Volume 291, number 2, 289–295 FEBS 10276 October 1991

ADONIS 001457939100985Q

Structure and organization of the gene encoding rat *S*-adenosylmethionine decarboxylase

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Received 20 August 1991

The gene for *S*-adenosylmethionine decarboxylase (AdoMetDC) was isolated from a rat genomic library using AdoMetDC cDNA as a probe Nucleotide sequence analysis shows that the rat AdoMetDC gene consists of 8 exons which encode a protein identical to that inferred by a rat AdoMetDC cDNA sequence The exons range in length from 43 to 1964 base pairs spanning 15672 bases of chromosomal DNA All of the exon/intron junctions were found to conform to the consensus splice donor and acceptor sequences Exon 8 corresponds to the 3′ noncoding region of the 2 species of AdoMetDC mRNA which are formed by alternative utilization of 2 polyadenylation signals separated from each other by 1272 nucleotides The transcription initiation site was located by S1 nuclease protection and by primer extension analysis, −325 nucleotides upstream of the translation initiation codon The promoter region of the rat AdoMetDC gene contains a TATA box at −29 base pairs No typical GC or CAAT boxes are located in the promoter, but six GC boxes and several putative binding sites for both tissue-specific and non-specific transcription factors are found in the proximal part of intron 1 Southern blot analyses reveal a complex hybridization pattern suggesting that there are multiple copies of the AdoMetDC gene in the rat genome

S-Adenosylmethionine decarboxylase, Gene, Rat

1. INTRODUCTION

S-Adenosylmethionine decarboxylase (AdoMetDC; EC 4 1.1.50) is a key enzyme in polyamine biosynthesis Its product, decarboxylated AdoMet, serves as an aminopropyl donor in spermidine and spermine synthesis [1,2] The mammalian enzyme is synthesized as an inactive precursor of about M_r 38 000, which is converted to the active enzyme with subunits of approximately M_r 31 000 and 7500 by a putrescine-stimulated mechanism [3–6] The larger subunit contains a covalently bound pyruvate prosthetic group [7,8], which is formed during cleavage by a serinolysis reaction [9] AdoMetDC expression is not tissue-specific, therefore this enzyme may be encoded by a housekeeping gene. However, unlike most enzymes of this class, the activity of AdoMetDC is highly subject to regulation by trophic stimuli [10,11]. Three classes of regulatory phenomena apparently underlie changes in AdoMetDC activity. These include changes in the amount of AdoMetDC mRNA either by increasing the transcription rate or by a stabilization of the mRNA [3,12,13] In addition, changes occur in the translation efficiency of the mRNA [14,15] and in the intracellular stability of the enzyme itself [16,17] One or more of these steps is negatively regulated by the cellular spermidine and spermine concentrations [17]. However, the exact mechanism by which the polyamines interfere with AdoMetDC synthesis is not well understood

As a first step toward an understanding of the mechanisms regulating AdoMetDC gene expression, we have analyzed the organization of the rat AdoMetDC gene Previously we have described the isolation of a processed pseudogene coding for rat AdoMetDC [18] The present work reports the molecular cloning and characterization of a rat gene that codes for a functional AdoMet decarboxylase

Abbreviations AdoMetDC, *S*-adenosylmethionine decarboxylase, AdoMet, *S*-adenosylmethionine, kb, kilobase pairs, PolIII, DNA-dependent eukaryotic RNA polymerase III

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2 EXPERIMENTAL

2 1 *Enzymes and chemicals*

Restriction enzymes were purchased from New England Biolabs and Boehringer-Mannheim The Sequenase sequencing kit was from the United States Biochemical Corporation and the radiolabeled nt were from Amersham Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer The rat genomic *Bam*HI partial library in Lambda DASH was purchased from Stratagene

2 2 *Isolation of genomic clones of rat AdoMetDC*

About 3×10^5 plaques of the rat genomic library were screened by plaque hybridization [19] The library was initially screened with a nick-translated 1013-bp *Pst*I–*Pst*I fragment of pSAMr1 corresponding to the coding sequence of the rat AdoMetDC cDNA [3] DNA was purified by cesium chloride density gradient centrifugation [19] Purified positive clones were characterized by restriction mapping,

and suitable restriction fragments were subcloned into pUC19 vector for further restriction mapping

