriction map of pSSRL. Dashed boxes represent the cDNA fragments that comprise the sequences shown below. (B) Nucleotide sequence of pSSRL 5′ region. 210 nt of the 5′ untranslated region are shown along with the first 138 nt of the coding sequence. ATG translational initiation codon is boxed and the TAG termination codon 5′ upstream the ATG is upperlined. The sequence of the synthetic oligonucleotide used for the library screening and primer extension analysis is underlined. (C) Nucleotide sequence of pSSRL 3′ region. Asterisks at positions −72 and −71 (in B) and 1756–1757, 1762–1766 (in C) mark the points of discrepancy with the sequence published by Horikawa et al. [5], as discussed in the text.

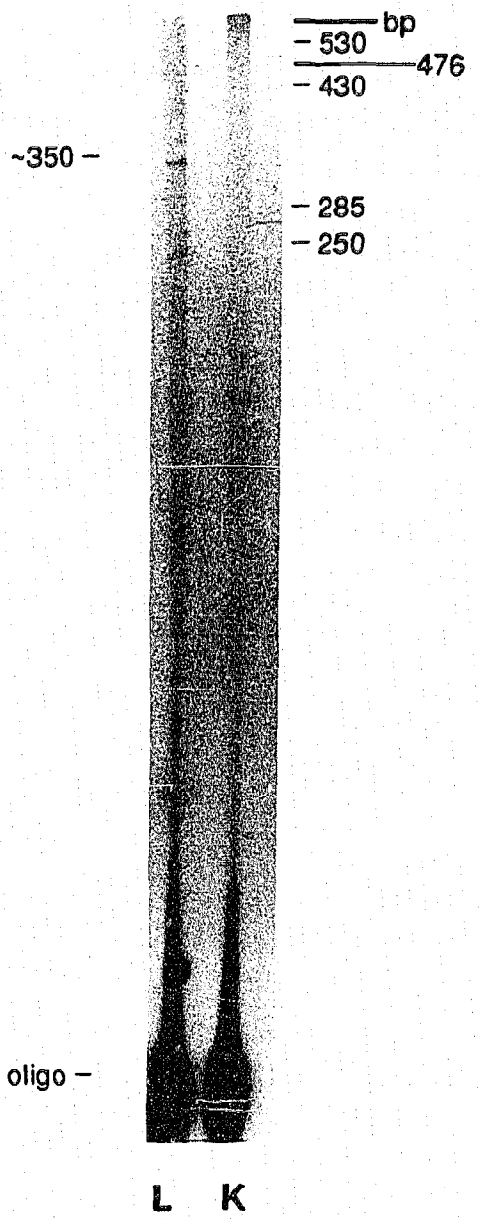

Fig. 2. Primer extension analysis of the mRNA corresponding to pSSRL cDNA. A synthetic primer complementary to nucleotides +61 to +78 of the AdoMet synthetase sequence was 5′ end ^{32}P-labelled and annealed with Poly(A)$^+$ RNA from rat liver (L) and kidney (K). Conditions were as described in section 2.

total RNA were denatured with glyoxal, separated by electrophoresis in 1.1% agarose gels and transferred to nylon membranes. Prehybridization and hybridization of the membranes were performed as described [10]. AdoMet synthetase probe (pSSRL) was labelled by nick translation to a specific radioactivity of 2×10^8 cpm/μg.

2.3. *Primer-extension analysis*

The primer extension procedure was performed essentially as previously described [11]. 5 pmol of the end-labelled oligonucleotide mentioned above were annealed to 5 μg of poly(A)$^+$ RNA either from rat liver or kidney by incubation at 85°C for 5 min and then at 42°C for 2 h in 0.1 M Tris-HCl buffer at 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 $MgCl_2$. 25 U of placental ribonuclease inhibitor and 50 U of AMV reverse transcriptase were added. Reverse transcription was carried out at 42°C for 90 min. After synthesis, nucleic acids were precipitated with ethanol and electrophoresed on 6% polyacrylamide gels containing 7 M urea.

2.4. *AdoMet synthetase purification on a thiopropyl-sepharose column*

A thiopropyl-sepharose column of 20 ml (12 × 1.5 cm) was prepared and equilibrated in 10 mM HEPES/Na, 10 mM $MgSo_4$, 1 mM EDTA, 50 mM KCl pH 8. High-M_r AdoMet synthetase (10 mg), purified as described by Cabrero et al. [3], was loaded on this column at a flow rate of 30 ml/h and 3 ml fractions were collected. The column was then washed with 50 ml of the equilibration buffer, followed by another 50 ml of the same buffer containing 5 mM cysteine. The elution was carried out with a 200 ml gradient from 5 mM to 20 mM cysteine in the equilibration buffer. AdoMet synthetase activity of all the fractions was assayed as described previously [3] and a pool of the active fractions was made. A sample of this pool was loaded on a 10% SDS-polyacrylamide gel under reducing conditions, and the electrophoresis was developed as described by Laemmly et al. [12].

