Isolation and Characterization of the Mouse Ornithine Decarboxylase Gene*

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Mouse ornithine decarboxylase (ODC) genomic clones were isolated from a bacteriophage λ genomic library representing mouse myeloma 653-1 cells which produce ODC due to amplification of an active ODC gene. Sequence analysis of the amplified ODC gene revealed that ODC mRNA is encoded by 12 exons, 10 of which (exons 3 to 12) code for the ODC protein. Exon 12 also corresponds to the 3' noncoding region of the two species of ODC mRNA which are formed by alternative utilization of two polyadenylation signals separated from each other by 422 nucleotides. The transcription initiation site was mapped by S1 nuclease protection and by primer extension analysis. The 5' flanking region is extremely rich in G+C and contains typical promoter motifs such as the TATA box and SP1 transcription factor binding sites. Joining the 5' flanking region to the Escherichia coli chloramphenicol acetyltransferase structural gene and its introduction into mouse cells resulted in the expression of a high level of chloramphenicol acetyltransferase activity. Comparing the sequence of the ODC gene to our previously published sequence of ODC cDNA revealed a disagreement between the sequences located 5' to the A AvaI site and demonstrated that this region of our previously reported cDNA represents a cloning artifact. The portion of the correct 5' noncoding region encoded by exon 1 is extremely rich in G+C and includes potential secondary structures which may be involved in translational regulation of ODC mRNA.

Ornithine decarboxylase (ODC; EC 4.1.1.17) is the first and a key enzyme in the biosynthesis of polyamines in mammalian cells, since it provides the only route for de novo synthesis of putrescine. Accumulating evidence suggests that ODC may be regulated at several control levels: production of mRNA (1-5), changes in the translatability of the mRNA (6, 7), changes in protein stability (8), and by post-translational interactions and modifications (6, 7, 9-12). High ODC activity and elevated levels of polyamines are characteristic of rapidly proliferating cells. Pharmacological and genetic studies demonstrated that growth-related cellular functions require sufficient intracellular concentration of polyamines; in fact, deficiency results in cessation of growth (13-18). We have recently demonstrated that mitogenic induction of quiescent BALB/c 3T3 mouse fibroblasts results in transcriptional activation of an ODC gene and consequently in accumulation of ODC mRNA (5). To further explore the mechanisms which regulate ODC at the transcriptional level, we have isolated ODC genes from a λ phage genomic library representing mouse myeloma 653-1 cells (2) which massively overproduce ODC due to amplification of an ODC gene. The gene analyzed in this study corresponds to the gene amplified in 653-1 cells. This ODC gene consists of 12 exons and 11 introns, and computer analysis demonstrated potential for secondary structure which may be involved in regulating the translation of ODC mRNA. The region upstream to the transcription initiation site contains typical promoter motifs such as a TATA box and CCGCCC SP1 transcription factor binding sites (20, 21). Fusing this upstream region in front of the bacterial chloramphenicol acetyltransferase coding region and its introduction into mouse cells directed the synthesis of a chloramphenicol acetyltransferase protein, demonstrating its function as an active promoter. Exon 12 which contains the translational termination TAG triplet, contains also two AATAAA polyadenylation signals separated from each other by 422 nucleotides, whose alternative usage accounts for the two species of ODC mRNA observed in mouse cells (1-4, 22). Moreover, since both species of mRNA are overproduced in 653-1 cells and only one ODC gene was amplified in these cells, we conclude that both species are encoded by a single ODC gene.

EXPERIMENTAL PROCEDURES

Cloning of the ODC Gene—High molecular weight DNA from 653-1 cells was prepared (33) and partially digested with EcoRI to yield fragments of about 20 kb which were then dephosphorylated and ligated into the EcoRI site of the λ-vector EMBL 4 (34). After packaging, recombinant phages were plated on the restrictive strain NM539 and screened with ODC cDNA as hybridization probe. Analysis of DNA—Cellular DNA and DNA of the isolated clones were digested with restriction endonucleases, fractionated by electrophoresis in 1% agarose, transferred to nitrocellulose, and hybridized to radioactive probes as before (2, 11).

