# Structure and activity of mouse *S*-adenosylmethionine decarboxylase gene promoters and properties of the encoded proteins

Kazuhiro NISHIMURA*, Marja LIISANANTTI†, Yasuhide MUTA*, Keiko KASHIWAGI*, Akira SHIRAHATA‡, Marja JÄNNE†, Katja KANKARE†, Olli A. JÄNNE† and Kazuei IGARASHI*[1]

*Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263, Japan, †Institute of Biomedicine, Department of Physiology and Department of Clinical Chemistry, University of Helsinki, FIN-00014 Helsinki, Finland, and ‡Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado 350-02, Japan

The promoter regions of two *S*-adenosylmethionine decarboxylase genes (AMD genes) were isolated from a mouse genomic library. One promoter was that of the *bona fide* mouse AMD gene (AMD1) whereas the other was that of the intronless AMD gene (AMD2). There was no sequence identity between the two promoters. The sequence of the AMD1 promoter was highly homologous to the human AMD1 and rat *Amd1B* promoters. After transient transfection in various cell lines, the AMD1 promoter was one to two orders of magnitude stronger than the AMD2 promoter. Similar results were obtained by using stably transfected mouse FM3A cells. In *S*-adenosylmethionine decarboxylase (AdoMetDC)-overproducing SAM-1 cells, the AMD1 gene was amplified over 5-fold. AdoMetDC encoded by the intronless AMD2 gene had two amino acid replacements (Met to Ile at codon 70 and Ala to Val at codon 139), compared with the protein encoded by the AMD1 gene, and exhibited decreased catalytic activity ($< 50\,\%$) and decreased processing activity when expressed in AdoMetDC-deficient *Escherichia coli* cells. When Ile-70 of the protein encoded by AMD2 was converted into Met, both the catalytic and processing activities recovered markedly, indicating that Met-70 adjacent to the proenzyme-processing site is important for both activities. The third AMD locus (AMD3) in FM3A cells contains a pseudogene, in which deletion of two bases generates a premature termination codon at position 57. Since the AMD2 promoter had only 1–10 % of the strength of the *bona fide* AMD1 gene and AMD2 protein possessed lower specific activity, the relative contribution of the AMD2-encoded enzyme to total AdoMetDC activity is small. Thus AdoMetDC activity in murine cells is thought to be due mainly to the product of the AMD1 gene.

## INTRODUCTION

*S*-Adenosylmethionine decarboxylase (AdoMetDC; EC 4.1.1.50) is a key enzyme in polyamine biosynthesis. Its product, decarboxylated *S*-adenosylmethionine, serves as an aminopropyl donor in spermidine and spermine synthesis [1,2]. With regard to the gene encoding AdoMetDC (the AMD gene), different numbers of chromosomal loci containing AMD sequences have been reported in human, rat and mouse. There is one active gene (AMD1) which has nine exons and eight introns and one AMD pseudogene (AMD2) in the human genome [3,4]. The rat genome was initially reported to possess two intron-containing active AMD genes (*Amd1A* and *Amd1B*) with distinct promoter sequences [5]. However, more recent studies have revealed that these two genes are alleles of a single gene and that differences in their 5′-flanking regions originated from a cloning rearrangement in *Amd1A* sequence [6]. In addition, three *Amd* pseudogene loci (*AmdP1*, *AmdP2* and *AmdP3*) have been characterized for the rat genome [5,6]. On the basis of AdoMetDC mRNA and cDNA analysis, the mouse genome is expected to contain at least two loci coding for AdoMetDC, and one of them has been shown to be an intronless but active AMD gene [7].

Although the nucleotide sequence of an intronless mouse AdoMetDC gene and that of mouse AdoMetDC cDNA have been published [7–9], there are no reports on the structure and sequence of the *bona fide* mouse AMD gene. In the present work, we have isolated promoter sequences corresponding to both the authentic mouse AMD gene and the intronless AMD gene [7] from a mouse genomic library and examined their relative strengths in different cell lines. In addition, we have determined how many AMD genes are expressed in mouse FM3A cells and which one of these genes is amplified in an AdoMetDC-overproducing murine cell line, SAM-1 [8]. Our results indicate that the promoter of the *bona fide* mouse AMD genes is one to two orders of magnitude more active than that of the intronless AMD gene and that amplification of the former locus has occurred in SAM-1 cells.

## MATERIALS AND METHODS

### Isolation of mouse AMD promoter sequences

A mouse genomic DNA library (129SvJ; Stratagene) was screened with a cDNA probe corresponding to the 5′-untranslated region (5′-UTR) of human AdoMetDC mRNA [10]. DNA–DNA hybridizations were performed using $^{32}$P-labelled probes under standard conditions [11]. Screening of approx. $8 \times 10^5$ phage plaques yielded over 10 positive signals, eight of which were plaque-purified and analysed by Southern blotting and restriction enzyme digestion. Isolation of phage DNA was carried out as described [12]. Southern hybridizations with probes covering various parts of human AdoMetDC cDNA revealed

Abbreviations used: AdoMetDC, *S*-adenosylmethionine decarboxylase; AMD gene, *S*-adenosylmethionine decarboxylase gene; CHO, Chinese hamster ovary; EGBG, ethylglyoxal bis(guanylhydrazone); ORF, open reading frame; RT-PCR, reverse transcriptase–polymerase chain reaction; UTR, untranslated region.

[1] To whom correspondence should be addressed.

The nucleotide sequences reported in this paper have been submitted to the DDBJ, EMBL and GenBank Data Bank under accession number(s) AB003152, AB006794 and AB006795.

that two of the phage clones were potential candidates for AMD genes. These two genomic clones termed gAMD1 and gAMD2 were 18 and 17 kb respectively. *Sac*I digestion yielded 2.7 and 1.6 kb fragments from gAMD1 and gAMD2 respectively, which hybridized to the 5′-UTR of human AdoMetDC cDNA. *Sac*I fragments were subcloned into pGEM-3Z (Promega) and sequenced. The nucleotide sequence was determined using the dideoxy chain-termination method [13] and the ALFExpess system (Pharmacia Biotech) or an automated fluorescent DNA sequencer (model DSQ-1000; Shimadzu, Kyoto, Japan). Nucleotide sequence comparisons were performed by using the BESTFIT program [14].

