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Certain changes in ornithine decarboxylase gene methylation accompany gene amplification

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The ornithine decarboxylase (ODC; EC 4.1.1.17) gene in parental, dexamethasone-resistant and 2-difluoromethylornithine (DFMO)-resistant human IgG-myeloma-cell lines was studied with the aid of methylation-sensitive restriction endonucleases and probes recognizing different parts of the gene. In all cell lines the promoter region of the ODC gene appeared to be heavily methylated, whereas the first long intron was unmethylated. Methylation analyses of several clones from the parental cell line revealed that these cells are heterogeneous with respect to the methylation status of the ODC gene, whereas all clones from DFMO-resistant cell lines displayed the same methylation pattern. Two of the parental clones represented a hypomethylated type very close to that exclusively found among the DFMO-resistant clones with ODC gene amplification. This typical methylation pattern was due to decreased methylation of a few CCGG sequences in the 3′-flanking region of the gene. It is possible that this kind of hypomethylation favours the initiation of the gene-amplification process in certain individual cells. This hypothesis was supported by the finding that no hypomethylation was present in the ODC gene of another human myeloma cell line that had acquired resistance to DFMO without gene amplification. In a dexamethasone-resistant cell line that overproduced ODC mRNA at normal gene dosage there were some minor differences between the methylation pattern of the ODC gene of different clones, but no such hypomethylation could be found in clones from the parental cell line. In dexamethasone-resistant cells the ODC gene was hypomethylated around the two *Hpa*II sites and three *Cfo*I sites in the coding region and also, as well as in cells with amplified ODC sequences, in the 3′-flanking region of the gene. Some hypomethylation in the distant 5′-flanking region was also observed.

INTRODUCTION

Ornithine decarboxylase (ODC) is the rate-controlling enzyme of polyamine biosynthesis, and it is regulated at many different levels. DNA methylation is apparently also involved in the regulation of gene expression of this very peculiar enzyme. The relationship between decreased methylation and enhanced gene expression is quite clear (Doerfler *et al.*, 1990) and is supported by experimental evidence, such as the inducibility of the mouse metallothionein-I gene (Compere & Palmiter, 1981) and the expression of human interferon (Fukunaga *et al.*, 1986), rat growth hormone (Strobl, 1986) and human interphotoreceptor retinoid-binding protein (Albini *et al.*, 1990) genes. The present theory states the significance of CpG islands to be associated with the 5′ ends of most of the genes, especially the so-called 'housekeeping' genes. This theory stresses that these CpG islands have to be unmethylated to allow transcription of the gene, and methylation of the island will silence the gene. This holds true also for human ODC-gene expression, because we have shown recently that methylation of cloned active human ODC gene (Hickok *et al.*, 1990) *in vitro* before transfection abolishes its transient expression in Chinese-hamster ovary cells (Halmekytö *et al.*, 1989). However, the *Bam*HI fragment containing the gene was methylated totally, and the contribution of the CpG-island methylation to decreased ODC expression cannot be determined in this experiment. Pilz *et al.* (1990) have also shown that the hypermethylated 'silent' ODC allele in Chinese-hamster ovary cells becomes activated as a result of spontaneous or 5-azacytidine-induced hypomethylation.

Several phenotypic changes in human cells have been shown to be associated with an altered methylation state of the ODC gene. (i) In chronic lymphocytic leukaemia, the ODC gene of leucocytes is apparently hypomethylated when compared with other leukaemia types or mononuclear leucocytes from healthy controls (Lipsanen *et al.*, 1988). (ii) Glucocorticoids and their synthetic congeners (e.g. dexamethasone) depress the ODC activity in cells of lymphatic origin. When human IgG-myeloma cells were adapted to grow in the presence of micromolar concentrations of dexamethasone, the ODC gene became hypomethylated, and this was associated with an enhanced accumulation of ODC mRNA and partial restoration of ODC activity (Leinonen *et al.*, 1987*a*). (iii) When human IgG-myeloma cells were grown in stepwise-increasing concentrations of DFMO, an irreversible inhibitor of ODC, they developed resistance to this drug through ODC-gene amplification (Leinonen *et al.*, 1987*b*). Hypomethylation was also shown to be connected with this process (Alhonen-Hongisto *et al.*, 1987*a*). On the other hand, in human multiple-myeloma cells, which acquire DFMO resistance by enhanced arginase activity without ODC-gene amplification (Alhonen-Hongisto *et al.*, 1987*b*), no signs of hypomethylation were detected.