2 3 *DNA sequence analysis*

Single-stranded and double-stranded DNA sequencing were performed by the dideoxy chain termination method after subcloning fragments into M13 or pUC19 vectors, respectively [20] All DNA fragments were sequenced on both strands with the exception of those regions which were identical to previously published AdoMetDC cDNA sequences [3,18] DNA sequences were analyzed using the Microgenie computer program (Spinco Division, Beckman Instruments, Inc, Palo Alto, CA)

2 4 *Genomic Southern blot analysis*

DNA was isolated from rat brain as described by Maniatis et al [19] Genomic DNA (10 μg) was digested with restriction endonucleases (5 units/μg), subjected to 1% agarose gel electrophoresis and blotted onto nitrocellulose membranes by the procedure of Southern [21] The membranes were then hybridized with [^{32}P]cDNA probes for AdoMetDC

2 5 *Primer extension*

Primer extension analysis was performed in modification of a procedure by Boorstein and Craig [22] An single-stranded DNA fragment from restriction endonuclease digest (a *Pvu*II–*Ppu*MI fragment 187 nucleotides in length) was used in a primer extension experiment The fragment was end-labeled with [γ-^{32}P]dATP and T4 polynucleotide kinase (New England Biolabs) and was annealed to RNA at 50°C for 3 h in 50 μl of 80% (v/v) formamide, 0 4 M NaCl, 10 mM PIPES (pH 6 4), 2 mM EDTA The hybrid was precipitated in ethanol and dissolved in 40 μl of a buffer containing 10 mM Tris-HCl (pH 8 3), 10 mM dithiothreitol, 6 mM $MgCl_2$, and 1 mM of each of the four dNTPs Primer extension reaction was done at 42°C for 1 h in the presence of 20 units of avian myeloblastosis virus reverse transcriptase (Promega) The product was electrophoresed on a 6% denaturing polyacrylamide gel The poly(A)RNA was prepared from Sprague–Dawley rat prostate as described previously [23,24]

2 6 *S1 Nuclease protection assay*

A 503 nucleotides single-stranded *Sca*I–*Ppu*MI fragment labeled at the 5′ end with [γ-^{32}P]dATP and T4 polynucleotide kinase was annealed to 15 μg of poly(A)RNA in 50 μl of 80% formamide (v/v), 0 4 M NaCl, 40 mM PIPES (pH 6 4), 1 mM EDTA at 58°C for 16 h [25] The hybridization mixture was digested with 300 units of S1 nuclease (Boehringer-Mannheim) in 0 5 ml of 0 28 M NaCl, 0 05 M sodium acetate (pH 4 5), 4 5 mM $ZnSO_4$, 16 μg/ml single-stranded calf thymus DNA at 30°C for 1 h The reaction mixture was extracted with phenol/chloroform, precipitated with ethanol, and electrophoresed on 6% denaturating acrylamide gel

→

Fig 2 Nucleotide sequence of the 5′-upstream region of the rat AdoMetDC gene Numbers on the left refer to nucleotide positions within the rat gene starting at the site (+1) of transcription initiation, and the nucleotides 5′ to residue 1 are indicated by negative numbers The protein translation is shown above the first exon, with dashes above the untranslated sequences The gene sequences homologous to the transcription factor DNA-binding sites are underlined and indicated These include one TATA box, one C/EBP-binding site, 6 GC boxes, 2 CCAAT elements, 2 octamer core-like sequence motifs, one 'AP-2-like' element, one SV40 core enhancer sequence and one 'BTE' element Five repetitive elements are also underlined including one MT-4 repeat, 2 type B1 repeats and one type B2 repeat in the 5′ flanking region, and one inverse type B2 repeat within the first intron The sequences representing the A and B boxes of the split polymerase III promoter are overlined The flanking direct repeats are underlined with dashes

3 RESULTS AND DISCUSSION

A genomic λ phage library was screened using nick-translated fragments of the AdoMetDC cDNA to isolate the rat AdoMetDC gene. The initial screening was performed using a probe covering the coding sequence of the cDNA Two recombinant colonies yielded positive hybridization signals and the clones λAdoMetDC 62 and λAdoMetDC 33 were isolated Restriction frag-

