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METHYLATION OF HUMAN ORNITHINE DECARBOXYLASE GENE BEFORE TRANSFECTION ABOLISHES ITS TRANSIENT EXPRESSION IN CHINESE HAMSTER OVARY CELLS

Maria Halmekytö, Ari Hirvonen, Jarmo Wahlfors, Leena Alhonen, and Juhani Jänne*

Department of Biochemistry and Kuopio Cancer Research Centre, University of Kuopio, P.O.B. 6, SF-70211 Kuopio, Finland

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SUMMARY: Different methylations of cloned human ornithine decarboxylase gene with restriction methylases *in vitro* before transfection greatly reduced the transient expression of ODC in Chinese hamster ovary cells. Single methylation of the gene with *Hpa* II (CCGG) methylase decreased the transiently expressed peak activity by about 50 %, single methylation with *Hha* I (CCGG) methylase by about 80 % whilst a double methylation at both *Hpa* II and *Hha* I restriction sites virtually abolished any transiently expressed ornithine decarboxylase activity. These results together with our earlier circumvential evidence indicate that the expression of mammalian ornithine decarboxylase is critically influenced by the methylation state of the gene.

Even though some controversies exist as regards the importance of gene methylation for its transcriptional activity (1), the majority of recent studies seems to indicate that hypomethylation in or around a given gene is associated with enhanced transcriptional activity of the gene (2-5). In our own studies we have obtained evidence indicating that methylation of mammalian ornithine decarboxylase (ODC) -related sequences may influence the expression and amplification of the gene. We found that genomic ODC sequences are differently methylated among several mouse and tumor cell lines (6) and that gene amplification may be preceded by an enhanced transcriptional activity associated with gene hypomethylation (7). Similarly, the development of acquired resistance to glucocorticoids in a human myeloma cells appears to be associated with ODC gene hypomethylation and enhanced accumulation of the enzyme's mRNA (8). In line with these observations, we found (9) that ODC sequences in mononuclear leukocytes obtained

*To whom correspondence should be addressed at Department of Biochemistry, University of Kuopio, P.O.B. 6, SF-70211 Kuopio, Finland.

The abbreviations used are: ODC, ornithine decarboxylase (EC 4.1.1.17); CHO cells, Chinese hamster ovary cells.

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from lymphocytic leukemia patients were distinctly less methylated than those from healthy volunteers. Interestingly, similar hypomethylation was likewise found in Erb-A1 sequences, but not in those encoding for some other proto-oncogene products (9).

A severe limitation of such descriptive studies is the fact that it is almost impossible to relate the observed gene hypomethylation to the actual expression of the gene, as ODC activity and also its expression greatly varies during the cell cycle being sensitive to any imaginable external stimuli.

We recently succeeded to isolate and clone a transcriptionally active ODC gene from a human myeloma cell line with amplified ODC sequences. ODC-deficient Chinese hamster ovary (CHO) cells transfected with the human ODC gene transiently expressed the gene between 12 and 60 h after the gene transfer. A prior methylation of the gene *in vitro* at *Hpa* II sites (CCGG) and especially at *Hpa* I sites (GCGC) greatly reduced the transient expression of ODC. A double methylation at both sites virtually abolished any expression of the enzyme. These experiments directly show that a methylation of CpG dinucleotides in ODC gene renders the gene transcriptionally inactive.

MATERIALS AND METHODS

Materials

Restriction endonucleases *Hpa* II and *Cfo* I (*Hha* I) were purchased from New England Biolabs (MA, USA) and the corresponding site-specific methylases (*Hpa* II and *Hha* I) from Boehringer (Mannheim, W-Germany). Labeled [α-^{32}P]dCTP (sp-activity 410 Ci/mmole) and [1-^{14}C] ornithine (sp-activity 61 Ci/mmole) were purchased from Amersham International (Amersham, U.K.). α-Difluoromethyl-ornithine was a generous gift from the Centre Recherche Merrell International (Strasbourg, France).