At the time when the above-mentioned experiments were carried out, the precise structure of human ODC gene was not known, and a more exact determination of methylation sites was not possible. Recently, the nucleotide sequence of the gene has been published by four groups, including our own (Fitzgerald & Flanagan, 1990; van Steeg *et al.*, 1989; Hickok *et al.*, 1990; Moshier *et al.*, 1990), thus offering means for the precise determination of methylation sites in the parental cell line, in cells with an amplified ODC gene or in cells displaying glucocorticoid resistance (Table 1). Here I report that hypomethylation of the ODC gene associated with gene amplification in human IgG-myeloma cells is localized primarily in the 3′-flanking region of the gene, and this kind of methylation status is already present in a small fraction of the cells of the parental cell line. I also show that a different type of hypomethylation is involved during the de-

Abbreviations used: ODC, ornithine decarboxylase (EC 4.1.1.17); DFMO, 2-difluoromethylornithine; SSC, standard saline citrate (0.15 M-NaCl/0.015 M-sodium citrate).

Table 1. Description of cell lines used in the present study

Cell line	Description	References
Sultan	IgG-myeloma-cell line; ODC gene more hypomethylated than in normal leucocytes	A.T.C.C. CRL 1484
Sultan 20D	IgG-myeloma-cell line; resistant to 20 mM-DFMO; ODC gene amplification; ODC-gene hypomethylation (3′ flanking and protein coding region)	Leinonen *et al.*, 1987*b* ; Alhonen-Hongisto *et al.*, 1987*a*
Sultan Dex	IgG-myeloma-cell line; resistant to 1 μM-dexamethasone; enhanced ODC mRNA accumulation; ODC-gene hypomethylation (all regions of the gene)	Leinonen *et al.*, 1987*a*
Fravel 4D	Multiple-myeloma-cell line; resistant to 4 mM-DFMO; normal ODC-gene methylation status in the protein-coding region; enhanced arginase activity	Alhonen-Hongisto *et al.*, 1987*b*

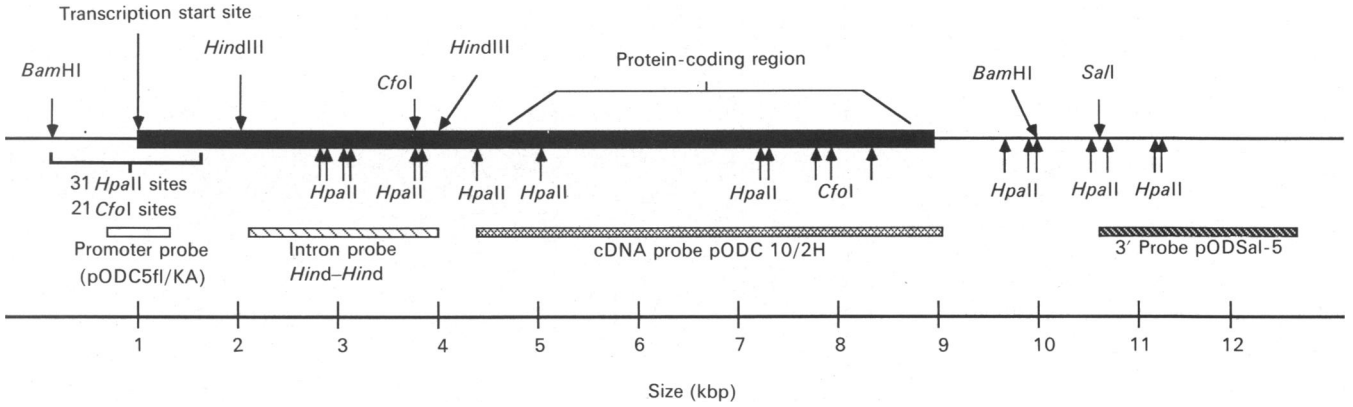

Fig. 1. Schematic model of a region in the human chromosome 2 containing the active ODC gene and flanking sequences