7604
DNA Sequence Analysis—The insert of the λ clone mODC-g1 was split into three primary subclones representing the 6.7-kb and 14-kb EcoRI fragments and a 1.1-kb BamHI fragment overlapping the two EcoRI fragments. The two EcoRI subclones were further dissected to generate smaller overlapping subclones. The subclones were in the Bluescript plasmid (Stratagene) which enabled the isolation of single-stranded plasmid DNA upon infection of plasmid containing cells with bacteriophage M13. Each subclone was linearized at either end and subjected to a limited digestion with EcoRI nucleases followed by S1 nuclease to generate a non-random series of deletions (23). Single-stranded DNA of the resulting deletions was hybridized to a 17-base pair sequencing primer and sequenced by the dideoxy chain termination method (24–26). Each nucleotide was sequenced at least twice.

S1 Nuclease Analysis—5-μg portions of total cellular RNA isolated from 653-1 cells by the guanidinium thiocyanate extraction (35) were hybridized with 5'-end-labeled or uniformly labeled probes in 15 μl of buffer containing 80% formamide, 0.4 M NaCl, 40 mM 1,4-piperazinediethanesulfonic acid (pH 6.4), and 1 mM EDTA. After 12 h of hybridization at 60 °C the sample was diluted with 300 μl of ice-cold buffer containing 40 mM potassium acetate (pH 4.5), 2.5 mM ZnCl2, 300 mM NaCl, 20% glycerol, and digested with 250 units of S1 nuclease (Pharmacia) for 30 min at 30 °C. Protected fragments were resolved by electrophoresis in polyacrylamide-urea denaturing gel and visualized by autoradiography. End-labeling was performed by klenow nusing dephosphorylated genomic fragment with [γ-32P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase. Uniformly labeled fragments were prepared by synthesizing the complementary strand using single-stranded template plasmid, sequencing primer, and Klenow enzyme in the presence of [α-32P]dCTP (600 Ci/mmol).

Primer Extension Analysis—A synthetic oligonucleotide complementary to nucleotides 113 to 130 of exon 1, corresponding to the 5' noncoding region of ODC mRNA, was end-labeled with [γ-32P]ATP and T4 polynucleotide kinase. 100 μl of total cellular RNA from 653-1 cells were hybridized with 0.2 pmol of the 5'-labeled oligonucleotide in 60 μl of solution containing 250 mM NaCl, 25 mM Tris-HCl (pH 7.5) for 1 h at 55 °C. The hybridization mix was diluted with 2 milliliters of buffer containing 500 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.1% sodium dodecyl sulfate and chromatographed on oligo(dT)-cellulose to select polyadenylated RNA and remove excess of nonadenylated primer. Extension of the annealed primer was with reverse transcriptase (Bethesda Research Laboratories) in the presence of dNTPs or dNTPs plus dideoxy-NTPs when sequencing of the RNA was required.

Computer Analysis—Nucleotide sequences were compared to the sequences in the Los Alamos and European Molecular Biology Laboratory data banks using the algorithm of Wilbur and Lipman (36). Potential secondary structures in ODC mRNA were determined using the method of Zuker and Stiegler (37).