Cloning of human ODC gene

High molecular weight DNA was isolated from human Sultan myeloma cells with amplified ODC sequences (10). Isolated DNA was partially digested with *Sau* 3A. Restriction fragments ranging from 9 to 20 kbp were isolated by electrophoresis on low melting point (LMP) agarose and ligated with EMBL3 arms (Promega). The genomic library was plated with *E. coli* strain LE 392. The transformants were screened with human ODC cDNA; pODC10/2H (11). Approximately 600.000 plaques were obtainted out of which about one hundred were positive. One clone containing the longest insert (about 19 kbp) was selected for restriction mapping and designated as λgODC/H2 (Fig. 1). A *Bam* HI -fragment (about 10 kbp) containing the whole human ODC gene (Fig. 1) was subcloned into pBR322 at *Bam*HI site and designated as pgODC/6 (Fig. 1). This plasmid was used for the transfection experiments.

Cell cultures

Mutant CHO cells deficient in ODC activity were kindly provided by Dr. E. Hölttä (University of Helsinki). These cells had been mutagenized by the suicide selection method introduced by Steglich and Scheffler (12). The cells were cultured in a medium containing one part of F12 and one part of Dulbecco's minimal essential medium (DMEM) supplemented with 5 % fetal calf serum.

Transfection of the cells

The circular plasmid (pgODC/6; Fig. 1) was used to transform the ODC-deficient CHO cells. A recently introduced protocol (13) was used in the transfection experiments. This method is based on the conventional calcium phosphate coprecipitation technique but employs low pH and low CO_2, and is reported to be extremely efficient for circular DNA (13). Exponentially growing cells were trypsinized and 1 x 10^6 cells were transfected with 25 µg of plasmid DNA using the buffers and conditions described in (13). After transfection the cells were grown in F12/DMEM medium and ODC activity was measured at 12, 24, 36, 48 and 60 h after the transfection. Mock-transfection was performed with 25 µg of sonicated herring sperm DNA.

Methylation of pgODC/6 in vitro

Bacterial DNA methyltransferases *Hpa* II and *Hha* I were used. Plasmid DNA (25 µg) was incubated for 4 h with 118 units (*Hpa*II) or 1275 units (*Hha*I) of the methyltransferases in the presence of 50 mM Tris-HCl, 10 mM EDTA, 5 mM 2-mercaptoethanol, 80 µM S-Adenosylmethionine.

Preparative and analytical methods

ODC activity was assayed by the method of Jänne and Williams-Asman (14). DNA was isolated by the method of Blin and Stafford (15). The restriction enzyme analyses were performed with nick-translated (16) pODC10/2H complementary to human ODC cDNA (11).

RESULTS

Lambda gODC/2H and pgODC/6 constructs are depicted in Fig. 1. The location of ODC gene (thick line) in λgODC/2H has been confirmed by direct sequencing (Wahlfors, Hirvonen, Alhonen, Jänne, J., Hickok and Jänne, O.A., unpublished).

Fig. 2 depicts a Southern blot analysis of pgODC/6 after methylation *in vitro*. As seen in lane 6, pgODC/6 is totally demethylated at CCGG sites as *Hpa* II (lane 6) and *Msp* I (lane 2) digestions yielded identical signal pattern consisting of a human-specific doublet 2.3-2.4 kbp in size (6). Although no methylation insensitive isoschizomer for *Hha* I exists, it is highly likely that also GCGC sites (lane 7) were demethylated. Methylation of pgODC/6 at CCGG sites (lane 1), at GCGC sites (lane 3) and at both sites (lanes 4 and 5) resulted in a formation of much larger (about the size of the gene) restriction fragments after digestion with *Hpa* II or *Hha* I.

To study the effect of a prior methylation on the expression of the human ODC gene in CHO cells, we first double methylated (with *Hpa* II and *Hha* I methyltransferases) the gene before the transfection and assayed the transient expression at 0 and 36 h after the transfection. As indicated in Table 1, a double methylation of the gene resulted in a more than 95 % inhibition of the enzyme activity in the recipient cells.