The ODC gene (from the transcription start site to the first polyadenylation signal) is shown as a long black box and flanking regions as a continuous line. Differently shaded boxes below the gene represent the probes recognizing the promoter region, the first intron, the exons included in the cDNA and the 3′-flanking region of ODC gene. The transcription start site and the restriction sites used in this work are marked with black arrows. The protein-coding region of the gene is also indicated.

velopment of glucocorticoid resistance in the same cell line. This decrease in DNA methylation appears to occur in the protein-coding region and in the 5′-flanking region, in addition to the 3′-flanking region of ODC gene, and none of the clones derived from the parental cell line show that type of hypomethylation.

EXPERIMENTAL

Cell cultures

The human myeloma cell lines were originally obtained from an IgG-myeloma patient (Sultan) or from multiple-myeloma patient (Fravel). Cell-culture conditions and adaptation of the cells to grow either in the presence of DFMO or dexamethasone are described elsewhere (Leinonen *et al.*, 1987*a*,*b*; Alhonen-Hongisto *et al.*, 1987*b*). Clones from parental, DFMO-resistant and dexamethasone-resistant cell lines were isolated from soft agar essentially as described by Polvinen *et al.* (1988).

Chemicals

[^{32}P]dCTP (sp. radioactivity > 400 Ci/mmol) and nick-translation kit were purchased from Amersham International (Amersham, Bucks., U.K.). The restriction endonucleases *Hpa*II, *Msp*I and *Cfo*I (= *Hha*I) were purchased from Boehringer, Mannheim, Germany; *Sal*I, *Sac*II, *Hin*dIII and *Bam*HI were from United States Biochemicals (Cleveland, OH, U.S.A.).

Analytical methods

Genomic DNA was extracted by the method of Blin & Stafford (1976). A 10 μg portion of isolated DNA was digested with restriction enzymes according to the instructions of the suppliers. *Hpa*II and *Msp*I both recognize the sequence CCGG, but *Hpa*II cleaves only when the internal cytosine is unmethylated. *Cfo*I is an isoschizomer of *Hha*I and it cleaves the sequence GCGC, provided that the internal cytosine is not methylated. *Bam*HI was sometimes used with methylation-sensitive enzymes in order to get fragments with either fixed 3′-ends or fixed 5′-ends. The restriction fragments were electrophoresed in 0.9 %-agarose gels, transferred to nitrocellulose filters (Southern, 1975) and hybridized with nick-translated (Rigby *et al.*, 1977) probes [sp. radioactivities (0.3–1.0) × 10^8 c.p.m./μg of DNA].

Labelled pODC10/2H complementary to human ODC cDNA (Hickok *et al.*, 1987) was used to detect fragments containing coding region of the ODC gene. The other probes used (Fig. 1) were restriction fragments from genomic clone λgODC/H2 (Halmekytö *et al.*, 1989). A *Sac*II fragment containing 370 bp 5′-flanking region, the first exon and 20 bp of the first intron was cloned into pBR322 and designated as 'pODC5fl/KA'. This probe was used for methylation analyses of the promoter region. A *Hin*dIII fragment containing 1980 bp of the first intron was used without cloning to detect restriction fragments from this part of the gene. A 2.3-kb *Sal*I fragment, starting 1560 bp from the 3′-end of the gene, was cloned into pBR322 and designated as 'pODSal-5'. This probe was used for methylation analyses of the 3′-flanking region. All the probes derived from the genomic ODC clone hybridized very unspecifically, owing to Alu repeats or other kinds of sequences typical of human DNA (Hickok *et al.*, 1990). This was overcome by washing the filters after hybridization for an additional 2 h at +80 °C (the washing solution was 0.1 % SSC/ 0.5 % SDS).

Total cellular RNA was isolated by the method of Auffrey & Rougeon (1980); 20 μg was fractionated on a 1.4% (w/v)-agarose/formaldehyde gel, transferred to Hybond N filters (Amersham) and hybridized with human cDNA probe pODC10/2H.

