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Mouse and human ornithine decarboxylase genes

Methylation polymorphism and amplification

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With the use of the isoschizomeric restriction endonucleases *Hpa*II and *Msp*I, we found that mouse tumour ornithine decarboxylase (ODC; EC 4.1.1.17) genes are extensively methylated. ODC genes in L1210 mouse leukaemia cells were apparently more methylated than in Ehrlich ascites carcinoma, as revealed by the use of *Hpa*II endonuclease, yet the digestion of genomic DNA isolated from these two murine tumour cell lines with *Msp*I, which cleaves at a CCGG sequence, also with internally methylated cytosine, resulted in an apparently identical restriction pattern. It is possible that the amplification of ODC genes in Ehrlich ascites-carcinoma cells in response to 2-difluoromethylornithine (DFMO) was associated with hypomethylation, or that less-methylated genes were amplified. A human myeloma (Sultan) cell line only revealed three separate hybridization signals when cleaved with *Hpa*II. One of these signals was amplified under the pressure of DFMO. When cleaved with *Msp*I, these three *Hpa*II fragments disappeared and were replaced by a double signal of 2.3–2.4 kilobase-pairs (kbp) in size. The amplified ODC sequences in the Sultan myeloma cell line apparently originated from chromosome 2, as indicated by a unique hybridization signal in a 5.8 kbp *Hin*dIII fragment specific for the human ODC locus on chromosome 2. A comparison of different human cells, the Sultan myeloma, a lymphocytic B-cell leukaemia (Ball), normal mononuclear leucocytes and leucocytes obtained from leukaemia patients, revealed interesting differences in the methylation of ODC genes. The use of two restriction endonucleases (*Hpa*II and *Cfo*I), the cleavage site for both of which contains a CG sequence and which only cleave when cytosine is unmethylated, indicated that ODC genes in the lymphocytic leukaemia cells were much less methylated than those in the normal leucocytes or in the Sultan cells.

INTRODUCTION

Studies with the cloned ornithine decarboxylase (ODC) probes have indicated that not only is the enzyme protein unqiue among mammalian enzymes, but also the molecular biology of ODC includes many interesting features. Mouse (McConlogue *et al.*, 1984; Kahana & Nathans, 1984; Alhonen-Hongisto *et al.*, 1985*a*) and human (Winqvist *et al.*, 1986; Leinonen *et al.*, 1987) cells apparently contain a multigene family for ODC. The genes for mouse ODC also easily undergo amplification under the pressure of 2-difluoromethylornithine (DFMO), a highly specific irreversible inhibitor of the enzyme (McConlogue *et al.*, 1984; Kahana & Nathans, 1984; Alhonen-Hongisto *et al.*, 1985*a*,*c*). We selected the first human cell line with amplified ODC sequences (Leinonen *et al.*, 1987) and by using mouse/human somatic-cell hybrids found that human ODC sequences are localized on chromosomes 2 and 7 (Winqvist *et al.*, 1986).

In mouse, the amplification and the subsequent loss of the amplified ODC-gene sequences appear to result in genetic rearrangements, alteration of the expression of cell-surface glycoproteins and changes of tumorigenicity (Alhonen-Hongisto *et al.*, 1985*b*).

With the use of isoschizomeric restriction endonucleases *Hpa*II and *Msp*I, which both cleave at CCGG sequence (*Msp*I, but not *Hpa*II, also cleaves when the internal cytosine is methylated), we have studied the methylation status of ODC genes in mouse and man. The two mouse tumour cell lines studied indicated that ODC genes are methylated, probably at numerous introns. In contrast with the approx. 20 signals appearing after digestion of mouse DNA with *Msp*I, similar digestion of human genomic DNA revealed only a double signal in restriction fragments of 2.3 and 2.4 kilobase-pairs (kbp). Interesting methylation polymorphism was found between a human B-cell leukaemia cell line (Ball) and a human myeloma cell line (Sultan). The cleavage of genomic DNA isolated from these cells with *Hpa*II or *Cfo*I (cleaving at GCGC, provided that cytosine is not methylated) clearly indicated that ODC sequences in the myeloma cell line were more methylated than those in the lymphatic leukaemia cells. The fact that there was a methylation polymorphism was confirmed by *Msp*I digestion yielding exactly identical cleavage patterns in both cell lines.

