Vol. 189, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1992 Pages 424-429

MOLECULAR CLONING OF THE MOUSE S-ADENOSYLMETHIONINE DECARBOXYLASE cDNA: SPECIFIC PROTEIN BINDING TO THE CONSERVED REGION OF THE mRNA 5'-UNTRANSLATED REGION

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Received October 15, 1992

SUMMARY: The nucleotide sequence of a cDNA encoding the proenzyme of mouse S-adenosylmethionine decarboxylase (AdoMetDC) including 257 nucleotides of the 5' untranslated region has been determined. Comparison of the nucleotide sequence of the mouse 5' untranslated region with those of other mammals shows it to be highly conserved. The 52 nucleotides upstream from the translation initiation codon are identical in human, rat, bovine and mouse. The polyamines, spermidine and spermine, have been shown to inhibit AdoMetDC mRNA translation. An RNA gel retardation assay demonstrated that a cytoplasmic extract from mouse brain forms an RNA-protein complex with the completely conserved 5' untranslated sequence and that the complex formation is highly dependent on the presence of spermine. Crosslinking by UV irradiation shows that the complex contains a 39-kDa protein interacting with the 5' untranslated sequence. These data demonstrate spermine-dependent specific protein binding to a highly conserved 5' untranslated region of an mRNA translationally regulated by polyamines.

S-Adenosylmethionine decarboxylase (EC 4.1.1.50) is a key enzyme in the biosynthesis of polyamines (1-3). The mammalian enzyme is known to be activated by putrescine and to contain a pyruvate prosthetic group (4-7). Complete cDNA sequences for eukaryotic AdoMetDCs have been reported from human (8), rat (8, 9), hamster (10), bovine (11) and yeast (12). These clones contained an open reading frame corresponding to a polypeptide of about M_r 38,000. Translation of mRNA isolated from rat prostate and of RNA transcribed from rat and human cDNAs in a reticulocyte lysate system indicated that the enzyme is synthesized as a proenzyme of this size which is then processed in a putrescine-stimulated reaction to yield two subunits of approximate M_r 32,000 and 6,000 (8, 13-15). Both polypeptides are precipitable by antiserum to AdoMetDC indicating that the smaller subunit does remain as part of the final enzyme (8). The longer polyamines, spermidine and spermine, do not affect this conversion, but there is good evidence from a number of laboratories that the activity of AdoMetDC is increased in cells depleted of spermidine and spermine by treatment with inhibitors. Conversely, the administration of these polyamines leads to a rapid decline in AdoMetDC activity (2, 5).

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0006-291X/92 $4.00

Determination of the content of AdoMetDC protein by radioimmunoassay has indicated that the changes in activity in response to the polyamines are mediated by changes in the amount of enzyme protein. These changes involve both regulation of AdoMetDC synthesis and the rate of degradation of the protein (16). The increased synthesis of AdoMetDC when polyamine concentrations are reduced is produced by at least two factors: an increase in the content of AdoMetDC mRNA and a change in the translation efficiency of the mRNA. The latter is firmly supported by experiments in which it was found that the synthesis of AdoMetDC in reticulocyte lysates was strongly inhibited by spermidine or spermine (17). Inhibition was produced by concentrations of these polyamines which did not reduce the synthesis of other proteins including albumin. Thus, it appears that spermidine and spermine selectively inhibit the translation of the AdoMetDC mRNA. This effect may result from an interaction between the mRNA and the polyamines themselves or with a protein influenced by polyamines (17). Several models can be considered. In one, the secondary structure in the mRNA alone is responsible, and its stability may be controlled by the intracellular polyamine concentration. A second model is more analogous to the mechanism proposed for the regulation of the translation of ferritin mRNA which involves the binding of a specific protein to the 5'-untranslated region (5'-UTR) (18, 19). Finally, more complicated models may involve the interaction of the leader sequence with downstream regions such as the coding sequence or the 3'-untranslated region.