RESULTS

A restriction map of the ODC locus in human chromosome 2 is shown in Fig. 1. This map is based on nucleotide sequence of the gene and the flanking regions (Hickok *et al.*, 1990). Some unpublished sequence data (about 2 kb of 3′-flanking region) and results from restriction mapping have also been used. Fig. 1 shows the gene itself and known parts of the 5′- and 3′-flanking regions, as well as the probes, deduced from the genomic clone λgODC/ H2 and used for hybridization analyses. Cleavage sites of restriction enzymes used are shown in the map as precisely as possible, but it is impossible to locate every site in the 5′ region of the gene separately, because this CpG island contains 21 *Cfo*I sites and 31 *Hpa*II/*Msp*I sites.

The probe pODC5fl/KA (Fig. 1) covers about 600 bp around the transcription start site, and it was used to study the methylation status of the promoter region of human ODC gene. Because of the large number of CCGG and GCGC sequences in this region, the exact methylation pattern could not be resolved by using this approach. However, rough estimations about the extent of methylation and differences between cell lines can be made. In the parental cell line and in the cell line with amplified ODC sequences, the promoter region appeared to be heavily methylated. The whole CpG island covering the most 5′-region of the first intron, the first exon and hundreds of basepairs of the region upstream from the transcription start site was in fact methylated at most of the sites recognized by the enzymes used. However, no differences between the parental cell line and DFMO-resistant cell lines were observed, indicating that the ODC-gene transcription rate was not enhanced as a result of the decreased methylation state of the 5′ regulatory regions.

The length of the first intron of the human ODC gene is 2852 bp, containing 18 *Hpa*II/*Msp*I sites and ten *Cfo*I sites; 11 of the most 5′ *Hpa*II sites and nine of the most 5′ *Cfo*I sites of the first intron belong to the CpG island, which extends beyond the first exon to the 5′-flanking region. As mentioned above, these sites were mostly methylated when analysed with the probe pODC5fl/KA. When the *Hin*dIII fragment from the first intron was used as a probe, it appeared that one, or a few, of the most 3′ CCGG and GCGC sequences of the GC-rich island were unmethylated in addition to the sites residing further away to the 3′ direction in the first intron. This applied to all types of cell lines, excluding a role for methylation of the first intron in the development of either gene amplification or glucocorticoid resistance.

The cDNA probe pODC10/2H was used to gain information about the methylation within the protein-coding region and 3′-flanking region of the gene. When *Bam*HI was included in the reaction, the 3′-ends of the fragments became fixed to the only *Bam*HI site in the 3′-flanking region. When the cDNA probe was used it became obvious that there were clear differences between the methylation status of the parental and both variant cell lines. However, digestions with *Msp*I or other methylation-insensitive enzymes revealed no differences between different cell lines, indicating that no genetic rearrangements were involved in the development of gene amplification or glucocorticoid resistance (results not shown). The methylation patterns of the Sultan parental-cell line, the cell line with amplified ODC sequences, the dexamethasone-resistant cell line and the multiple-myeloma cell line Fravel are shown in Fig. 2. All the *Hpa*II/*Msp*I and *Cfo*I sites belonging to the protein-coding region appeared to be fully methylated in the parental Sultan cell line. That also held true for the 3-flanking region, where only the two last *Hpa*II/*Msp*I sites (see Fig. 1) were partially hypomethylated. This hypomethylation did not occur in the ODC gene of the multiple-myeloma cell line Fravel with acquired resistance to DFMO without ODC gene amplification or enzyme overproduction (Fig. 2).

Analyses of several clones derived from the Sultan parental-cell line revealed that this cell line was a heteropopulation consisting of at least four different clones having different types of hypomethylation in the 3′-flanking region of their ODC genes (Fig. 3*a*). The clone number 1 was obviously the most methylated one. All the sites analysed in the protein-coding region and in the 3′-flanking region were totally methylated until an unknown site extending about 1 kb to the 3′ direction from the last *Hpa*II/*Msp*I site shown in Fig. 1. The rest of the clones derived from the parental cell line were less methylated when compared with clone 1, having their seven most 3′ *Hpa*II/*Msp*I sites shown in Fig. 1 hypomethylated to different extents. The most interesting clones were numbers 4 and 5, the less methylated ones, resembling clones from a cell line with amplified ODC sequences. In these clones the five *Hpa*II/*Msp*I sites next to the 3′-end of the gene (Fig. 1) were partially hypomethylated, whereas the other sites (including *Cfo*I sites) in the known 3′-flanking region and in the protein-coding region were methylated.