### AMD promoter constructs

*Sac*I fragments of gAMD1 and gAMD2 were cloned into pGL3-Basic vector (Promega) to generate p(−1396/+1311)AMD1-LUC and p(−1160/+400)AMD2-LUC vectors (where LUC stands for luciferase). To remove exon 1 and intron I sequences from p(−1396/+1311)AMD1-LUC or exon 1 and phage sequences from p(−1160/+400)AMD2-LUC, the constructs were digested with *Nhe*I, which cleaves at nt +33 of AdoMetDC 5′-UTR and at the polylinker of pGL3-Basic. Religation of the plasmids yielded constructs p(−1396/+33)AMD1-LUC and p(−1160/+33)AMD2-LUC. A shorter promoter construct was made by double digestions with *Sac*I and *Msc*I for p(−1396/+33)AMD1-LUC, followed by blunt-ending and religation to generate p(−366/+33)AMD1-LUC. The p(−441/+33)AMD2-LUC was constructed similarly from p(−1160/+33)AMD2-LUC using *Sac*I and *Kpn*I.

### Isolation of AMD2 and AMD3 promoter regions from FM3A cells

A method, referred to as cassette-ligation-mediated polymerase chain reaction (PCR) [15], was adopted for cloning of promoter regions of intronless AdoMetDC genes using Cassettes & Cassette Primers (Takara, Kyoto, Japan). Chromosomal DNA from FM3A cells [11] was digested with *Eco*RI or *Xba*I. Then PCR was performed using *Eco*RI or *Xba*I cassette. Additional two-step PCRs were performed using Cassette Primer C1 and S1 primer (complementary to nt 213–236 from the transcription start site of the AMD gene) the first time and Cassette Primer C2 and S2 primer (complementary to nt 119–142 or 72–94 from the transcription start site of the AMD gene plus *Eco*RI- or *Xva*I-recognition sequence) the second time. The products were digested with *Eco*RI and *Xba*I and inserted into the multiple cloning sites of M13mp18 (Takara). The resulting clones (pM13AMD2 and pM13AMD3) were sequenced.

### Preparation of reporter gene constructs for stable transfections

Pica Gene™ cassette vector (pPGV-B) and control vector (pPGV-C) were purchased from Nippon Gene (Tokyo, Japan). AMD1, AMD2 and AMD3 promoter regions were obtained by PCR using 5′-end primers with *Sac*I site (AMD1, 5′-TCCACCAGTTGAGCTCATGGAGTA-3′; AMD2, 5′-TTTGAGCTCATAACCCCATGGGACAAGGAA-3′; AMD3, 5′-TTGTGGAGCTCCCTGAGCCTGAGG-3′) and 3′-end primer (5′-GAGGCAGATACAGTTCAGTCTCTT-3′). The products were digested with *Sac*I and *Nhe*I and inserted into the same site of pPGV-B. The plasmids containing the *neo*$^r$ gene (pPGV-B-LUC, pPGV-AMD1-LUC, pPGV-AMD2-LUC and pPGV-AMD3-LUC) were then constructed by inserting the *Bam*HI fragment of pMAMneo (Clontech) into the *Bam*HI site of the above plasmids. pPGV-SV40-LUC was prepared by replacing the 1.2 kb *Sac*I–*Hin*dIII fragment of pPGV-B with the 1.5 kb *Sac*I–*Hin*dIII fragment of pPGV-C, containing the promoter region of SV40, and by inserting the *neo*$^r$ gene as described above.

### Culture of FM3A and SAM-1 cells

SAM-1 cells were isolated from FM3A mammary carcinoma cells (Japan Health Science Foundation) as described previously [8], using an AdoMetDC inhibitor, ethylglyoxal bis(guanylhydrazone) (EGBG) [16,17]. FM3A cells and SAM-1 cells ($1 \times 10^4$/ml) were cultured in ES medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) as described by Ayusawa et al. [18]. SAM-1 cells were maintained by subculturing them in the presence of 50 $\mu$M EGBG.

### Transient transfection

NIH/3T3 and COS-1 cells were purchased from American Type Culture Collection (Rockville, MD, U.S.A.). BW1 cells (a mouse hepatoma cell line) and N2A cells (a mouse neuroblastoma cell line) were gifts from Dr. G. L. Hammond (Ontario Regional Cancer Center, London, Ontario, Canada) and Dr. E. Castrén (University of Kuopio, Kuopio, Finland) respectively. The cells were maintained in Dulbecco's minimal essential medium containing penicillin (25 units/ml), streptomycin (25 units/ml) and 10% (v/v) fetal calf serum. Cells were transfected using the calcium phosphate precipitation method as described previously [19,20]. In short, $(1.5–2.0) \times 10^6$ cells were plated on a 10 cm dish 24 h before the addition of the precipitate containing 15 $\mu$g of promoter construct DNA and 5 $\mu$g of pSV$\beta$-gal DNA (Pharmacia Biotech) as an internal control for transfection efficiency. The cells received fresh medium 18–20 h after transfection and were cultured for an additional 24–26 h before harvesting. Luciferase and $\beta$-galactosidase activities and protein concentrations in cell extracts were measured as previously described [20].

### Transfection of FM3A cells

*Sca*I-digested plasmids (pPGV-B-LUC, pPGV-SV40-LUC, pPGV-AMD1-LUC, pPGV-AMD2-LUC and pPGV-AMD3-LUC) were transfected into FM3A cells by electroporation by the method of Kimura et al. [21]. Transfectants were obtained by culturing for 10 days in 0.5% semisolid agar medium containing 5% fetal calf serum and 1 mg/ml geneticin disulphate (Life Technologies). Luciferase activity was measured using $2 \times 10^6$ cells and the Pica Gene™ assay kit (Nippon Gene) according to the manufacturer's protocol.