As a first step toward an understanding of the mechanisms regulating AdoMetDC mRNA translation, we have cloned a mouse cDNA and examined the possible involvement of a specific protein factor interacting with the 5' leader sequence of the mRNA.

MATERIALS AND METHODS

A mouse brain cDNA library (Clontech, Palo Alto, CA) in λgt10 vector was screened with a nick-translated 1013-bp *Pst*I-*Pst*I fragment of pSAMr1 corresponding to the coding sequence of the rat AdoMetDC (8). The initial screening yielded 2 positive clones, H1 and H2. Their sequencing showed that they are copies of two different mRNAs. H1 (937 bp) corresponds to the shorter mRNA (8) and contains about half of the coding sequence. This sequence is followed by a poly A tail. H2 corresponding to the 3' region of the larger mRNA (8, 9) is 2235 bp longand starts from the same internal *Eco*RI site as H1.To obtain cDNA corresponding to the 5' region of the mRNA, mouse brain poly(A)$^+$RNA was reverse-transcribed, and amplified by PCR using 5' primer (5'-AAGAGACTGAACTGTATCTGC-3') base sequence of the rat 5'-UTR and 3' primer (5'- GATCAGCATCTAGAAAGAGCA-3') derived from the mouse cDNA. Both contain additional *BamH*I sites. The resulting PCR product was ligated into puC19 and double-stranded DNA was sequenced by the dideoxy chain termination method using Sequenase^{TM} (USB Inc.). The PCR clone (PCR1) was 1338 bp long covering 257 bp of 5' untranslated region and 1081 bp of coding region. PCR1 overlaps the H1 and H2 by 459 nucleotides.

Using the pGEM-4Z vector, two constructs were made from λgtSAMr1 (9): pGEM-4Z-729 covering the 5'-UTR and 456 bp of the coding region, and pGEM-4Z-1164 covering 50 bp of the 5'-UTR and 1114 bp of the coding region.

The mRNA probes were made by linearizing pGEM-4Z-729 and pGEM-4Z-1164 plasmids with *Sfu*I, and RNAs were synthesized with SP6 polymerase to produce 291-base (p291), or 72-base (p72) run-off transcripts, respectively. Transcription reactions were performed according to the Promega protocol. Labeled RNA transcripts were made by inclusion of [α-^{32}P]CTP at 150 Ci/mmol (1 Ci = 37 GBq).

A mouse brain cytosolic extract (S100) was prepared by the procedure of Dignam *et al.* (20). Binding reactions were carried out with the S100 cytoplasmic extract (8 μg of protein) and 10,000 cpm (0.2-0.5 ng) of [^{32}P]RNA in 10 mM Hepes, pH 7.6/3 mM $MgCl_2$/40 mM

KCl/5% glycerol/1 mM dithiothreitol in a volume of 20 µl. After incubation at 25 ^{o}C for 30 min, 1 unit of RNase T1 was added for 10 min, followed by 5 mg of heparin per ml for 10 min, both at the temperature of the binding reaction. For the RNA-protein UV cross-linking assay, the reaction mixtures were cross-linked with 254 nm UV irradiation (250 mJ/cm^2) using a Stratalinker UV cross-linking apparatus and were then separated by electrophoresis on 10 % SDS-polyacrylamide gels under reducing conditions. For the RNA gel shift assay, electrophoresis of RNA-protein complexes was carried out using a low-ionic-strength-PAGE as described by Konarska and Sharp (21). Gels were dried and exposed to film at -70^{o}C.