The variant cell line Sultan 20D with an amplified ODC gene appeared to be clearly less methylated than the parental cell line. Analyses with the cDNA probe pODC 10/2H and the 3′-probe pODSal-5 revealed partial hypomethylation within all seven *Hpa*II/*Msp*I sites in the known 3′-flanking region (10–40%), three *Cfo*I sites and the most 5′ *Hpa*II/*Msp*I site in the protein-coding region (about 10%), whereas the two other *Hpa*II/*Msp*I sites in the coding region remained methylated. Analysis of six different clones of the cell line with amplified ODC sequences

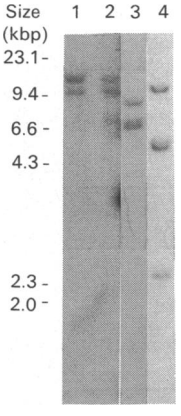

Fig. 2. Restriction-enzyme analysis of ODC sequences in genomic DNA isolated from parental and variant human myeloma-cell lines (Sultan and Fravel)

Isolated DNA (10 μg) from multiple-myeloma-cell line Fravel (1), IgG-myeloma-cell line Sultan (2), Sultan-cell line with ODC gene amplification (3) and dexamethasone-resistant Sultan-cell line was digested with *Hpa*II and processed as described in the Experimental section. Hybridizations were performed with the cDNA probe pODC 10/2H. Molecular-size markers are shown to the left. Lane 3 represents a shorter exposure time (20 h) than other lanes (7 days) because of the 100-times-higher ODC-gene dosage in the cell line with gene amplification.

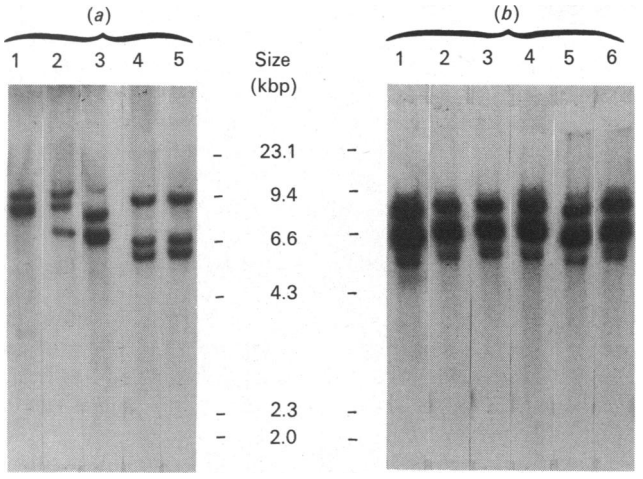

Fig. 3. Restriction-enzyme analysis of ODC sequences in genomic DNA isolated from clones derived from a human myeloma-cell line (Sultan)

Isolated DNA (10 μg) from parental clones (*a*) or clones with amplified ODC sequences (*b*) was digested with *Hpa*II and processed as described in the Experimental section. Hybridizations were performed with cDNA probe pODC 10/2H. Molecular-size markers are shown in the middle. Panels (*a*) and (*b*) represent different exposure times, as the signals in parental clones are about 100 times weaker than in clones with amplified ODC sequences.