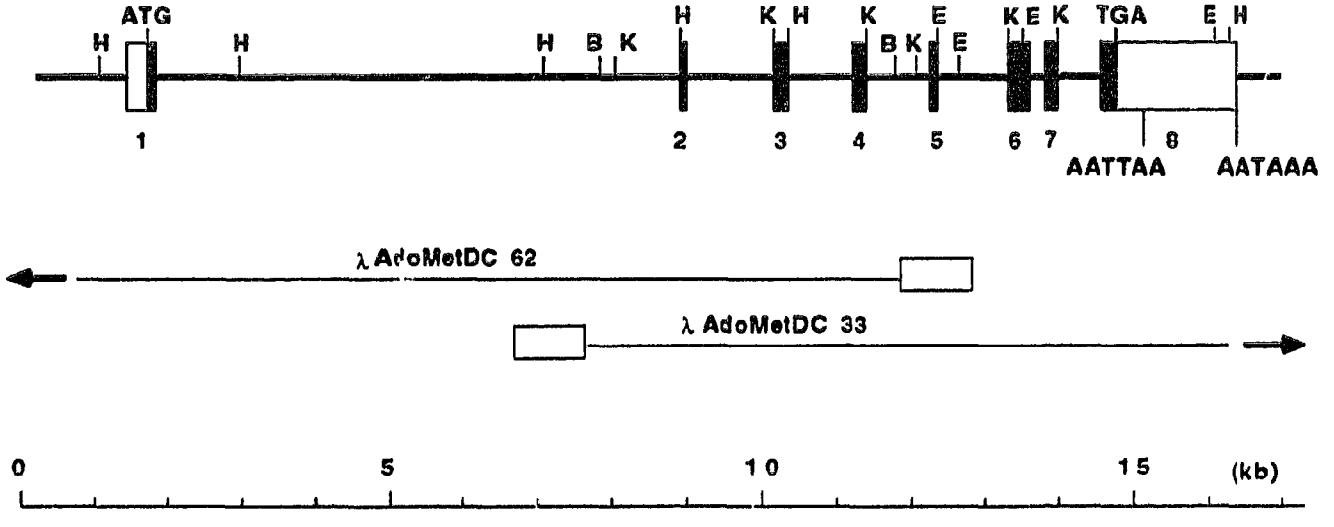
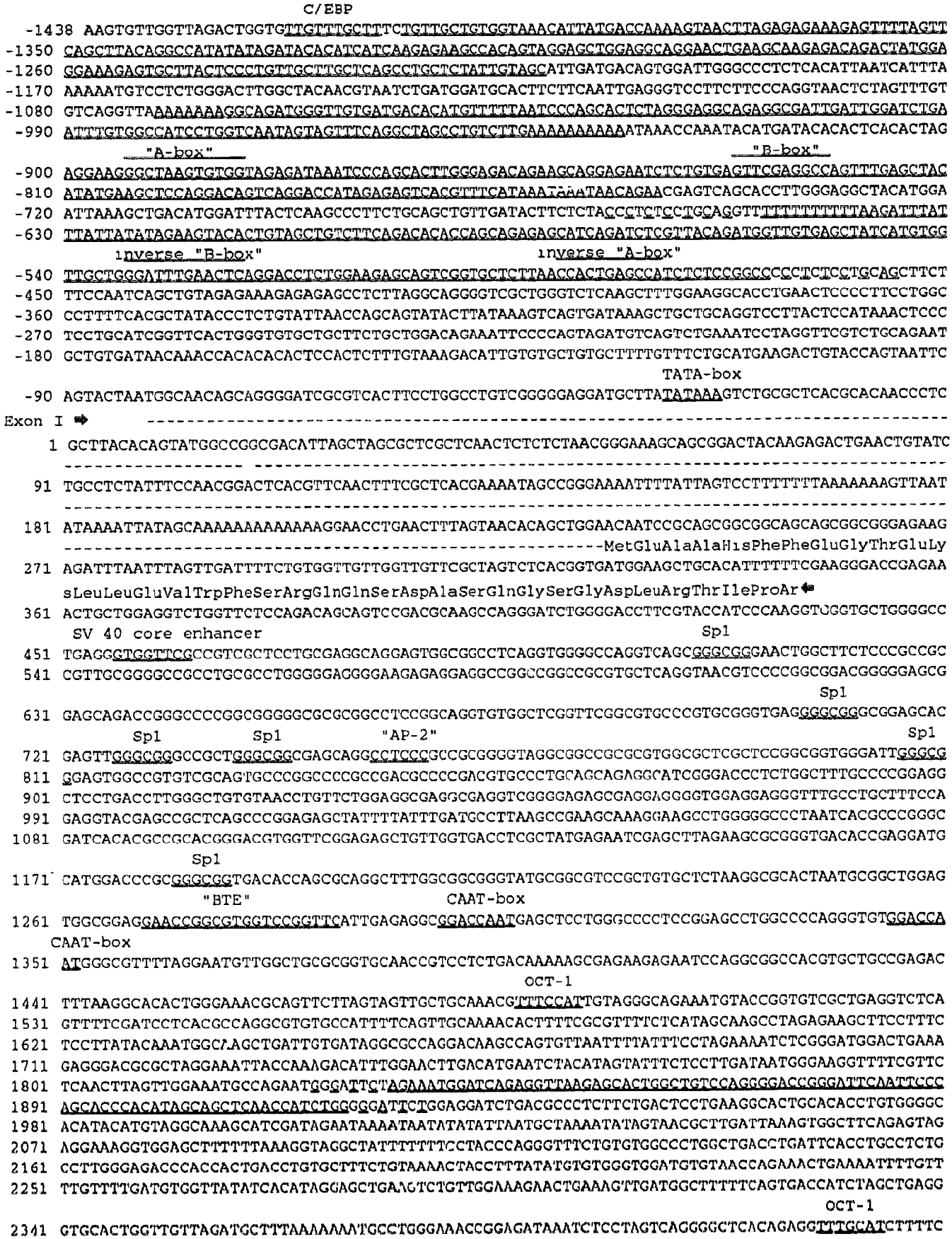