RESULTS AND DISCUSSION

Screening of a mouse brain cDNA library with a 1013 bp rat AdoMetDC cDNA fragment (PstI/PstI digested pSAMr1)(8) led to the isolation of cDNA clones H1 and H2. Sequencing studies showed that these clones did not represent the entire cDNA but were incomplete at their 5' ends. To extend the sequence at the 5' end, reverse transcription followed by PCR was performed using an upstream primer identical to the rat RNA sequence (9) and a downstream primer complementary to the mouse RNA sequence. A PCR product of expected size (1338 bp) was obtained which overlapped the H1 and H2 by 459 nucleotides. The entire nucleotide sequence of the mouse AdoMetDC cDNA has been deposited in the EMBL data library under the accession number Z14986. The cDNA contained 257 nucleotides of 5' untranslated region, 1002 nucleotides coding for a protein of 334 amino acids and 1852 nucleotides of the 3' untranslated region. The nucleotide sequence of the coding region of the mouse AdoMetDC cDNA exhibited about 96 % homology with the human (8) and bovine (11) sequences and about 97 % homology with the rat (8, 9) and hamster (10) sequences. When the deduced amino acid sequence of mouse AdoMetDC proenzyme was compared with human , rat, hamster and bovine equivalents, the mouse sequence showed 8 amino acid differences with the human sequence, 7 differences with the bovine sequence, and only 4 and 2 differences with the rat and hamster sequences, respectively. Thus most of the differences in the nucleotide sequence are silent. The high level of conservation of the AdoMetDC sequence may reflect the multiple functions of the enzyme protein including the autocatalytic processing of the proenzyme, enzymatic activity, regulation by putrescine and rapid protein turnover.

The 3' untranslated region sequences within these species are 80-85 % homologous. However, there is a striking conservation of the 5' untranslated sequences between the human, bovine, rat, hamster and mouse. The comparison of the 5'-UTR sequences shows that there are only two regions where the homology ceases (Fig. 1). The first one is the run of A residues (between nt 123 and 138 in the mouse) the length of which differs from 10 in the bovine to 16 present in the mouse. The second homology interruption in the human and bovine mRNAs compared with those of the rat and mouse corresponds to mouse positions 59-70. The length of 4the published hamster (10) leader sequence is 169 nt, and it is also highly conserved containing only 6 mismatches as compared with the corresponding sequence of the mouse. The 5'-UTR sequence of mouse AdoMetDC mRNA showed 91 - 98 % homology with the corresponding rat, human, bovine and hamster sequences. However, the sequences downstream from the run of A residues are practically identical giving the homology percentage of 98-99. Thus this region represents a potential target for polyamine-mediated translational regulation.

To investigate *in vitro* whether the leader sequence of AdoMetDC mRNA binds to a specific cytoplasmic factor, we analyzed the formation of RNA-protein complexes by