### Analysis of AMD genes by PCR and Southern blotting

Chromosomal DNA from FM3A and SAM-1 cells was isolated by standard methods [11]. PCR analysis was performed using 1 $\mu$g of chromosomal DNA and the primers shown in Figure 3. For Southern blotting, 20 $\mu$g of DNA was digested with *Hin*dIII or *Xba*I, fractionated by gel electrophoresis on 1% agarose, transferred to Gene Screen *Plus*™ membrane (Du Pont–New England Nuclear) and hybridized with a $^{32}$P-labelled DNA probe. The following probes were used for Southern blotting; 1.2 kb *Pvu*II–*Xba*I fragment of human AdoMetDC cDNA and 1.4 kb *Sac*I–*Nhe*I, 0.7 kb *Eco*RI and 0.7 kb *Xba*I fragments of the AMD1, AMD2 and AMD3 promoters respectively.

### Analysis of the amounts of AdoMetDC mRNAs encoded by AMD1 and AMD2 genes

Total RNA was isolated from $2 \times 10^7$ cells by the method of Cathala et al. [22]. The amount of AdoMetDC mRNA encoded


**Figure 1 Comparison of the proximal promoter sequences in mouse (M), rat (R) and human (H) *bona fide* AMD genes**

The three sequences of the proximal promoters (approx. 550 nt) are aligned using the BESTFIT program [16]. Nucleotides that are identical in all three sequences are marked by dots beneath the human sequence. DNA elements for transcriptional factors analysed by GENETYX-MAC 8.0 (Software Development Co.) are shown above the mouse sequence. Rat and human sequences are those of *Amd1B* and AMD1 genes respectively [3,5]. The transcription start site is numbered +1. The 5′-flanking nucleotides are shown in lowercase and those of 5′-UTR in uppercase letters.

by AMD1 and AMD2 genes was estimated by the reverse transcriptase (RT)-PCR method [23]. The 5′-end and 3′-end primers used for AMD1-derived AdoMetDC mRNA were 5′-GAGTAATCAGTCAGCCAGATCAAACA-3′ (nt 829–854 from the transcription start site of the AMD1 gene) and 5′-GAATATGCCCTAGAAAGTGGAGGTGC-3′ (complementary to nt 1781–1806 from the transcription start site of the AMD1 gene). Replacement of the underlined A of the 5′-end primer by C and that of underlined GC of the 3′-end primer by AA was performed, and the primers produced were used for the detection of AMD2-derived AdoMetDC mRNA. After 25 cycles of PCR, the products were analysed by gel electrophoresis on 1% agarose. The yield of DNA was proportional to the amount of RNA added. Under standard conditions, 0.5 $\mu$g of total RNA was used for RT-PCR.

### Expression of proteins encoded by AMD1, AMD2 and AMD3 genes in *Escherichia coli* and assay for AdoMetDC activity

The open reading frames (ORFs) of AMD1, AMD2 and AMD3 were amplified by using PCR with primers 5′-TCTCACGCATATGGAAGCTGCACATT-3′ and 5′-CTTCATGGATCCTAATCAACTCTG-3′. After digestion with *Nde*I and *Bam*HI, 1.0 kb *Nde*I–*Bam*HI fragments containing AdoMetDC ORF were inserted into the corresponding restriction sites of pT7-7 [24]. For heterologous expression of enzyme proteins, *E. coli* HT551 cells deficient in the AMD gene [25] were transfected with pT7-7, containing the ORF of AdoMetDC, and pGP1-2 [26], containing the gene for heat-inducible T7 RNA polymerase. *E. coli* HT551 cells were cultured in Luria–Bertani medium in the presence of 30 $\mu$g/ml putrescine. After heat induction at 42 °C for 15 min at a cell density of $A_{600} = 0.5$, the cells were cultured further at 37 °C for 120 min. AdoMetDC activity was measured using the 16000 ***g*** supernatant as described previously [27].

### Immunoblot analysis of AdoMetDC

Antiserum against rat prostate AdoMetDC was raised in rabbits as described [28]. Immunoblotting was performed by the method of Neilson et al. [29], and protein concentration was determined by the method of Lowry et al. [30].

### Purification of AdoMetDC expressed in *E. coli*

Purification of AdoMetDC was performed as described previously [24] with some modifications. Cell extract prepared by a French press was treated with 1% (w/v) streptomycin, and the precipitate was removed. Then protein precipitated between 35 and 65%-satd. $(NH_4)_2SO_4$ was collected. AdoMetDC was purified from the fraction by affinity chromatography using methylglyoxal bis(guanylhydrazone)–Sepharose as previously described [24].

## RESULTS

### Promoter sequences in the mouse AMD genome

Screening of a mouse genomic DNA library with a probe corresponding to the 5′-UTR of human AdoMetDC mRNA yielded many positive hybridization signals. With the use of defined cDNA probes, restriction enzyme mapping and partial sequencing, only two phage clones were found to contain AMD genes. These two clones were termed gAMD1 and gAMD2, and the 1401 and 1165 nt sequences of the 5′-flanking region of gAMD1 and gAMD2 were determined. The 5′-flanking region of the gAMD1 sequence shows significant similarity to the promoter regions of human AMD1 [3] and rat *Amd1B* [5] genes, as illustrated in Figure 1. Even though there are regions of high sequence identity in the upstream promoter regions, extending to over 1000 nt from the transcription start site, the greatest sequence conservation among the mouse, rat, and human promoters is within the proximal 500 nt, with over 60 % of the nucleotides in this region being identical in the three promoters. A computer search for potential transcription-factor-binding sites in the promoter region of gAMD1 revealed the presence of DNA elements for a number of factors, such as Sp1, CRE, AP-2, Ets-1 and EGR-1. The first 51 nt sequence of the 5′-flanking region of gAMD2 is identical with that reported by Persson et al. [7] (GenBank/EMBL Data Bank accession number Z23077). On the basis of these data, we concluded that gAMD1 corresponds to the promoter of the *bona fide* mouse AMD gene and that gAMD2 is the 5′-flanking sequence of the intronless mouse AMD gene [7].