RESULTS AND DISCUSSION

Isolation and Sequencing of an Active ODC Gene—As reported previously, the mouse genome contains a family of ODC-related genes (2, 4, 11) of which only one can be positively identified as an active gene, since its amplification gave rise to α-difluoromethylornithine (a suicide inhibitor of ODC (14)) resistance in mouse plasmacytoma cells (2, 4). In order to isolate this active ODC gene, high molecular weight DNA from α-difluoromethylornithine-resistant 653-1 cells (2) was partially digested with EcoRI and ligated into bacteriophage λ-EMBL 4. 500,000 plaques of the resulting library were screened with ODC cDNA as hybridization probe, yielding two types of hybridizing clones, both of which contain the 6.7-kb EcoRI fragment previously demonstrated as the amplified ODC DNA fragment in 653-1 cells (2, 11) (Fig. 1). In addition to the 6.7-kb fragment, the first type of clones contains a 14-kb EcoRI fragment also amplified in 653-1 cells (Fig. 1) and the second type contains a 9-kb EcoRI fragment equally represented in 653-1 cells and in the parental 653 cells (Fig. 1). Since the first group of clones corresponds to the ODC gene amplified in 653-1 cells, one of these clones designated mODC-g1 was selected for sequence analysis. For this purpose, several overlapping subclones were prepared in the Bluescript plasmid (Stratagene), and each was subjected to limited unidirectional digestion with exonuclease III followed by S1 nuclease to generate a successive set of deletions (23). Single-stranded plasmid DNA was prepared from each deletion by infecting plasmid-containing cells with M13 bacteriophage and sequenced by the dideoxy chain termination method (24–26). Schematic representation of the ODC gene and its nucleotide sequence are presented in Fig. 2. As shown, the ODC gene consists of 12 exons and 11 introns which obey the gt-ag splice rule (19) (Table I). The first two exons and 16 nucleotides of the third represent the 5' noncoding region of ODC mRNA (Fig. 2 and Table II). The rest of exon 3, which corresponds to the first and second exons, and its nucleotide sequence are presented in Fig. 2. Since both ODC mRNAs are overproduced in 653-1 cells (lane 1) and from their parental cells (lane 2) was digested with EcoRI, fractionated in 1% agarose gel, transferred to nitrocellulose, and hybridized with nick-translated DNA of phage mODC-g1 (A, lane 1). Marker molecular weights in kilobase pairs are indicated.

FIG. 1. Restriction analysis of two ODC genomic clones and of ODC sequences in mouse genomic DNA. A, purified DNA of two of the ODC genes isolated by screening of the genomic library was digested with EcoRI and fractionated in 1% agarose gel. The molecular weight of the two λ arms (19.3 and 9.2 kb) and that of the ODC 14-kb and 6.7-kb fragments are indicated. B, the DNA fragments shown in A were transferred to nitrocellulose and hybridized with a fragment of ODC cDNA spanning the region between Aval and SalI which represents the first and second exons. C, total genomic DNA isolated from 653-1 cells (lane 1) and from their parental cells (lane 2) was digested with EcoRI, fractionated in 1% agarose gel, transferred to nitrocellulose, and hybridized with nick-translated DNA of phage mODC-g1 (A, lane 1). Marker molecular weights in kilobase pairs are indicated.
**Fig. 2. Structure and sequence of the mouse ODC gene.** The structure of the genomic clone mODC-g1 as deduced from its complete sequence is displayed at the top. Exons are indicated by boxes with their number on top, the numbers below the line represent length in kilo-base pairs. The position of the TATA box, ATG initiation codon, TAG termination codon, and that of the two AA-TAAA polyadenylation signals are indicated. The restriction endonuclease sites shown below the line are as follows: A, AuaI; H, HindIII; R, EcoRI; S, Sun. The sequence of the 7100-nucleotide-long ODC gene is also presented. The numbers on the left refer to the nucleotide number starting with a guanosine at the site of transcription initiation as number 1 (marked with a dot on top). Underlined are the transcription factor spl binding sites and the TATA box at the upstream region, the ATG initiation codon in exon 3, the TAG termination codon in exon 12, and the two AATAAA polyadenylation signals in exon 12. The 3' end of each of the two mouse ODC mRNAs is marked by a short vertical line. The 5' upstream region and the exons are represented by capital letters and the introns by lowercase letters.

bp fragment was protected with the first probe (Fig. 3, lanes 1 and 2) and a 196-bp fragment with the second probe (lane 3). To further establish that the 5' end of exon 1 represents the transcription initiation site the 5' end of ODC mRNA was mapped by primer extension analysis. A 32P-labeled oligonucleotide complementary to nucleotides 113 to 130 was hybridized with 653-1 RNA, and the primer was extended with reverse transcriptase in the presence of deoxynucleotides only (Fig. 4, lanes 1 and 2, respectively), and the length of the extended products was determined by fractionation in a denaturing gel.
the figure, we were able to determine four transcription initiation sites clustered in a region of 13 nucleotides; the predominant site (accounting for 90% of the initiations) corresponds to a G residue and is numbered as position +1 in Fig. 2. The three other minor initiation sites are mapped to +5, -4, and -8 (Fig. 4). The region 5’ to the transcription initiation site contains typical promoter motifs such as a TATA box at -26 and three CCGCCC transcription factor SP1 binding sites (28, 29) at -108, -179, -328, and its inverted form GGGCGG at -207. The 5’ flanking region is also rich in G+C, particularly between -85 and -335 (81%), and then its A+T content increases (Fig. 2). Interestingly, one CCGCCC sp1 binding site and two of its inverted forms GGGCGG are present also in exon 1 corresponding to the 5’ noncoding region of ODC mRNA (positions 28, 98, and 111, respectively).