Fig. 3 shows the effect of a prior methylation of ODC gene on the time course of transient expression in recipient CHO cells. As shown, ODC was transiently expressed between 12 to 60 h after the transfection. A single methylation with *Hpa* II methyltransferase reduced the peak activity by about 50 % whereas similar methylation with *Hha* I resulted in a clearly greater depression of the enzyme activity. Double

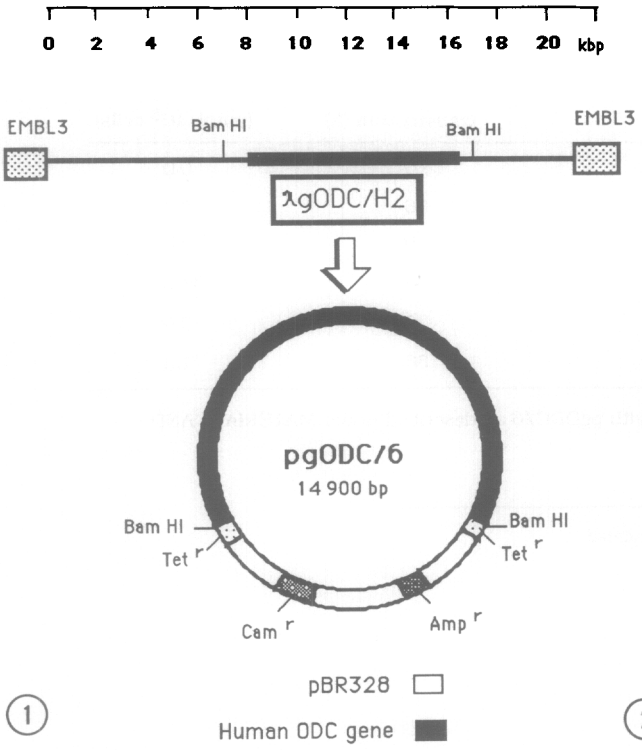


Fig .1. Structures of λgODC/2H and pgODC/6. The thick line in λgODC/2H shows the location of the ODC gene.

Fig. 2. Southern blot analysis of unmethylated and methylated pgODC/6. Lane 1, the plasmid was methylated with *Hpa* II methyltransferase and digested with *Hpa* II restriction endonuclease; lane 2, methylated with *Hpa* II methyltransferase and digested with *Msp* I restriction endonuclease; lane 3, methylated with *Hha* I methyltransferase and digested with *Hha* I restriction endonuclease; lane 4, double methylated with *Hpa* II and *Hha* I methyltransferases and digested with *Hpa* II restriction endonuclease; lane 5, double methylated and digested with *Hhal* restriction endonuclease; lane 6, unmethylated plasmid, digested with *Hpa* II restriction endonuclease; lane 7, unmethylated, digested with *Hha* I restriction endonuclease. The filter was probed with pODC10/2H. The molecular size markers are shown to the right. kbp, kilobasepairs.

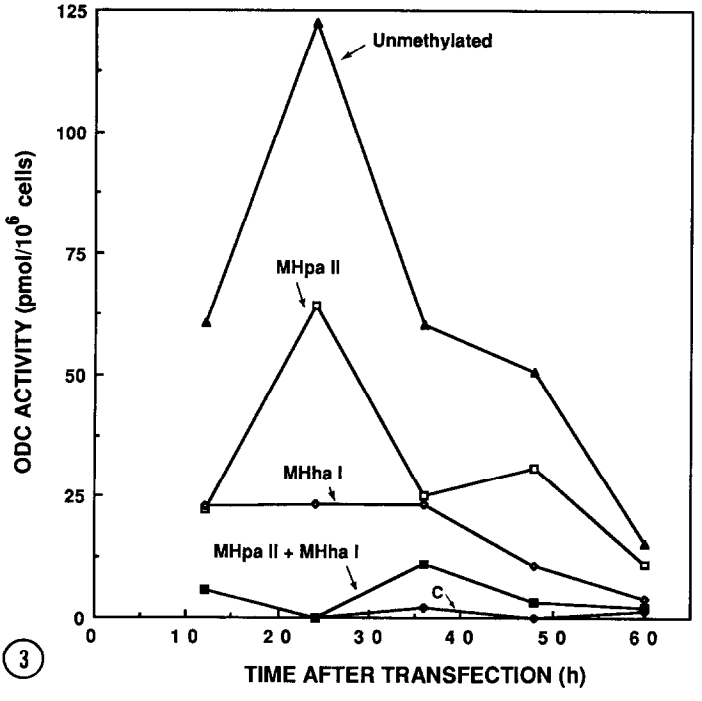
methylation reduced the enzyme activity virtually to the background level of the mock-transfected control cells (Fig. 3).