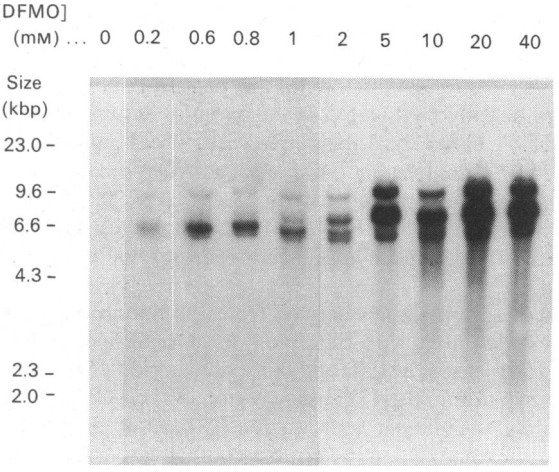

Fig. 4. Restriction-enzyme analysis of ODC sequences in genomic DNA isolated from human myeloma-cell line (Sultan) exposed to increasing concentrations of DFMO

Isolated DNA (10 μg) was digested with *Hpa*II and processed as described in the Experimental section. Hybridization was performed with cDNA probe pODC 10/2H. The DFMO concentrations tolerated by each cell line are shown above the lanes. Molecular-size markers are shown to the left.

revealed an identical methylation pattern when the cDNA probe was used (Fig. 3*b*). The results appeared to be identical irrespective of the particular probe used (results not shown).

When DNA from cell lines resistant to different concentrations of DFMO was analysed with restriction enzymes *Hpa*II or *Cfo*I, it became obvious that hypomethylation can be detected at the same time as the ODC-gene dosage begins to grow (Fig. 4). In fact, the *Hpa*II pattern seen in the cell line growing readily in the presence of 0.2 mM-DFMO was identical with that found in clones 4 and 5 derived from the Sultan parental-cell line. This suggests that the fraction of cells with this kind of methylation

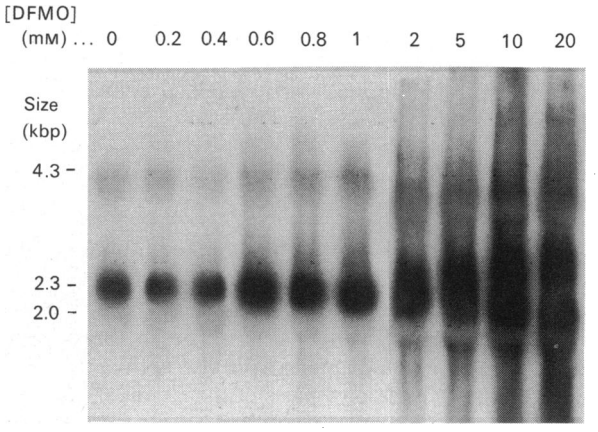

Fig. 5. Amounts of ODC mRNA in the human myeloma cell line (Sultan) exposed to increasing concentrations of DFMO

RNA was extracted from human myeloma cells grown in the presence of the DFMO concentrations shown above the lanes and processed as described in the Experimental section. Hybridization was performed with the cDNA probe pODC 10/2H. Molecular-size markers are shown to the left.

pattern may have a growth advantage, thus becoming enriched in the cell population. At this stage, the amplification process had already started, the gene dosage of the resistant cell line being about doubled in comparison with the parental cell line (Fig. 4). When the concentration of DFMO was raised stepwise to 1 mM, two changes took place. Firstly, the ODC-gene dosage increased, but quite moderately. Secondly, the gene became even more hypomethylated. This can be seen in Fig. 4, where additional small *Hpa*II fragments appeared. When *Cfo*I was used, a new small fragment could also be seen (results not shown). These new restriction fragments were due to partial hypomethylation of either the most 5′ *Hpa*II site in the coding region or one of the three *Cfo*I sites in the coding region (Fig. 1). After this final stage of the hypomethylation process, a very drastic burst of ODC-gene dose occurred when DFMO was increased from 2 to 5 mM (Fig. 4). The gene dosage appeared to grow until the DFMO concentration reached 20 mM, being about 100 times higher than that in the parental cell line. Additional methylation changes did not occur during the later stages of gene amplification. When ODC mRNA levels were measured at different concentrations of DFMO (Fig. 5), it appeared that the enhanced accumulation of ODC message was entirely due to an increased gene copy number. As can be seen in Figs. 4 and 5, at DFMO concentrations of 0.2 mM the cells were apparently already hypomethylated, but the mRNA contents remained at the parental level. The amount of ODC message did not rise clearly until the tolerated DFMO concentration was 0.6 mM and the gene had undergone a 10-fold amplification.