**Table I**

| Exon-intron junctions of the mouse ODC gene |
|------------------------------------------------|
| The sequence presented was determined by sequencing the entire ODC gene (see Fig. 2). Exon and intron sequences are represented by capital and lower-case letters, respectively. IVS stands for intervening sequence. The numbers below each IVS refer to their length. |
| TGGAG gtaggagggacgcgg - IVS-2 - tgggctattcactag TGTT |
| 1298 nucleotides |
| CGTG gtaaatctgttatt - IVS-2 - cttctctcttacag AAGC |
| 80 nucleotides |
| CTCCT gtaagtacgggaac - IVS 3 - cttggcttatccag GAGC |
| 232 nucleotides |
| CAAG gtaagactgtcacc - IVS 4 - gctctctcttcctacag ACTG |
| 143 nucleotides |
| CAAA gtaggcttctcgtga - IVS 5 - tgtactccacactag GTTG |
| 186 nucleotides |
| TGAG gtaggtctgatgga - IVS 6 - cttgtgctatctag CTCG |
| 101 nucleotides |
| GCCA gtagagtactgcac - IVS 7 - tttctctgtgctag ACAG |
| 160 nucleotides |
| AGAG gtaattagacatc - IVS 8 - ttcctctctcttag ATCA |
| 544 nucleotides |
| GACC gtaggtgggtgagg - IVS 9 - gatcgggttctctag ATGA |
| 81 nucleotides |
| GAAC gtaggtgctgcagat - IVS 10 - gctctctctctcctag AGAC |
| 82 nucleotides |
| TGGG gtaggtgtagattg - IVS 11 - cttctctctctctag GCAA |
| 517 nucleotides |

**Functional Identification of the ODC Promoter**—To test whether this upstream region displays promoter activity, a psfI fragment spanning the region between +9 and -1940 was joined to the coding region of the *Escherichia coli* chloramphenicol acetyltransferase (30) with position +9 adjacent to the 5’ of the chloramphenicol acetyltransferase gene, and introduced into mouse NIH/3T3 cells by DNA-mediated gene transfection (31) together with the plasmid pCH110 (Phar- macia) which contain the LacZ coding region under the regulation of the SV40 early promoter. A simian virus 40 promoter-chloramphenicol acetyltransferase construct was used.

**Table II**

| Exons of the mouse ODC gene and their relation to the functional regions of ODC mRNA |
|-------------------------------------------------------------------------------------|
| The length of the exons of the mouse ODC gene is presented and their relation to the 5’,3’ noncoding and the coding region of the two species of ODC mRNA is indicated. |
| Exon 1 | 196 nucleotides |
| Exon 2 | 98 nucleotides |
| Exon 3 | 118 nucleotides |
| Exon 4 | 174 nucleotides |
| Exon 5 | 173 nucleotides |
| Exon 6 | 135 nucleotides |
| Exon 7 | 82 nucleotides |
| Exon 8 | 84 nucleotides |
| Exon 9 | 163 nucleotides |
| Exon 10 | 113 nucleotides |
| Exon 11 | 215 nucleotides |
| Exon 12 | 473 nucleotides (mRNA I) |
| 895 nucleotides (mRNA II) | 142 coding |
| 753 noncoding |

**Fig. 3.** Mapping of the transcription initiation site by S1 nuclease analysis. 5-µg portions of total cellular RNA from 653-1 cells was annealed to the indicated probes, the RNA-DNA hybrids were digested with nuclease S1 and fractionated by electrophoresis in denaturing polyacrylamide gel (see "Experimental Procedures"). The probes used were: 1, 5’ end-labeled Aval-BamHI fragment; 2, uniformly labeled Aval- BamHI fragment; 3, uniformly labeled BamHI-BamHI fragment (see diagram on the right). The molecular weights indicated on the left represent HpaII digest of pBR322 DNA. 5p, base pairs.
Mouse Ornithine Decarboxylase Gene

as promoter activity control plasmid. 48 hours post-transfection chloramphenicol acetyltransferase activity was determined in cellular extract after normalizing for transfection efficiency by monitoring β-galactosidase activity. As shown in Fig. 5, chloramphenicol acetyltransferase activity was monitored in the transfected cells. The availability of the ODC gene and its promoter region will serve as a major tool in