**Table 1 Activities of AMD promoter constructs in various cell lines**

The cell lines were transfected with the indicated AMD promoter constructs as described in the Materials and methods section. The reporter gene activities are expressed relative to that of the promoterless pGL3-Basic vector which was set as 1. Mean ± S.D. values for four to six separate experiments were determined as indicated; in some cases, mean values from quadruplicate dishes in a single experiment are shown. n.d., not determined.

| AdoMetDC promoter construct | Relative reporter gene activity (arbitrary units) | | | |
|---|---|---|---|---|
| | NIH/3T3 cells | N2A cells | BW1 cells | COS-1 cells |
| pGL3-SV40-LUC | 2214 | 18.5 | 78 | 7020 |
| p(−1396/+33)AMD1-LUC | 123 ± 28 | 15.4 ± 9.1 | 6.0 ± 1.5 | 117.0 ± 20 |
| p(−366/+33)AMD1-LUC | 31 | 9.0 ± 1.0 | n.d. | n.d. |
| p(−1160/+33)AMD2-LUC | 0.54 ± 0.13 | 0.20 ± 0.04 | 0.30 ± 0.24 | 0.8 ± 0.1 |
| p(−441/+33)AMD2-LUC | 1.78 | 1.0 | n.d. | n.d. |

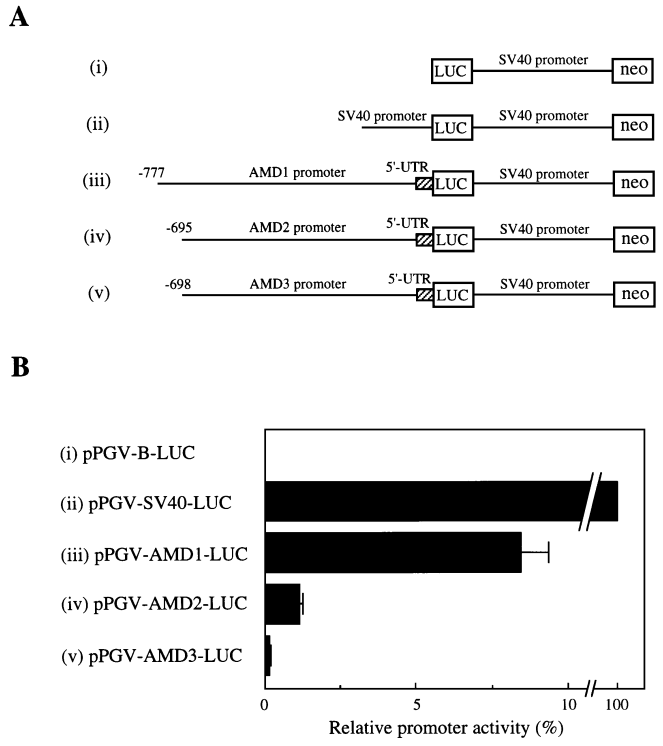


**Figure 2 Promoter activities for AMD constructs in FM3A cells**

(**A**) Schematics showing the various AMD reporter constructs used to study promoter activities. The luciferase gene (LUC) is preceded by 33 nt of AdoMetDC 5′-UTR. (**B**) Luciferase activity is shown as mean ± S.D. and is expressed relative to that of pPGV-SV40-LUC (= 100 %, 26600 relative luminescence units/min per mg of protein).

### AMD genes in mouse FM3A cells

To identify how many functional AMD genes exist in mouse FM3A cells, we analysed the nucleotide sequences of 5′-flanking regions of putative AMD genes. One of the sequences was identical with the promoter sequence of the *bona fide* mouse AMD gene. Two other 5′-flanking sequences were determined using clones isolated by genomic DNA walking [15] from the region coding for AdoMetDC 5′-UTR. One of these sequences corresponded to the promoter sequence of the intronless mouse AMD gene [7]. The third potential promoter sequence was unrelated to the 5′-flanking sequences of gAMD1 and gAMD2 and was tentatively considered to represent a third mouse AMD gene (AMD3). The results with FM3A cells together with those obtained in the genomic library screening thus revealed the presence of multiple AMD loci in the murine genome.

### Activity of mouse AMD promoters

The strengths of the promoters were measured using both transiently and stably transfected cell lines. In transient transfections, three mouse cell lines of dissimilar origin [fibroblasts (NIH/3T3), neuroblastoma cells (N2A) and hepatoma cells (BW1)] and simian kidney epithelial cells (COS-1) were used to compare gAMD1 and gAMD2 promoter strengths. In the case of stable transfectants, mouse FM3A cells were used to examine activities of AMD1, AMD2 and AMD3 promoters.

In every cell line and with all the constructs examined, the AMD1 promoter was much stronger than the AMD2 promoter. The activities of AMD2-derived promoter constructs were extremely low (Table 1). To study the promoter activities under long-term stable conditions, the plasmids pPGV-AMD1-LUC, pPGV-AMD2-LUC and pPGV-AMD3-LUC (Figure 2A) were employed to generate stably transfected mouse FM3A cell lines. In these FM3A cell lines, the rank order of promoter strength was AMD1 > AMD2 > AMD3, with their relative activities being 1:0.14:0.02 (Figure 2B). The strength of the AMD1 promoter in stable transfectants was 7 % of that of a reporter gene driven by the strong SV40 promoter (Figure 2B).