The amplified ODC sequences, under the pressure of DFMO, in the human myeloma cell line were apparently derived from the ODC locus on human chromosome 2, as indicated by the amplified signal from a unique *Hin*dIII restriction fragment.

Abbreviations used: ODC, ornithine decarboxylase (EC 4.1.1.17); DFMO, 2-difluoromethylornithine; kbp, kilobase-pairs.

EXPERIMENTAL

Cell cultures

Mouse L1210 leukaemia cells and Ehrlich ascites-carcinoma cells were grown in RPMI 1640 medium supplemented with 5% (v/v) pooled human serum (Transfusion Service, Finnish Red Cross, Helsinki, Finland) and antibiotics (penicillin and streptomycin). The selection of DFMO-resistant mouse tumour cells with amplified ODC sequences was accomplished as described by Alhonen-Hongisto *et al.* (1985*a*,*c*).

The human myeloma cell line (Sultan) was originally obtained from a IgG-myeloma patient. The human lymphocytic (B-cell) leukaemia cell line (Ball) was generously given by Dr. Leif Andersson. Both cell lines were cultured in the same medium used for the mouse cell lines. ODC-overproducing Sultan cells were selected by exposing the myeloma cells to stepwise-increasing concentrations of DFMO (Leinonen *et al.*, 1987).

Peripheral mononuclear leucocytes were isolated by the method of Seppänen *et al.* (1980).

Chemicals

DFMO was generously given by Centre de Recherche Merrell International (Strasbourg, France). [^{32}P]dCTP (sp. radioactivity 410 Ci/mmol) was purchased from Amersham International (Amersham, Bucks., U.K.). The restriction endonucleases *Hpa*II, *Msp*I, *Cfo*I and *Eco*RI were purchased from Boehringer Mannheim, West Germany, and *Bam*HI and *Hin*dIII from Promega Biotec, Madison, WI, U.S.A.

Analytical methods

Genomic DNA was extracted by the method of Blin & Stafford (1976). Isolated DNA was digested with *Hpa*II, *Msp*I, *Cfo*I, *Eco*RI, *Bam*HI or *Hin*dIII according to the instructions of the suppliers. The restriction fragments were electrophoresed in 0.9% -agarose gels and transferred on to nitrocellulose filters (Southern, 1975). The hybridization analyses for ODC were performed with nick-translated (Rigby *et al.*, 1977) pODC16 complementary to mouse ODC cDNA (Jänne *et al.*, 1984) or with the pODC10/2H complementary to human ODC cDNA (Winqvist *et al.*, 1986). The specific radioactivities of the ^{32}P-labelled probes were 0.5–1.0 ($\times 10^8$) c.p.m./μg of DNA. The partial restriction maps of the probes can be found in the references cited.

The hybridization analyses for *N-myc* sequences were performed with the radioactive insert of the pNb-1 clone of the human *N-myc* oncogene (Schwab *et al.*, 1983).

RESULTS

Fig. 1 shows the cleavage pattern by *Hpa*II and *Msp*I of genomic DNA isolated from mouse L1210 leukaemia cells and hybridized with pODC16 DNA. As shown in Fig. 1, ODC genes in this tumour cell line appear to be heavily methylated, as *Hpa*II (which cleaves at an unmethylated CCGG sequence) only cleaved three major fragments (15.0, 8.7 and 6.2 kbp) (Fig. 1, lane 1), whereas digestion with the isoschizomeric *Msp*I (cleaving also when the internal cytosine of the CCGG sequence is methylated) yielded about 20 signals (Fig. 1, lane 2). The great number of signals obtained after the digestion with *Msp*I, in comparison with those yielded with *Hpa*II, is, of course, an indication of extensive methylation of