Fig 1 Structure of the rat AdoMetDC gene Two overlapping rat genomic clones (λAdoMetDC 62, λAdoMetDC 33) spanning over 20 kb were isolated and characterized as described in Section 2 *Top*, a composite map of the rat AdoMetDC gene is shown with restriction sites located as indicated H, *Hin*dIII; E, *Eco*RI, K, *Kpn*I, B, *Bam*HI The exons are depicted by boxes, and they are numbered starting from the 5′-end of the gene The coding sequences are shown by solid boxes and the noncoding sequences are shown by open boxes The introns and flanking sequences are depicted by solid lines. Exons were located by restriction mapping and sequencing A scale in kilobases is shown below.


C/EBP
"A-box"
"B-box"
inverse "B-box"
inverse "A-box"
TATA-box
Exon I
SV 40 core enhancer
Sp1
"AP-2"
"BTE"
CAAT-box
OCT-1

ment mapping and Southern blot analysis revealed that these clones partially overlap

A schematic representation of the rat AdoMetDC gene is presented in Fig. 1 The overlapping λ clones span over 20 kb. Restriction digests of the clones using the enzymes *Eco*RI, *Hin*dIII and *Kpn*I established a partial restriction map of the rat AdoMetDC gene locus The linear order of the exons and intervening intron sequences was deduced from a detailed sequence analysis of subcloned genomic AdoMetDC-specific fragments. The nucleotide sequence of the 5'-upstream region is shown in Fig. 2 The entire nucleotide sequence of the rat AdoMetDC gene has been deposited in the EMBL data library under the accession number M64274 The gene consists of 8 exons and 7 introns which obey the gt-ag splice rule [26] (Fig 3) The 5' noncoding region of the AdoMetDC mRNA is represented by 325 nucleotides in the first exon (Fig 2 and Table I) The rest of exon 1, and exons 2 through 7, as well as the first 135 nucleotides of exon 8 encode the AdoMetDC protein The nucleotide sequence is identical to the coding region of the AdoMetDC cDNA [3,18] The remaining part of exon 8 corresponds to the 3' noncoding region of the two AdoMetDC mRNAs detected in rat cells [3] which apparently are formed by alternative utilization of 2 polyadenylation signals separated from each other by 1272 nucleotides [18]

The transcriptional start site of the rat AdoMetDC gene was determined by a combination of primer extension and S1 nuclease mapping procedures because the 5' end of the rat AdoMetDC cDNA is truncated [18] The result indicated that AdoMetDC mRNA transcription is initiated at a G residing 325 nucleotides upstream from the translation start site (Fig 4). This is in agreement with the finding that ΨAdoMetDC borders by the flanking direct repeat at the same nucleotide ([18] and Fig. 5)