Six clones derived from the Sultan variant cell line capable of growing in the presence 1 μM-dexamethasone were analysed in the same way as the other cell lines. The analysis of the promoter region revealed very heavy methylation in general, even though some kind of hypomethylation was apparent. The largest *Hpa*II fragment was 1.7 kb in all the clones, instead of 2.1 kb in the clones from the parental cell line (results not shown). Because all *Hpa*II/*Msp*I sites in the first intron were also found to be unmethylated in dexamethasone-resistant cells, this hypomethylation must occur about 1.5 kb upstream from the transcription start site. Other techniques, such as genomic sequencing (Church & Gilbert, 1984) or ligation-mediated PCR (Pfeifer *et al.*, 1989) need to be used to exactly localize this/these hypomethylated sites.

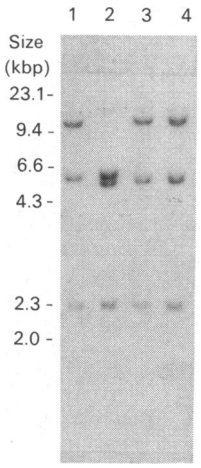

Fig. 6. Restriction-enzyme analysis of ODC sequences in genomic DNA isolated from clones derived from the Sultan-cell line growing in the presence of 1 μM-dexamethasone

Isolated DNA (10 μg) was digested with *Hpa*II and processed as described in the Experimental section. Hybridization was performed with cDNA probe pODC 10/2H. Molecular-size markers are shown to the left. Lanes: 1, D1; 2, D2; 3, D3; 4, D4.

Analyses with the cDNA probe pODC 10/2H and the 3′-probe pODSal-5 revealed three different methylation patterns among the clones from dexamethasone-resistant cell line (Fig. 6). The variation in *Hpa*II patterns arose from methylation differences in the protein-coding region and in the 3′-flanking region, but in all the clones the three *Cfo*I sites and the two most 3′ *Hpa*II/*Msp*I sites in the protein-coding region were partially (about 50%) hypomethylated. Analyses with methylation-insensitive *Eco*RI did not reveal any differences between the clones or between parental and resistant cell lines, indicating that genomic rearrangements in or around ODC gene were not involved (results not shown).

Even though the observed hypomethylation in this case concerned all regions of the ODC gene, overall genomic hypomethylation was not detected by ethidium bromide staining of *Hpa*II or *Cfo*I digests of genomic DNA from parental and resistant cell lines. However, Leinonen and others (P. Leinonen, L. Alhonen, R. Laine, O. A. Jänne & J. Jänne, unpublished work) had earlier shown that proto-oncogene *erb-Al* also becomes hypomethylated in Sultan-myeloma cells chronically exposed to dexamethasone.

DISCUSSION

Specific hypomethylation of the ODC gene is apparently related to gene amplification and glucocorticoid resistance in the human IgG-myeloma-cell line (Alhonen-Hongisto *et al.*, 1987*a*; Leinonen *et al.*, 1987*a*), but not to DFMO resistance in a multiple-myeloma-cell line (Alhonen-Hongisto *et al.*, 1987*b*). Whether the hypomethylation is a cause, or a consequence, of glucocorticoid resistance is not known. However, it is quite obvious that initiation of the gene-amplification process is connected with the methylation state present in a fraction of the parental Sultan-cell line. This hypomethylation in the 3′- flanking region, and later in the protein-coding region of the ODC gene, is not connected to enhanced transcriptional activity, as the levels of ODC message are tightly coupled to the gene dosage.

The mechanism leading to the primary stage of ODC-gene amplification after hypomethylation is not clear. However, when these cells were adapted to grow in the presence of 2 mM-DFMO, additional *Hpa*II (5.5 kb) and *Cfo*I (4.2 and 4.8 kb; results not shown) bands appeared. The small *Cfo*I bands were caused by hypomethylation in the protein-coding region, followed by a dramatic increase in gene dosage when a DFMO concentration of 5 mM was reached. When a DNA sequence containing these particular hypomethylated *Cfo*I sites was analysed with the aid of a computer, a motif identical with the amplification-promoting sequence in mouse (Wegner *et al.*, 1990) was found. This sequence has been shown to stimulate DNA amplification provided that it is not methylated. It is possible, even in the case of the human ODC gene, that unmethylation of this sequence or its surroundings enhances the amplification process that leads to the ability to grow in the presence of DFMO concentrations up to 40 mM.