Fig. 4. Mapping of the transcription initiation site by primer extension analysis. Total cellular RNA (100 µg) from 653-1 cells was annealed to 3²P-labeled synthetic oligonucleotide (10¹ cpm/pmol) complementary to nucleotides 113 to 130 of exon 1 which represent the 5' noncoding region of ODC mRNA and polyadenylated RNA selected by chromatography through the oligo(dT)-cellulose column. 80% of the material used for primer extension sequencing reaction with reverse transcriptase and in the presence of the appropriate mixtures of dNTPs and dideoxy-NTPs (lane 1). 20% were extended by reverse transcriptase in the presence of dNTPs (lane 2). Lane 3 displays the sequence of the DNA which was obtained using the synthetic oligonucleotide as sequencing primer. The nucleotide sequence of the region encompassing the initiation sites is presented on the right with the initiations marked by asterisks.

Fig. 5. Functional identification of the promoter region of the ODC gene. NIH/3T3 mouse fibroblasts were transfected with a plasmid (pSF64) containing the chloramphenicol acetyltransferase structural gene under the regulation of the indicated promoters. 48 h post-transfection chloramphenicol acetyltransferase activity was determined in cellular extracts after normalizing for transfection efficiency by monitoring β-galactosidase activity (38) directed by a co-transfected plasmid which contains the LacZ gene under the regulation of the early simian virus 40 promoter. 1, extract from cells transfected with a plasmid containing a 1.9-kb ODC pstI fragment (-1940 to +9) tailored upstream to the chloramphenicol acetyltransferase gene with the +9 position adjacent to the chloramphenicol acetyltransferase gene. 2, extract from cells transfected with the pSF64-CAT. 3, extract from cells transfected with a plasmid in which the chloramphenicol acetyltransferase is regulated by the early simian virus 40 promoter. 4, extract from untransfected cells. The letters on left stand for: CM, chloramphenicol; A, 1-acetate chloramphenicol; B, 3-acetate chloramphenicol; and C, 1,3-diacetate chloramphenicol.

Fig. 6. Potential secondary structure in the 5' noncoding region of ODC mRNA. A potential configuration is shown as predicted by computer analysis. The individual free energy of each hairpin in kilocalories/mol is indicated. G-C interactions are indicated by thick connecting lines. The numbers refer to the nucleotide numbers of ODC mRNA, the sequence of its 5' noncoding region is displayed.
studies aiming at the identification and characterization of specific sequences and putative cellular factors which by interaction mediate the mitogenic activation of the ODC gene in mammalian cells.

5' Noncoding Region of ODC mRNA—Comparison between the sequence of exons 1, 2, and the beginning of 3 with that of the 5' noncoding region of our previously reported mouse ODC cDNA (11) revealed extensive disagreement in the region 5' to the AvaI site (position 138 in exon 1 and position -172 in the cDNA). Since in the present study the 5' end of ODC mRNA was carefully mapped, including by direct sequencing of the mRNA, and since a subclone of the cDNA representing the region 5' to the AvaI site failed to hybridize with ODC mRNA (not shown), we concluded that this region of the cDNA represents a cloning artifact. Computer analysis of the correct sequence of exon 1 predicted possible secondary structures in this region of the mRNA (Fig. 6). Of particular interest is the first hairpin (position 1 to 118) which displays high free stabilizing energy (~69 kcal/mol) and whose formation is preferred over interaction with any other region of the mRNA. In considering this observation together with previous studies which demonstrated that polyamines negatively affect ODC mRNA translation (6, 7, 32), it is tempting to speculate that polyamines exert their effect on the translatability of ODC mRNA by stabilizing such potential secondary structures. This possibility will be tested in cells and in vitro using a authentic full length ODC cDNA isolated during the course of the present study.

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Mouse Ornithine Decarboxylase Gene 7609