### Structures of the AMD genes

The downstream sequences flanked by the AMD1, AMD2 and AMD3 promoters in FM3A cells were investigated by using PCR. When PCR was performed with primers 2a–2c (nucleotide

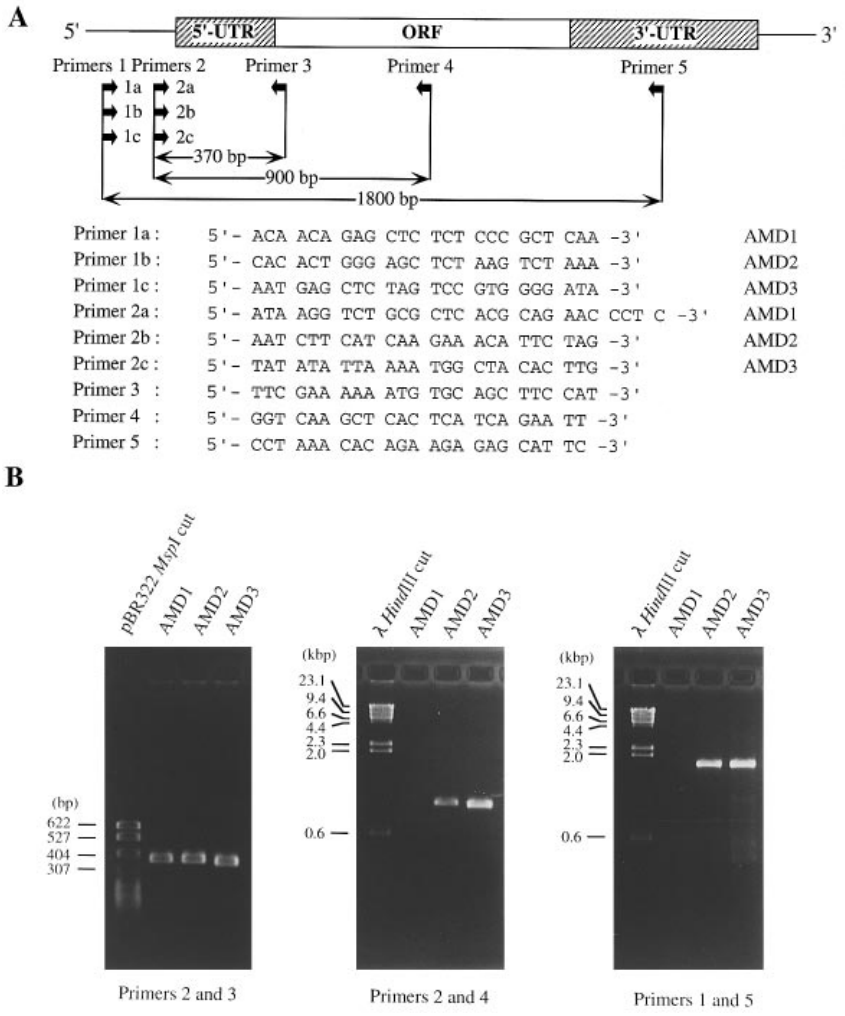


**Figure 3 PCR products generated from AMD1, AMD2 and AMD3 loci using various primer combinations**

(**A**) Localization of the primers complementary to specific promoter regions of AMD1, AMD2 and AMD3 genes. (**B**) Results from PCR analysis performed using the indicated primer combinations. Numbers on the left depict molecular size markers (bp). The target gene for PCR and the primers used are shown above and below the gels respectively.

sequence specific for a given promoter region) and primer 3 (complementary to the nucleotide sequence for the first eight encoded amino acids) (Figure 3A), three PCR products were generated. However, the product from the AMD3 promoter was somewhat shorter than those from AMD1 and AMD2, suggesting that the 5′-UTR synthesized from the AMD3 locus is not identical with the others (Figure 3B). When PCR was performed using primers 2a–2c and primer 4 (complementary to nucleotide sequence 534–556 of AdoMetDC ORF) or using primers 1a–1c (another set of promoter sequence specific primers) and primer 5 (complementary to nucleotide sequence of AdoMetDC 3′-UTR), PCR products were only generated for AMD2 and AMD3 (Figure 3). These results indicate that AMD2 and AMD3 are co-linear with the AdoMetDC cDNA sequence and correspond to intronless genes or pseudogenes. Partial nucleotide sequence analysis of gAMD1 isolated from the genomic DNA library proved that it possesses exon 1 and exon 2 of the AMD gene separated by intron I which is highly homologous with that of the rat *Amd1B*.

The complete nucleotide sequences of AdoMetDC 5′-UTRs originating from the AMD1, AMD2 and AMD3 genes were then determined. The transcription start site was identified with the aid of the gAMD1 and gAMD2 sequences and that of Ado-MetDC cDNA [8]. The 5′-UTRs of the AMD1 and AMD2 genes were very similar to each other with the respective nucleotide numbers being 326 and 328. There were only five mismatches between the two 5′-UTR sequences (DDBJ, EMBL and GenBank accession numbers AB003152 and AB006794). As expected from the PCR experiments, the size of the 5′-UTR from the AMD3 locus was smaller (315 nt) than that of the other two (DDBJ, EMBL and GenBank accession number AB006795).