We repeated the experiment, now using unmethylated and double methylated ODC gene, in order to ascertain whether we can directly show the human gene at 24 h after transfection and whether the methylated gene has remained methylated. *Msp* I digestion of DNA obtained from the recipient CHO cells at 24 h after the transfection indicated that in both cases the gene was incorporated into the cells (human specific 2.3-2.4 kbp doublet after *Msp* I digestion), as shown in Fig. 4 (lanes 1 and 3). Double digestion of the isolated

Table 1. Effect of a prior methylation (*Hpa* II and *Hha* I) on the transient expression of human ODC gene in CHO cells.

Transfected gene	Time after transfection (h)	ODC activity (pmol/10^6 cells)
None (mock-	0	0.0
transfected)	36	0.0
Unmethylated	0	0.0
	36	37.6
Double methylated	0	0.0
	36	1.3

The transfection was carried out with pgODC/6 as described under MATERIALS AND METHODS.



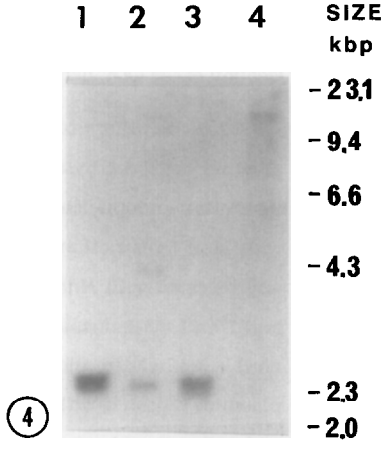


Fig. 3. Transient expression of human ODC gene in CHO cells without or with a prior methylation. The transfection was performed with unmethylated, with CCGG methylated (MHpa II), with GCGC methylated (MHha I) or with double methylated (MHpa II + MHha I) gene. C, mock-transfected control.

Fig. 4. Southern blot analysis of genomic DNA obtained from CHO cells 24 h after transfection. The cells were transfected either with unmethylated or double methylated (*Hpa* II and *Hha* I methyltransferases) pgODC/6. Lanes 1 and 2; DNA from cells transfected with unmethylated plasmid and digested with Msp I (lane 1) or with *Hpa* II and *Hha* I (lane 2). Lanes 3 and 4; DNA from cells transfected with double methylated plasmid and digested with *Msp* I (lane 3) or with *Hpa* II and *Hha* I (lane 4). The filter was probed with pODC10/2H. The molecular size markers are shown to the right. kbp, kilobasepairs.

DNA with *Hpa* II and *Hha* I restriction endonuclease indicated that the unmethylated gene had remained unmethylated and the methylated gene remained methylated, as also shown in Fig .4 (lanes 2 and 4).

DISCUSSION

The present results indicate that pgODC/6 contains a functional human ODC gene which is transiently expressed in ODC-deficient CHO cells. It is noteworthy that the construct only contains about 1 kbp sequencies in excess into both 5' and 3' directions of the gene (Fig. 1). This implies that the regulatory sequences of the human ODC gene reside close (within 1 kbp) to the 5' end of the gene which is in agreement with recent findings indicating that in mouse ODC gene a shorter than 300 nucleotides long fragment upstream from the start site for transcription contains full promoter activity (17). Our results likewise indicate that at least the transient expression of human ODC gene is critically influenced by methylation at CpG dinucleotides. This situation very much resembles that described for human Ha-*ras* oncogene which upon methylation *in vitro* looses its transforming activity (18). In contrast to ODC gene, single methylations at *Hpa* II or *Hha* I sites in Ha-*ras* oncogene were largely ineffective but double methylation at both sites reduced the transforming activity by 80 % (18). Similarly, *in vitro* methylation of the 5' flanking region of human γ-globin gene prevented transcription (19).

Even more relevant to our results are the present findings of Shimada *et al.* (20) who found that a prior methylation of *Hha* I sites, but not *Hpa* II sites, of a dihydrofolate reductase minigene, reduced the transfection frequency of dihydrofolate reductase deficient recipient CHO cells by 90 % (when selected directly for dihydrofolate reductase-positive cells).

Together with our earlier experimental evidence (6-9) the present findings strongly support the view that mammalian ODC gene belongs to those genes the activity of which is profoundly influenced by methylation of some critical CpG dinucleotides. In addition, the present transfection system with an efficient transient expression is an invaluable tool for further studies aimed to elucidate the structural requirements for ODC expression.

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