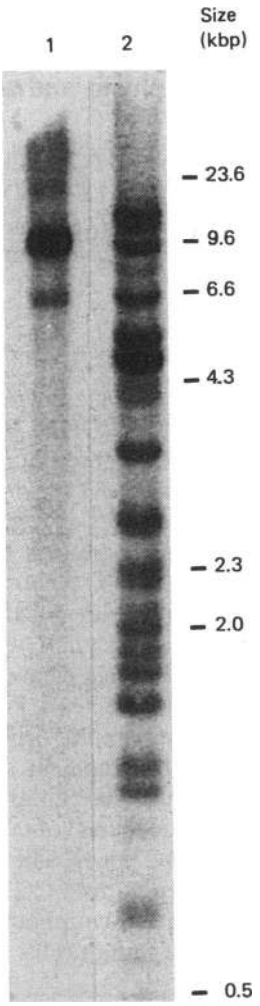

Fig. 1. Restriction-enzyme analysis of ODC sequences in genomic DNA isolated from L1210 leukaemia cells

Isolated DNA (10 μg) was digested either with *Hpa*II (lane 1) or *Msp*I (lane 2), fractionated by electrophoresis, transferred on to hybridization membranes and hybridized to nick-translated pODC16 DNA. Molecular-size markers are shown to the right.

mouse ODC genes or sequences flanking them, but also reflects the multigene family of this enzyme. The methylation of the genes is probably located at introns, as the cDNA of ODC only contains two *Hpa*II sites in its coding segment (McConlogue *et al.*, 1984; Kahana & Nathans, 1984).

Similar restriction-endonuclease analyses with the isoschizomers *Hpa*II and *Msp*I performed with genomic DNA isolated from mouse Ehrlich ascites-carcinoma cells indicated that ODC genes in this tumour-cell line were less methylated (assuming that the size of the mouse ODC gene is 6–8 kbp) than those in L1210 leukaemia cells. *Hpa*II digestion of parental Ehrlich ascites-carcinoma DNA resulted in small-M_r fragments. Lane 3 (Fig. 2) shows the hybridization signals after *Hpa*II digestion of genomic DNA isolated from Ehrlich ascites-carcinoma cells resistant to 20 mM-DFMO and bearing amplified ODC genes (Alhonen-Hongisto *et al.*, 1985*a*). Although difficult to judge, one may get the impression that the amplifed ODC genes were less

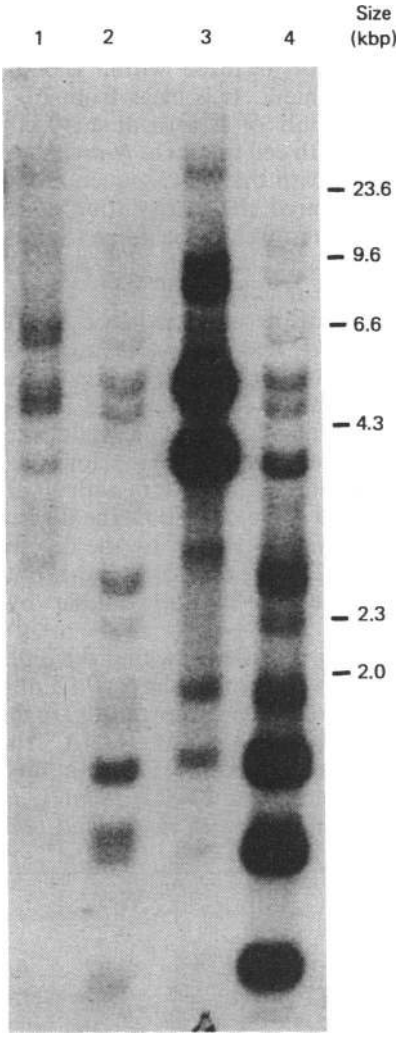

Fig. 2. Restriction-enzyme analysis of ODC sequences in genomic DNA isolated from parental Ehrlich ascites-carcinoma cells or from carcinoma cells containing amplified sequences for ODC