3. RESULTS AND DISCUSSION

3.1. *Isolation of cDNA coding for rat liver AdoMet synthetase and primer extension analysis*

A lambda gt11 rat liver cDNA library was screened with a synthetic oligonucleotide complementary to 61–78 nucleotide residues of the AdoMet synthetase cDNA sequence previously reported [5]. Out of 200 000 independent recombinants, we obtained five positive clones which contained inserts ranging in size between 0.9 and 3.0 kbp. All of them were subcloned into PUC18 and the largest one, designated pSSRL, was subjected to further analysis. The sequencing strategy of the cDNA was performed according to both the reported restriction map [5] and that shown in Fig. 1. The nucleotide sequence was determined on both strands. A sequence comparison between our cloned cDNA (pSSRL) and that already published (RLAS) indicates that the former is about 1200 bp longer. pSSRL contains two additional fragments of 138 bp and 1029 bp beyond the 5′ and 3′ ends of RLAS respectively. The sequences of both fragments are shown in Fig. 1. The 3′ additional extension does not contain the poly(A) tail, and no regulatory element has been found. Differences between pSSRL and RLAS sequences in the overlapping region are marked by asterisks.

The 5′ non-coding region of RLAS contains 72 nucleotides. From nucleotide residue −70, both pSSRL and RLAS sequences are identical. However, they differ in two bases located at positions −72/−71. Since these residues are positioned just at the 5′ end of RLAS, it cannot be excluded that Horikawa's group have interpreted two nucleotides of the cloning vector as part of AdoMet synthetase sequence. As shown in Fig. 1, in the pSSRL 5′ non-coding region another ATG start codon upstream the putative ATG reported in RLAS has not been detected. Moreover, the presence of a TAG translational termination codon in the same frame upstream from that ATG has been observed (Fig. 1). Therefore,

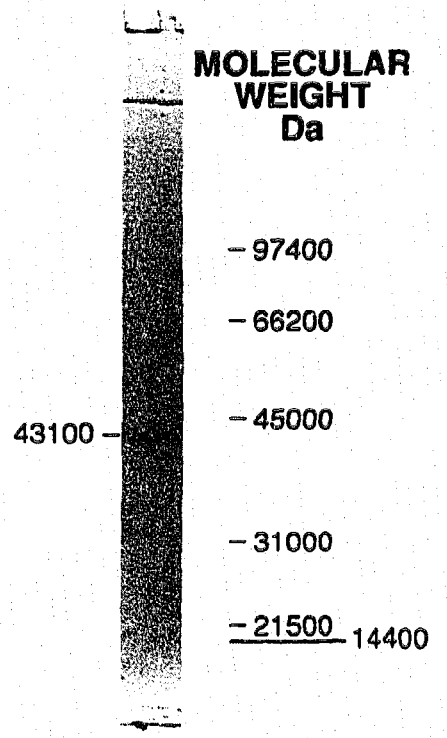

Fig. 3. SDS-polyacrylamide electrophoresis of high-M_r AdoMet synthetase purified on a thiopropyl-sepharose column. 50 μg of high-M_r AdoMet synthetase purified on a thiopropyl-sepharose column was loaded on a 10% SDS-polyacrylamide electrophoresis gel under reducing conditions. The molecular weight standards used were: phosphorylase B (97 400 Da), bovine serum albumin (66 200 Da), ovalbumin (45 000 Da), carbonic anhydrase (31 000 Da), soybean trypsin inhibitor (21 500 Da) and lysozyme (14 400 Da).