It is premature to propose that this kind of hypomethylation always precedes the gene-amplification process of various genes in different cell lines. This conclusion would be easy to draw in case of malignant cell lines by alluding to many reports about hypomethylation in various cancers (Feinberg & Vogelstein, 1983*a*,*b*; Gama-Sosa *et al.*, 1983; Goelz *et al.*, 1985; Feinberg *et al.*, 1988). However, when fibroblastic Chinese-hamster ovary cells were analysed by Pilz *et al.* (1990), there appeared to be methylation differences between a wild-type cell line and a DFMO-resistant cell line with ODC-gene amplification. Hypomethylation in the cell line with amplified ODC sequences is located at two *Hpa*II/*Msp*I sites and two *Ava*I sites in the protein-coding region and also at one *Hpa*II/*Msp*I site and one *Ava*I site in the 3′-flanking region. Whether this kind of hypomethylation can be found in all cell lines capable of amplifying their ODC sequences remains to be resolved. Even though the specific hypomethylation is certainly not the only factor involved in ODC-gene-amplification process, we have shown that multiple-myeloma cell line Fravel with a heavily methylated 3′-flanking region of ODC gene does not undergo gene amplification in the presence of DFMO, but instead acquires resistance to the drug by overproducing arginase.

Chronic exposure to dexamethasone caused by hypomethylation all over the ODC gene and enhanced accumulation of ODC mRNA in the Sultan-myeloma-cell line. DNA methylation decreased also in the 5′-flanking region, and this could be the most obvious reason for enhanced transcription of the gene. However, the promoter region itself remained heavily methylated in these cells. It is of course possible that some CpG sites not detectable with the restriction enzymes used have been unmethylated and are the real cause of enhanced ODC-gene expression. This is in fact the only reasonable explanation, because according to the CpG-island theory heavy methylation would not allow transcription at all. That the promoter region of ODC gene in parental and variant cell lines was extremely methylated came as quite a surprise, but Jones *et al.* (1990) have shown that CpG islands can become methylated to an abnormally high degree in immortalized cell lines when compared with normal tissues. This observation has also been made by Antequera *et al.* (1990). More specifically, de Bustros *et al.* (1988) reported that, in spite of genomic hypomethylation in human neoplasms, the *CALC1* gene (segregates with chromosome 11) was hypermethylated also within its CpG-rich 5′-region.

It would be tempting to presume that the heavy methylation of the ODC gene's 5′-region in the Sultan-cell line is only a consequence of growth in cell culture. The methylation state of the normal precursor of the Sultan-cell line is unknown. It is however possible that this kind of hypermethylated state may also be typical of certain myelomas and may make a considerable contribution to gene amplification. Moreover, hypermethylation of the 5′-region of the ODC gene might even be the primary cause of gene amplification. It is possible that, because of the 5′ hypermethylation, the transcription level of the gene under optimal

conditions is just high enough to provide a minimum amount of ODC, but far too low when ODC inhibitor is added. The pressure for gene amplification would in these cells be higher than in normal cells or other types of cancer cells, thus providing means to survive under the selection pressure. The amplification process itself does not seem to cause decreased methylation in the key sites in the regulatory regions of the gene, because the transcription rate remains tightly coupled to the gene dosage, as recently shown (Hyttinen *et al.*, 1991).

Whatever the ultimate cause of enhanced ODC mRNA accumulation in the dexamethasone-resistant cell line, the typical methylation status found in these cells is not present in the parental cell line. Even though the decreased methylation level in this cell line does not involve the whole genome, the observed hypomethylation of the *erb-Al* proto-oncogene reveals that enhanced expression of this, and some other, growth-related genes may also be involved, thus indicating the complexity of this phenomenon.

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