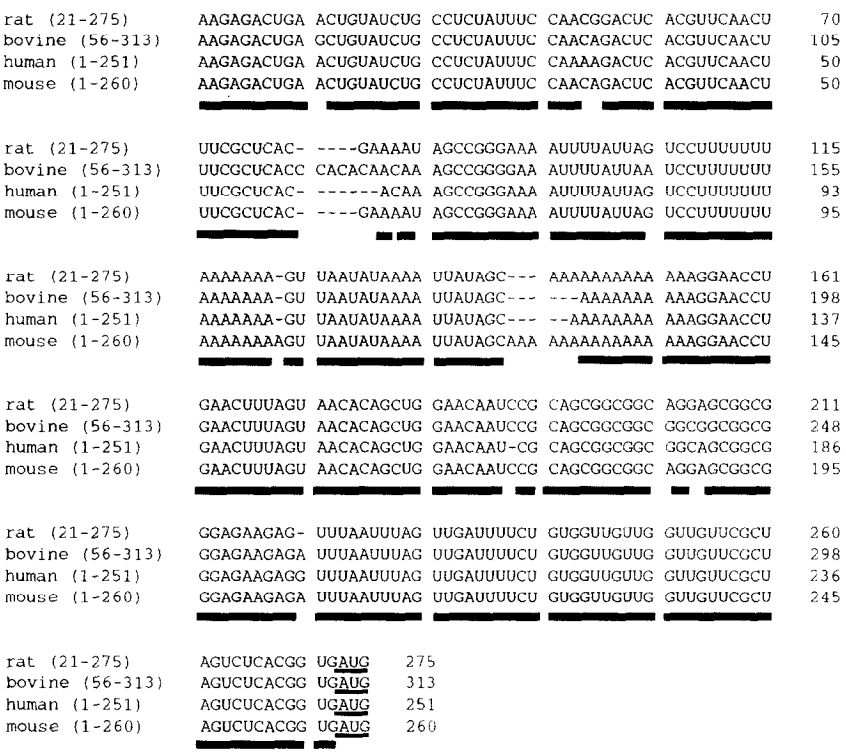

Fig. 1. Comparison of mammalian AdoMetDC mRNA 5'-UTR. Positions of identity in all four species are identified by black horizontal bars under the sequence. Translational initiation codons (AUG) are underlined.

nondenaturing gel electrophoresis (21). A cytoplasmic protein extract prepared from mouse brain was incubated with ^{32}P-labeled transcripts corresponding to the 5' region of AdoMetDC mRNA. To remove unspecific RNA-protein complexes, the samples were digested with RNase T1 and subsequently incubated with heparin (18). Only RNA fragments that are tightly associated with protein remain protected from RNase digestion and are resolved by nondenaturing polyacrylamide gel electrophoresis. The experiments clearly revealed the formation of a complex between a cytoplasmic factor and the p291, and that the RNA-protein binding is highly dependent on the presence of spermine (Fig. 2). The specificity of binding was tested in competition gel shift assays using unlabeled p291 as a specific competitor and a 721-base pGEM-RNA as a nonspecific competitor (Fig. 2). With a 50-fold molar excess of the specific competitor, the complex begins to disappear. In contrast, pGEM-RNA is not competitive even at higher concentrations (200-fold molar excess). This suggests that the binding of the factor is specific.The complex formation can be abolished by the pretreatment of S100 extract with proteinase K or by heat-inactivation which indicates that the binding factor is at least partly protein in nature.

In order to determine whether the sequence responsible for the protein binding is located in that part of 5'untranslated region which is identical in all four species (human, rat, mouse and bovine), i.e. 52 ribonucleotides upstream from the translation initiation codon, p72 containing 50 ribonucleotides from 5'-UTR and19 ribonucleotides downstream from AUG was