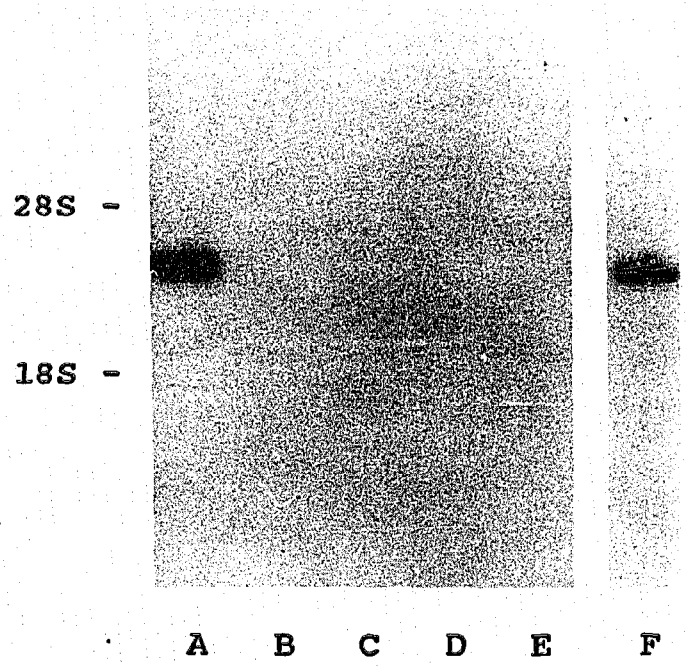

Fig. 4. Northern blot analysis of AdoMet synthetase mRNA in rat tissues and in human liver. Total RNA extracted from the tissues listed below was hybridized to the pSSRL probe. The RNA sources were: liver (A), kidney (B), spleen (C), heart (D), and brain (E) from rat and human liver (F). Lanes A–E of the blot were exposed for autoradiography for one day. The exposition of lane F lasted for 3 days.

the possibility of a larger form of AdoMet synthetase produced by the presence of another translational site can be excluded.

To further delineate the length of the 5′-untranslated sequence of AdoMet synthetase mRNA, a primer extension analysis was carried out. The oligonucleotide primer shown underlined in Fig. 1 was annealed to Poly(A)$^+$ RNA isolated from both rat liver and rat kidney and was extended towards the 5′ end of the corresponding mRNA by reverse transcription. As shown in Fig. 2, in the liver sample, the primer extension reaction yielded a product of about 350 nt, indicating that the 5′ non-coding region of the mRNA is about 270 nt long. Consequently, pSSRL contains almost the entire 5′-untranslated sequence of AdoMet synthetase mRNA. On the other hand, no fragment was extended when Poly(A)$^+$ RNA from kidney was used. A cDNA clone for a kidney AdoMet synthetase has been reported, that in Northern analysis was able to detect two mRNA species in this tissue, but did not hybridize to the liver RNA [13]. Since the liver and kidney AdoMet synthetase cDNA sequences seem to be different, the result obtained with the kidney sample on primer extension was expected. The primer extension analysis also determines in a precise way the occurrence of only one mRNA species coding for this protein in rat liver and further excludes the possibility of an alternative transcriptional initiation site, as has been proposed to take place in kidney [13].

3.2. *AdoMet synthetase purification on a thiopropyl-sepharose column*

The results shown above unequivocally establish that the molecular mass of AdoMet synthetase is that deduced from the cDNA sequence published [5]. However, in SDS-PAGE the purified protein shows a larger band, of about 48.5 kDa [3].

Due to the known importance of AdoMet synthetase sulfhydryl groups [14,15] in maintaining the enzyme structure and activity, the high-M_r form of the enzyme was purified as described in [3], to then be chromatographed on a thiopropyl-sepharose column. After its elution with a cysteine gradient, AdoMet synthetase activity was determined (data not shown), the active fractions pooled and a sample loaded on a 10% SDS-PAGE gel under reducing conditions. The molecular weight of the protein band obtained by this procedure was about 43 kDa (Fig. 3), a result that is consistent with the M_r deduced from the cDNA sequence. The reason for the modification in the electrophoretic be-

haviour of the enzyme afte: its chromatography on the thiopropyl-sepharose column is not clear, but might be related to the role of sulfhydryl groups in maintaining the activity and structure of the enzyme [14,15].

3.3. *Northern blot analysis*

pSSRL was used as a probe for Northern analysis of AdoMet synthetase mRNA in various rat tissues and in human liver (Fig. 4). In rat, a strong hybridization signal corresponding to an mRNA species of about 3.4 kb was detected only in liver, in agreement with data previously reported [5,13] and with primer extension results. A single hybridization band of similar size was obtained when total RNA from human liver was analyzed, although this signal was weaker than that observed in RNA samples from rat liver. A high homology between rat and human liver AdoMet synthetase mRNA sequences could be expected, since an extensive similarity among Escherichia coli, yeast and rat liver has also been found [5,16–18]. Thus, this finding indicates that a rat liver cDNA can be used as a probe for studies on AdoMet synthetase mRNA regulation in human liver. This might be important in view of the known alterations in AdoMet metabolism in human liver diseases [2,19–21].

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