Isolated DNA (10 μg) from parental cells (lanes 1 and 2) or from cells with amplified ODC sequences (lanes 3 and 4) was digested either with *Hpa*II (lanes 1 and 3) or with *Msp*I (lanes 2 and 4) and processed as described in the legend to Fig. 1.

methylated than those in the parental cell line (Fig. 2, lane 1), as most of the amplified hybridization signals resided in smaller restriction fragments (Fig. 2, lane 3) than those in the parental cell line (Fig. 2, lane 1). However, the digestion with *Msp*I of both the parental and the variant cell line yielded almost identical restriction patterns, suggesting that the possible differences in the *Hpa*II digestion pattern were due to methylation polymorphism. The fact that digestion with *Msp*I yielded fragments smaller than the approx. 2.5-kb ODC transcript indicate that the methylated sequences are within ODC genes.

A cleavage of high-M_r DNA from the Sultan cells resulted in a pattern strikingly different from those obtained with the mouse tumour cells. As illustrated in Fig. 3(*a*) (lane 1), digestion with *Hpa*II produced three major hybridization signals residing in fragments of 11.2, 8.9 and 6.9 kbp (the fragment size calculations are averages from four separate blot analyses). On treatment with *Msp*I, these three signals disappeared and a major hybridization signal appeared at a restriction fragment of 2.3–2.4 kbp (Fig. 3*b*, lane 2). In fact, when different exposure times were used, this signal was apparently resolved to a double signal. One may also notice that there was an apparent summation of the signal intensities at the 2.3–2.4 kbp fragment appearing after the cleavage with *Msp*I (Fig. 3*a*). Fig. 3(*b*) shows similar isoschizomeric restriction analysis of DNA from DFMO-resistant Sultan cells. It is obvious that the amplified ODC sequences mainly resided in the 6.9-kbp *Hpa*II fragment (Fig. 3*b*, lane 1) and that the cleavage with *Msp*I again moved the hybridization signals to the 2.3–2.4-kbp *Msp*I fragments (Fig. 3*b*, lane 2). As in the mouse ascites-carcinoma cells, the amplification of ODC