Next, the nucleotide sequences of the protein-coding regions of AMD2 and AMD3 were determined. The protein-coding sequence of AMD1 is that of mouse AdoMetDC cDNA which has been published [8,9]. As reported previously [7], the AMD2 gene potentially encodes a pro-AdoMetDC protein consisting of 334 amino acid residues. AdoMetDC encoded by AMD2 had two amino acid replacements (a Met to Ile at codon 70 and an Ala to Val at codon 139) compared with the protein encoded by the AMD1 gene [8,9]. In the case of the AMD3 gene, there were two nucleotide deletions (between nt 468 and 469) which produce a change in reading frame, and consequently a termination

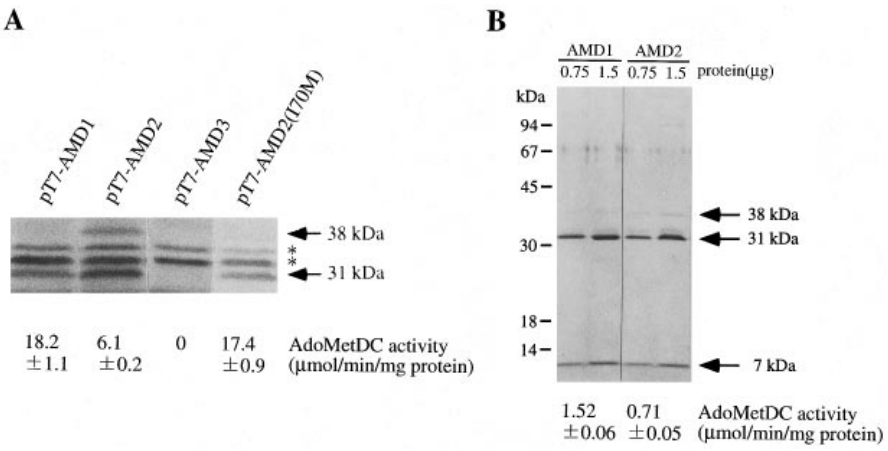


**Figure 4 Activities of AdoMetDC in *E. coli* cells transformed with AMD1, AMD2 and AMD3 genes, and of purified enzymes**

(**A**) Immunoblot analysis and catalytic activity of AdoMetDC expressed in *E. coli*. The plasmids pT7-AMD1, pT7-AMD2, pT7-AMD3 and pT7-AMD2(I70M) were used to transform *E. coli* HT551 cells devoid of the AMD (*speD*) gene. AdoMetDC activity was measured using 10 $\mu$g of protein per assay and the relative activity is shown as mean $\pm$ S.D. Asterisks indicate non-specific bands in the immunoblot. (**B**) Purified AdoMetDCs encoded by AMD1 and AMD2 were electrophoresed on a 12% acrylamide slab gel and stained with Coomassie Brilliant Blue R-250. Numbers on the left depict mobility of molecular mass markers in kDa. Catalytic activity measurements were carried out using 0.2 $\mu$g of purified enzyme protein and are shown as mean $\pm$ S.D.

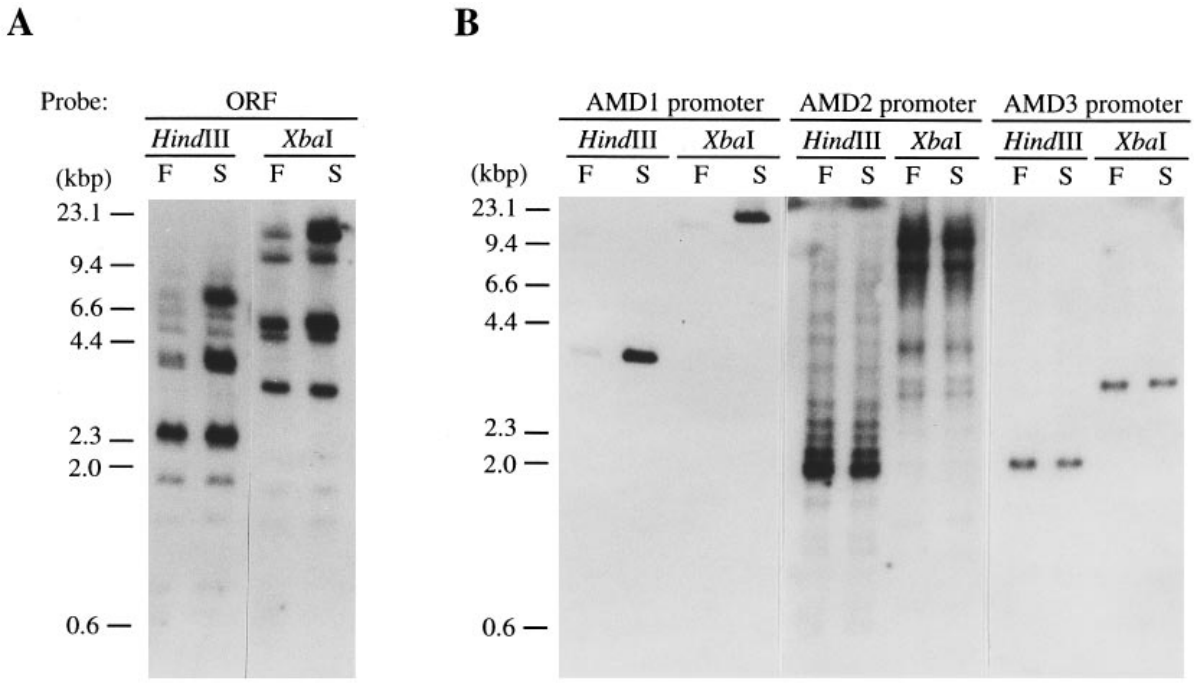


**Figure 5 Southern-blot analysis of AMD gene dosage in parental and AdoMetDC-overproducing mouse cells**

AMD gene copy number in FM3A (F) and SAM-1 (S) cells was examined by Southern blotting as described in the Materials and methods section using *Hin*dIII- or *Xba*I-digested mouse DNA and the probes indicated at the top.

codon (TGA) appeared at codon 57 of the protein-coding region. Otherwise, the nucleotide sequences of the AMD1 and AMD3 ORFs were remarkably similar.