The promoter regions of housekeeping genes are frequently characterized by one or more of the following features segments with a high content of G+C residues (GC boxes), especialy within the first 100 bp of the 5'-flanking DNA [27], absence of a typical TATA box just upstream of the start site [28], and the presence of octamer-like core sequences [29,30] The region of the rat AdoMetDC gene 5' to the transcription initiation

Table I

Exons of the rat AdoMetDC gene and their relation to the functional regions of AdoMetDC mRNA

Exon I	435 nucleotides	325 noncoding
		110 coding
Exon II	87 nucleotides	87 coding
Exon III	127 nucleotides	127 coding
Exon IV	103 nucleotides	103 coding
Exon V	43 nucleotides	43 coding
Exon VI	238 nucleotides	238 coding
Exon VII	156 nucleotides	156 coding
Exon VIII	672 nucleotides (mRNA I)	135 coding
		537 noncoding
	1944 nucleotides (mRNA II)	135 coding
		1809 noncoding

Total	mRNA I	1861 nucleotides
		5' noncoding 325 nucleotides
		coding 999 nucleotides
		3' noncoding 537
	mRNA II	3117 nucleotides
		5' noncoding 325 nucleotides
		coding 999 nucleotides
		3' noncoding 1793 nucleotides

The length of the exons of the rat AdoMetDC gene is presented and their relation to the 5' 3' noncoding and the coding region of the 2 species of AdoMetDC mRNA indicated

site, however, contains a TATA box at −29 but no GC-boxes or octamer-like motifs (Fig. 2) The only additional consensus sequence in the 5'-flanking region revealed by a computer search was TTGTTTGCTT, which is similar to the previously characterized HBV enhancer E site [31] and C/EBP-binding site (CCAAT/enhancer-binding protein) of OTC enhancer [32]

The sequence of the first intron, however, contained several known enhancer elements and recognition sequences for transcription factors Recently, a functional role has been demonstrated for first intron sequences of the human β-actin gene [33] and the mouse α-1 type I collagen gene [34] The proximal region of the first intron contains six SP1-recognition sequences and two CAAT box-like sequences (GGACCAAT) Moreover, there are two octamer core-like sequence motifs (TTTCCAT and TTTGCAT) and two repeats of the hexanucleotide 'CCTCCC' ('AP-2-like') which have been suggested to have enhancer function in some viral

CAAG gtgggtgctggggcc	IVS-1	7460 nucleotides	gtttgttggtgacag ATCC
TCAG gtaggtgctgtgcat	IVS-2	1093 nucleotides	ctttttctgttgcag TGAG
TCAA gtaagtacgcagact	IVS-3	970 nucleotides	tctctttttttttaag AGCT
CCAA gtaagtaaacctaga	IVS-4	1146 nucleotides	cctctttcctcacag ACGG
GTTG gtatgtggttttagt	IVS-5	1104 nucleotides	tttacttgtacatag GTAC
GGAT gtgagtggctcgcag	IVS-6	100 nucleotides	taacttttctctaag GGAA
TCAG gtaccttgacctctg	IVS-7	639 nucleotides	ttttcttttccccag AGTT

Fig 3 Exon intron organization of the rat AdoMetDC gene Exon and intron sequences are represented by capital and lower-case letters, respectively. IVS stands for intervening sequence The numbers after each IVS refer to their length