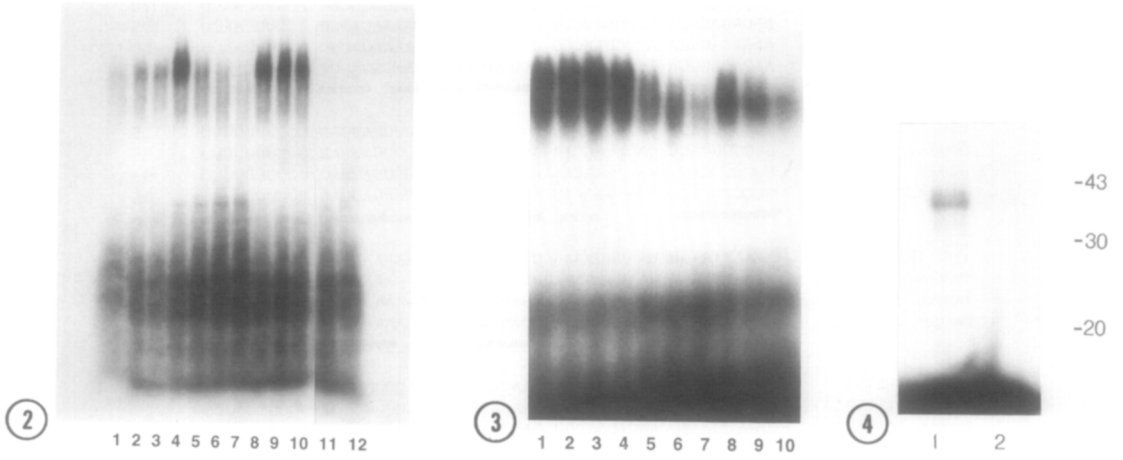

Fig. 2. Gel shift assay with the p291 transcript. RNA-protein complex formation between AdoMetDC 5'-UTR and mouse brain cytoplasmic protein, the effect of polyamines and the specificity of the complex formation. The binding reaction was performed with 8 μg of cytoplasmic extract (S100) and about 10,000 cpm of the ^{32}P-labeled p729 in 10 mM Hepes, pH 7.6, 3 mM $MgCl_2$, 40 mM KCl, 10% glycerol, 1 mM DTT for 30 min at room temperature, after which 1 U of RNase T1 was added for 10 min, followed by 5 mg of heparin per ml for 10 min, both at the temperature of the binding reaction. Electrophoresis of the RNA-protein complexes was carried out on a 4% nondenaturing polyacrylamide gel. Lane 1, no polyamine; lane 2, 1 mm putrescine; lane 3, 1 mm spermidine; lane 4, 1 mm spermine; lane 5, 1 mm spermine and 50-fold molar excess of unlabeled p291 (specific); lane 6, 1 mm spermine and 100-fold molar excess of p291; lane 7, 1 mm spermine and 200-fold excess of p291; lane 8, 1 mm spermine and 50-fold molar excess of unlabeled 720-base pGEM-RNA (unspecific); lane 9, 1 mm spermine and 100-fold excess of 720-base pGEM-RNA; lane 10, 1 mm spermine and 200-fold molar excess of 720-base pGEM-RNA; lane 11, same as in lane 4 but S100 extract treated with proteinase K (100 μg/ml) before incubation; lane 12, same as in lane 4 but S100 extract heat-inactivated before incubation (boiled for 1 min).

Fig. 3. Gel shift assay with p72 transcript. RNA-protein binding reactions with p72 were perfomed as described in the legend to Fig. 2 using mouse brain S100 extract (8 μg protein). Lane 1, 1 mm spermine ; lane 2, 1 mm spermine and 50-fold molar excess of unlabeled 720-base pGEM-RNA (unspecific); lane 3, 1 mm spermine and 100-fold excess of 720-base pGEM-RNA ; lane 4, 1 mm spermine and 200-fold molar excess of 720-base pGEM-RNA; lane 5, 1 mm spermine and 50-fold molar excess of unlabeled p72 (specific); lane 6, 1 mm spermine and 100-fold molar excess of p72; lane 7, 1 mm spermine and 200-fold excess of p72; lane 8, 1 mm spermine and 50-fold molar excess of unlabeled p291 (specific); lane 9, 1 mm spermine and 100-fold excess of unlabeled p291; lane 10, 1 mm spermine and 200-fold molar excess of unlabeled p291.

Fig. 4. UV-Crosslinking and SDS-PAGE of the RNA-protein complex formed with p72-RNA and S100 extract from the mouse brain (lane 1). Lane 2, same as in lane 1 but S100 extract treated with proteinase K (100 μg/ml) before incubation. The complex formation reaction and UV-crosslinking were carried out as described under "Materials and Methods". Molecular mass markers are indicated in kDa.

used in the binding reaction (Fig. 3). Because p72 is also able to form a complex with the cytosolic factor, and both p291 and p72 are effective competitors for that binding, in contrast to a 721-base pGEM-RNA, it is apparent that the binding site is located inside the 72-base sequence.

To identify a protein binding to the 5'-sequence, p72 was incubated with S100 extract from the mouse brain followed by the UV-crosslinking and SDS-PAGE under reducing

conditions (Fig. 4). The p72 probe labeled a mouse brain cytoplasmic protein that migrated at 39 kDa. When the binding reaction was performed with proteinase K treated extract, the 39 kDa RNA complex did not appear.

The binding of a cytoplasmic protein to the 5'-UTR of AdoMetDC mRNA in the presence of spermine may serve as a mediator of a polyamine-dependent depression of AdoMetDC translation. The exact localization of the target sequence and the purification of the protein will help in determining the quantitative affinity of the mRNA-protein interaction and the functional consequences of the complex formation.

ACKNOWLEDGMENTS

This research was supported by grants from the National Research Council for Natural Sciences, Academy of Finland and the Cancer Society of Finland.

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