### AdoMetDC activity encoded by AMD1 and AMD2 genes

The activities of the AdoMetDC proteins encoded by the AMD1 and AMD2 genes were measured using the pT7-7 plasmid containing the ORF of AdoMetDC in *E. coli* HT551 cells devoid of the AMD (*speD*) gene. The ORFs in AMD1, AMD2 and AMD3 sequences were fused right after the Shine–Dalgarno sequence [31] of pT7-7 and the resulting plasmids used to transform *E. coli* HT551. The AdoMetDC enzyme is synthesized as a proenzyme (molecular mass 38 kDa) which is converted to $\alpha$ (molecular mass 31 kDa) and $\beta$ (molecular mass 7 kDa) subunits, which are produced by an autocatalytic process in the

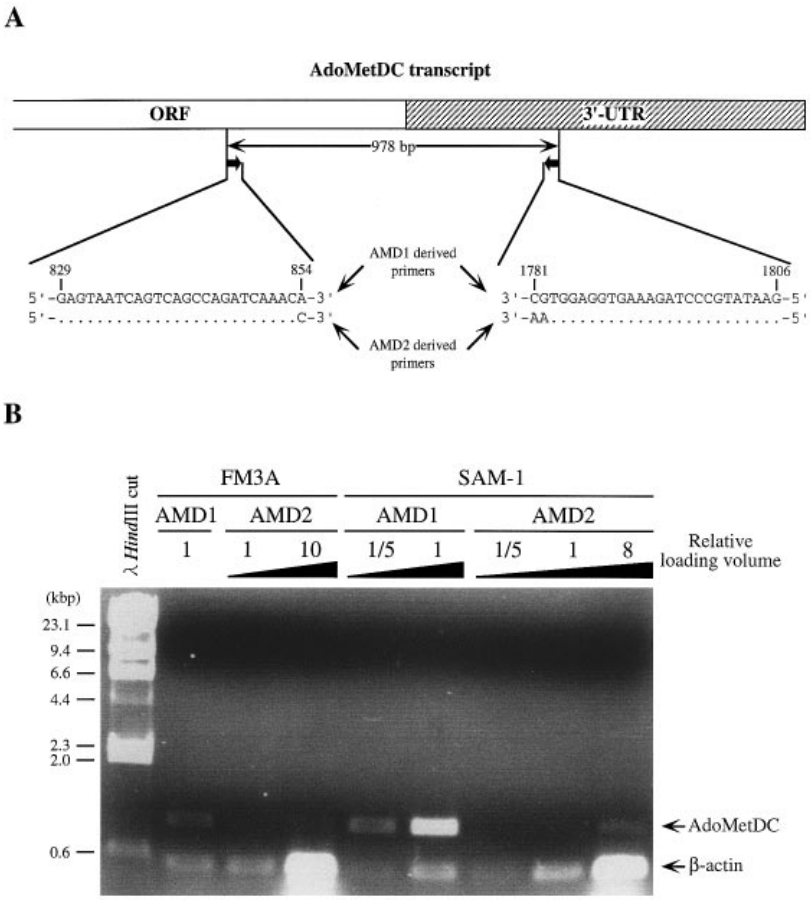


**Figure 6 Amount of AdoMetDC mRNA estimated by RT-PCR**

(**A**) Primers used for RT-PCR. (**B**) The amount of 978 nt DNA obtained by RT-PCR was parallel to that of AdoMetDC mRNA derived from AMD1 and AMD2. As a control, the amount of $\beta$-actin mRNA was simultaneously measured (582 nt DNA). The 5′-end and 3′-end primers used for $\beta$-actin mRNA were 5′-GTACCACCATGTACCCAGGCATTGC-3′ (nt 905–929 from the translation start site of the $\beta$-actin mRNA) and 5′-CTTCCTGTAACCACTTATTTCATGG-3′ (complementary to nt 1462–1486 from the translation start site of the $\beta$-actin mRNA). Numbers on the left depict molecular size markers (kbp). Relative loading volume is shown at the top.

presence of putrescine [10,32,33]. AdoMetDC encoded by the AMD1 gene had about three times higher activity than that encoded by AMD2 (Figure 4A). Furthermore some unprocessed proenzyme still remained in the AMD2-expressing cells (Figure 4A). As expected from the nucleotide sequence, there was no measurable AdoMetDC activity in *E. coli* HT551/pT7-AMD3. The processing site of pro-AdoMetDC is located between residues Glu-67 and Ser-68, and the amino acid sequence from 65 to 72 (Leu-Ser-Glu-Ser-Ser-Met-Phe-Val) is conserved among human, rat, mouse and *Xenopus* proteins [3,10,27,34,35]. There is an Ile rather than a Met at codon 70 in AMD2. Therefore we converted Ile-70 into Met-70 in AMD2 and measured the AdoMetDC activity in *E. coli* HT551. As a result of the Ile to Met substitution, the specific activity of AdoMetDC was increased and the unprocessed proenzyme could not be seen (Figure 4A), indicating that Met-70 is important both for processing of the proenzyme and for catalytic activity.

The specific activity of AdoMetDC encoded by AMD1 and AMD2 was examined using purified proteins. As shown in Figure 4(B), both the AMD1- and AMD2-encoded proteins were purified to near-homogeneity. Only a small amount of proenzyme was found in AMD2-encoded AdoMetDC. The specific activity of AMD2-encoded AdoMetDC was about one-half of that of the enzyme encoded by AMD1 (Figure 4B).

### Amplification of the AMD1 genes in AdoMetDC-overproducing SAM-1 cells

Since the description of AdoMetDC-overproducing mouse FM3A cells, termed SAM-1 cells [8], AdoMetDC-overproducing Chinese hamster ovary (CHO) cells have also been reported [36]. Although we could not detect AMD gene amplification in SAM-1 cells [8], amplification of the hamster AMD gene was observed in AdoMetDC-overproducing CHO cells [36]. When we first isolated the SAM-1 cells through their resistance to EGBG, the $IC_{50}$ of EGBG for cell growth was 20 $\mu$M. At present, the $IC_{50}$ of EGBG is 200 $\mu$M. Because of their greater tolerance to EGBG, and the presence of multiple AMD loci in mouse FM3A cells, the issue of AMD gene amplification in SAM-1 cells was re-examined.