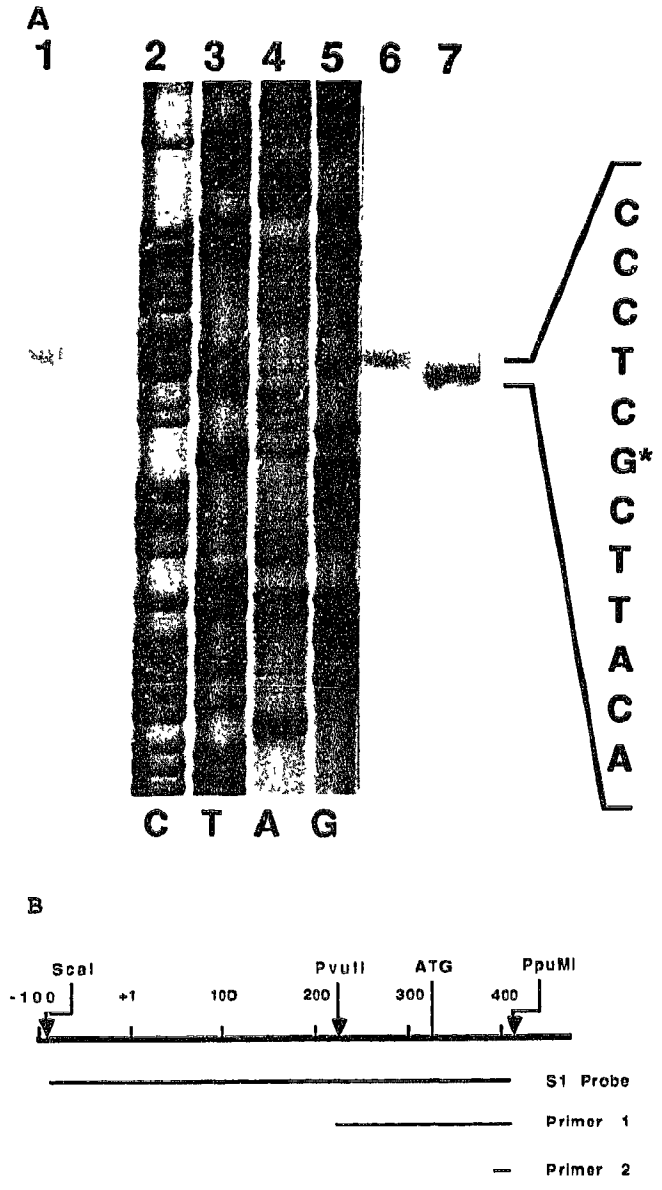


Fig 4 Transcription start site of rat AdoMetDC gene Transcription start site was determined by primer extension analysis and S1 nuclease mapping as described in section 2 A, lane 5, primer extension analysis End-labeled primer 1 *Pvu*II–*Ppu*MI (187 nucleotides) (Fig 4B) complementary to nucleotides 229 to 417 of exon 1 was annealed to 15 μg of poly(A)RNA from rat prostate A, lane 6, S1 nuclease analysis An anti-sense strand of 503 nucleotides in length (*Sca*I-*Ppu*MI) (Fig 4B, complementary to nucleotides −87 to +417) labeled at the *Ppu*MI terminus and 15 μg of poly(A)RNA from rat prostate was used for hybridization reaction The reaction products were analyzed by polyacrylamide gel electrophoresis and sizes determined by running in parallel DNA fragments of a sequencing reaction (lanes 1–4) performed with oligonucleotide complementary to nucleotides 400–417 (Fig 4B, primer 2).

genomes [35] and have been shown to be necessary for the function of certain cellular promoters [36,37]. It may be noteworthy in this context that the sequence GTGGTTCG, present at the 5′ end of intron 1, shares 7 out 8 nucleotides with the SV40 enhancer core sequence GTGG$^{AAA}_{TTT}$G [38,39] This core sequence has been found in a number of enhancer elements of cellular genes, and the cellular factors which interact with the core element have been described [40,41] It has been suggested that the core element plays a key role in activity of the enhancers [39,42,43]

Recently, a novel *cis*-acting regulatory element has been demonstrated (designated BTE for basic transcription element) which is required for a high level expression of the rat P-450c gene [44] The first intron of rat AdoMetDC gene contains a palindromic element GAACCGGCGTGGTCCGGTTC, which is highly related to BTE Thus, the putative binding domains are mainly located in the first intron of the gene

AdoMetDC is very highly regulated in the cell and responds to a wide variety of stimuli affecting growth Thus promotion of cell growth by mitogens, hormones, growth factors and oncogenic transformation induces AdoMetDC activity Supposing that the binding sites in the first intron are functional, they could explain the induction of AdoMetDC gene by a large repertoire of stimuli promoting growth (for reviews, see [10,11]) However, clarification of the roles of these *cis*- and *trans*-elements in the expression of the AdoMetDC gene requires further investigation

In the determined sequence, there are 10 known repetitive elements, 6 of which are located inside the gene and 4 in the 5′ flanking region The repeats are mainly incomplete and exist in both orientations. Some of the type B1 and B2 repeats are flanked by typical short direct repeats suggesting that they have been inserted as transposons Two of them contain putative PolIII promoter boxes A and B [45,46] (Table II)