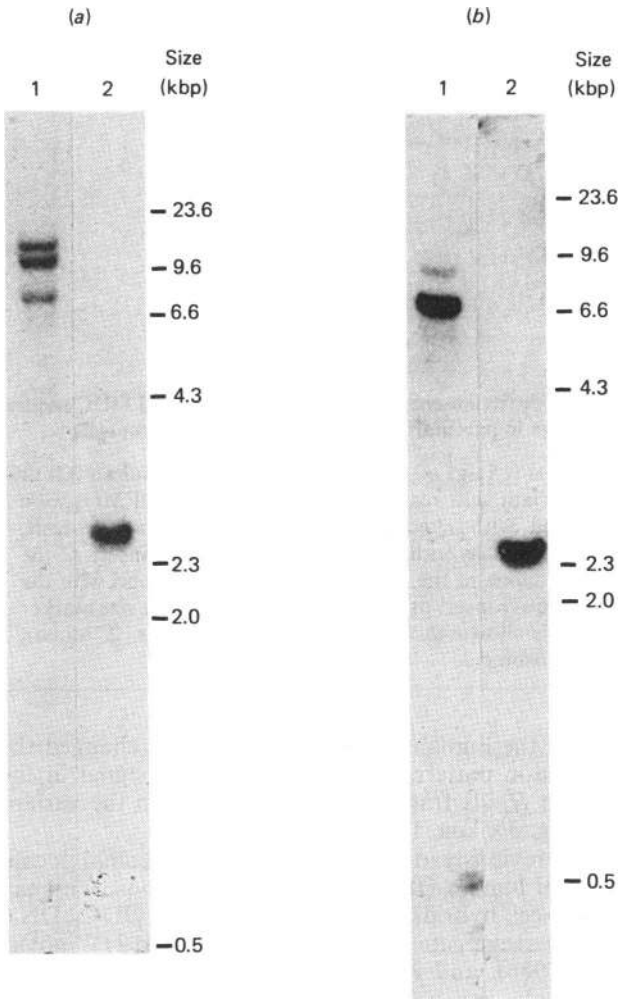

Fig. 3. Restriction-enzyme analysis of ODC sequences in genomic DNA isolated from human myeloma cell line (Sultan)

Isolated DNA (10 μg) from parental cells (*a*) or from cells with amplified ODC sequences (*b*) was digested either with *Hpa*II (lane 1 in *a* and *b*) or with *Msp*I (lane 2 in *a* and *b*) and processed as described in the legend to Fig. 1. The two analyses are from separate experiments, as the amplification was so pronounced that the signals in the parental cells under these conditions were almost invisible.

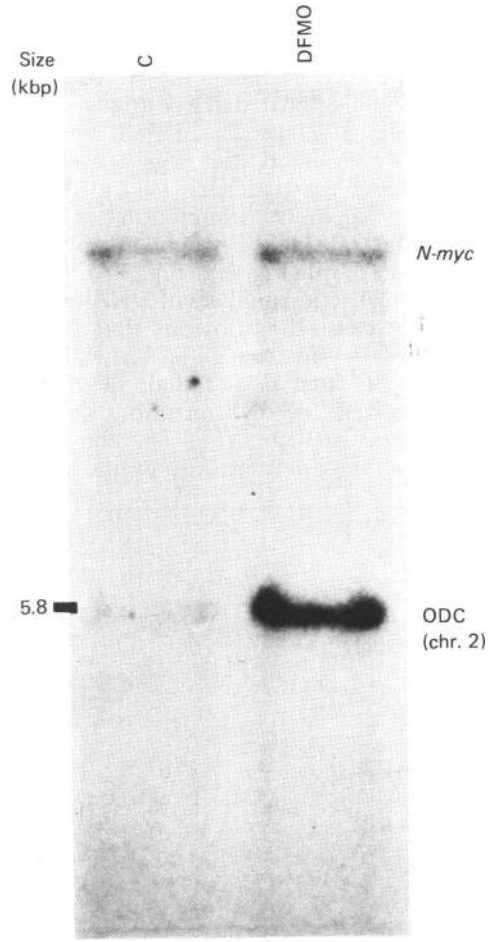

Fig. 4. Restriction-enzyme analysis of *N-myc* and ODC sequences in parental and DFMO-resistant Sultan cells

Samples (15 μg) of DNA from the parental Sultan (C) or the variant cells resistant to 1 mM-DFMO (DFMO) were digested with *Hin*dIII, fractionated by electrophoresis, blotted and hybridized with the radioactive insert of the pNb-1 clone of the human *N-myc* oncogene and with the radioactive insert of the pODC10/2H clone. A size marker (kbp) is shown to the left. Abbreviation: chr. 2, human chromosome 2.

genes in the human Sultan cells may have changed the methylation pattern, as the hybridization signal in the 11.2-kbp *Hpa*II fragment was not visible in the variant cells (Fig. 3*b*, lane 1).

We previously determined the chromosomal localization of human ODC genes. By using mouse × human somatic-cell hybrids, we found that human ODC cDNA sequences segregate with chromosomes 2 and 7 (Winqvist *et al.*, 1986), and in *Hin*dIII-digested human genomic DNA only one positive signal, residing in a 5.8-kbp restriction fragment, was seen. This signal is specific to the ODC locus at human chromosome 2 (Winqvist *et al.*, 1986).