When AdoMetDC cDNA was used as the hybridization probe for Southern-blot analysis, the increase in AMD gene in the SAM-1 cells was estimated to be about 2-fold (Figure 5A). However, when the AMD1 promoter sequence was used as the

probe in Southern-blot analysis, the 3.7 kb *Hin*dIII and ~ 20 kb *Xba*I fragments recognized by this sequence were found to be amplified over 5-fold in SAM-1 cells (Figure 5B), which was parallel to the increase in the amount of AdoMetDC mRNA in the same cells. In contrast, when AMD2 or AMD3 promoter sequences were employed as probes, no difference in the intensity of hybridizable bands was observed between the parental and AdoMetDC-overproducing cells (Figure 5B). There was no difference in the intensity of $\beta$-actin gene in both cells (results not shown). The nucleotide sequence of the AMD1 promoter in SAM-1 cells was identical with that in normal FM3A cells. Thus the reason for AdoMetDC overproduction in SAM-1 cells was amplification of the AMD1 gene.

### Measurement of the amount of AdoMetDC mRNA by RT-PCR

It has been reported that the amount of AdoMetDC mRNA from AMD2 is higher than that from AMD1, especially in the liver [7], and that regulatory elements of the promoter activity exist in the 3′-flanking region of several genes [37–40]. In our assay of the promoter activity, regulatory elements in the 3′-flanking region of AMD genes, if any, are ignored. Thus the amount of AdoMetDC mRNA derived from AMD1 and AMD2 was measured by RT-PCR. As shown in Figure 6, the amount of AdoMetDC mRNA from AMD1 was 10 times that from AMD2 in FM3A cells, and 40 times that in SAM-1 cells. The results indicate that the amount of AdoMetDC mRNA is nearly parallel to the promoter activity of AMD gene measured by luciferase activity (see Figure 2).

## DISCUSSION

At least four AMD loci have been reported to exist in the rat genome [5,6], with the initial understanding that there are two intron-containing rat genes (*Amd1A* and *Amd1B*) along with three pseudogenes (*AmdP1*, *AmdP2* and *AmdP3*). Even though later experiments proved that *Amd1A* and *Amd1B* are alleles of the same gene [6], it was still important to determine how many AMD loci exist in the mouse genome and how many of these are potentially expressed in cells. This was particularly pertinent after the report [7] that there is an intronless but active AMD gene in the murine genome, the product of which is not completely identical with that predicted from AdoMetDC cDNA sequence [8,9]. Screening of the mouse genomic library yielded two promoter sequences which correspond to the *bona fide* mouse AMD gene (AMD1) and the intronless AMD gene (AMD2) [7]. Moreover, by using genomic DNA walking with several restriction enzyme cassettes, one pseudogene (AMD3) was found in addition to AMD1 and AMD2.

In agreement with the results of others [7], we found that AdoMetDC encoded by the AMD2 gene has two amino acid replacements (Met to Ile at codon 70 and Ala to Val at codon 139) compared with that encoded by the AMD1 gene [7]. However, both the proenzyme-processing activity and the catalytic activity of AdoMetDC encoded by AMD2 were lower than those of the protein encoded by AMD1. Since one of the amino acid substitutions (Met to Ile at codon 70) is close to the processing site, Ile-70 was replaced by Met and the two activities were examined by expressing the AdoMetDC in *E. coli*. Both functions increased significantly after the modification, and Met-70 is thus important for both the specific activity of mature AdoMetDC and processing of pro-AdoMetDC between Glu-67 and Ser-68. Amino acid sequence identity in AdoMetDC proenzymes of mouse (AMD1 and AMD2), human, rat, hamster, bovine and *Xenopus* is very high, between 99.4 and 83.6 %. In addition to the sequence for proenzyme processing (codons 65–72, Leu-Ser-Glu-Ser-Ser-Met-Phe-Val), the residues important for catalytic activity (Glu-12, Glu-15, Lys-82 and Cys-84), putrescine stimulation of activity (Glu-180 and Glu-258) and putrescine stimulation of proenzyme processing (Glu-15, Glu-180 and Glu-258) [32,33] are all conserved. Ala-139 is replaced by Val in the AMD2-encoded protein; this substitution is not located in regions involved in the above functions and thus may not significantly modify properties of the encoded protein.

AdoMetDC-overproducing SAM-1 cells were isolated from parental FM3A cells by their resistance to EGBG, an inhibitor of AdoMetDC [16,17]. Immediately after the isolation, we examined gene amplification by Southern-blot analysis using AdoMetDC cDNA as a probe. At that time, no significant amplification of the AMD gene was demonstrated. However, gene amplification was clearly observed during the course of the present experiments, as has also been reported for AdoMetDC-overproducing CHO cells [36]. This may be explained by the following reasons: (i) the degree of gene amplification increased gradually since the SAM-1 cells were continuously cultured in the presence of EGBG; and (ii) there are multiple AMD loci in the mouse genome and therefore our initial analysis of AMD gene copy number in SAM-1 cells, using AdoMetDC cDNA probe, was not specific for AMD1 sequences and possibly underestimated the extent of amplification in this particular locus.

In conclusion, we have shown in this work that there are two AMD genes in the mouse genome that encode catalytically active enzymes: the AMD1 gene, which corresponds to human AMD1 and rat *Amd1B* genes respectively [3,5], and the AMD2 gene, which is devoid of intronic sequences [7]. A comparison of promoter strengths of murine AMD1 and AMD2 genes in five cell lines of dissimilar origin and species revealed that the activity of the AMD1 promoter is one or two orders of magnitude higher than that of the AMD2 promoter. Accordingly, the amount of AdoMetDC mRNA derived from AMD1 was much higher than that from AMD2 in mouse FM3A and SAM-1 cells. This marked difference in the promoter strength combined with the lower catalytic activity of the AMD2-encoded enzyme suggests that it is the AMD1 gene that is mainly responsible for the production of active AdoMetDC in murine tissues.

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