Fig 6 shows a genomic Southern blot analysis of the AdoMetDC gene. The 0.5-kb *Eco*RI–*Pst*I fragment of AdoMetDC cDNA, which contains the coding regions of exon 7 and exon 8, was used as a probe Three bands that hybridized strongly to rat AdoMetDC cDNA

Table II

Location of the repetitive elements in the 5′ flanking region and within rat AdoMetDC gene

Genetic region	Position in the sequence	Homologous element	%	Ref
5′ Flanking	−(1411–1212)	MT repeat (MT-4)	75%	[27]
5′ Flanking	−(1071 934)	type B1 repeat	77%	[28]
5′ Flanking	−(900–748)	type B1 repeat	70%	[28]
5′ Flanking	−(651–469)	inverse type B2 repeat	89%	[29]
I Intron	1834 2019	type B2 repeat	70%	[29]
I Intron	2620 2700	inverse type B1 repeat	80%	[28]
I Intron	3392–3448	type B1 repeat	85%	[28]
I Intron	6875 6928	type B1 repeat	85%	[28]
V Intron	12117 12238	type B1 repeat	78%	[28]
VII Intron	13472–13562	inverse B2 repeat	76%	[29]

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                    FLANKING REPEAT
ψAdoMetDC           TAAAAAATAAGATTAGCTTACACAGCATGGCGGGCAACATTAGCTA
                                  IIIIIIIIII IIIII III IIIIIIIIII
AdoMetDC gene       TCACGCACAACCCTCGCTTACACAGTATGGCCGGCGACATTAGCTA
                                  ⬆ TRANSCRIPTION START SITE

ψAdoMetDC           AC TCTCACTCAA     CTCTGACAAGAAAGCAGCAGACTACATGA
                     I  III IIIII     IIII II  IIIIIIIII IIIIIII II
AdoMetDC gene       GCGCCTCGCTCAACTCTCTCTAACGGGAAAGCAGCGGACTACAAGA
                                          ⬆ cDNA 272 nt to ATG

ψAdoMetDC           GACTGAACTGTATCTGCCTTTAGTTCCAACAGACTCACGTTCAACT
                    IIIIIIIIIIIIIIIIII II IIIIIII IIIIIIIIIIIIIII
AdoMetDC cDNA       GACTGAACTGTATCTGCCTCTATTTCCAACGGACTCACGTTCAACT
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Fig 5 Comparison of the 5′ noncoding region of the rat AdoMetDC gene exon 1 and the 5′ region of the ψAdoMetDC A direct repeat flanking 5′ end of the ψAdoMetDC [18] and the 5′ sequence of the cDNA (λgtSAMr1) [18] homologous with the AdoMetDC gene are underlined The transcription start site is marked by an arrow

under stringent conditions were detected in all the digests of chromosomal DNA, suggesting that several copies of the pseudogenes exist in the rat genome (see also [3,18]).

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

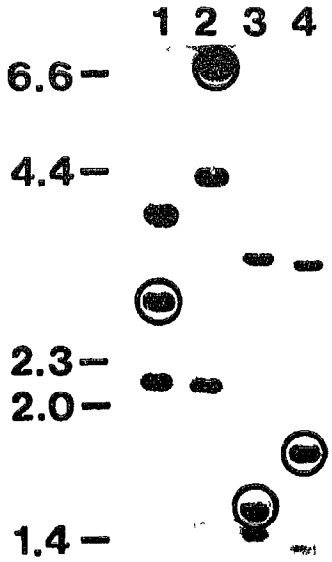

Fig 6 Analysis of the rat AdoMetDC gene by Southern blotting Ten micrograms of rat genomic DNA were cleaved with the restriction endonucleases *Eco*RI (1), *Hin*dIII (2), *Pst*I (3), and *Pvu*II (4) and hybridized with the *Eco*RI-*Pst*I fragment (495 bp) from a cDNA clone (pSAMr1) [18] including the coding sequence (residues 208–333) and 120 nucleotides of 3′ untranslated sequence. The restriction fragments, which correspond to the sequences of the functional AdoMetDC gene, are circled The migration positions of size markers (phage λ*Hin*dIII fragments) are shown in kb

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