Fig. 4 shows a restriction-enzyme analysis of human DNA isolated either from the parental Sultan cell line or the variant cell line overproducing ODC. DNA was digested with *Hin*dIII and hybridized with the human pODC10/2H plasmid. As seen in Fig. 4, the amplified sequences clearly resided in the 5.8-kbp fragment, specific to the human chromosome 2. The filter was also hybridized with the radioactive insert of the pNB-1 clone of the human *N-myc* oncogene, as ODC and *N-myc* sequences are both localized within the short arm of human chromosome 2. It is clear from Fig. 4 that the hybridization signals of *N-myc* at a 10-kbp fragment were similar in both cell lines, i.e. *N-myc* sequences were not co-amplified with the ODC sequences.

We next compared the methylation status of ODC genes of the human myeloma cell line (Sultan) with that of another human tumour cell line, Ball, a B-cell-type acute-lymphatic-leukaemia cell line. Three restriction enzymes were used: the isoschizomeric *Hpa*II and *Msp*I, cleaving at CCGG, and *Cfo*I, cleaving at GCGC, but only when the cytosine is unmethylated. The results of this restriction analysis are depicted in Fig. 5. The ODC genes in the human leukaemia cells (Ball) were strikingly less methylated than those of the myeloma cells (Sultan), as revealed by the digestion pattern with *Hpa*II and *Cfo*I. Lanes 1 (Ball) and 4 (Sultan) show the digestion pattern with *Cfo*I. However, as shown by the digestion pattern with *Msp*I (lane 2, Ball; lane 5, Sultan), both cell lines yielded the same 2.3–2.4-kbp double hybridization signal.

In further support of the view that the different *Hpa*II and *Cfo*I digestion patterns in these two human cell lines were due to methylation polymorphism are the following experimental findings. High-M_r DNA from Ball or Sultan cell lines was digested with five different restriction endonucleases: *Eco*RI, *Bam*HI, *Hpa*II, *Cfo*I and *Msp*I. When cleaved with *Eco*RI and *Bam*HI, which

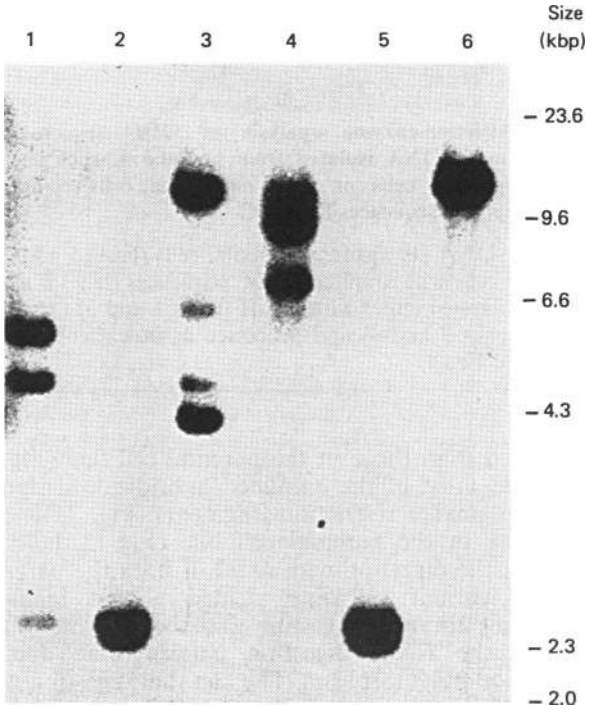

Fig. 5. Restriction-enzyme analysis of ODC sequences in genomic DNA isolated from human Ball and Sultan cells

Isolated DNA (10 μg) from Ball (lanes 1, 2 and 3) and Sultan (lanes 4, 5 and 6) cell lines were digested with *Hpa*II (lanes 1 and 4), with *Msp*I (lanes 2 and 5) or with *Cfo*I (lanes 3 and 6) and processed as described in the legend to Fig. 1.

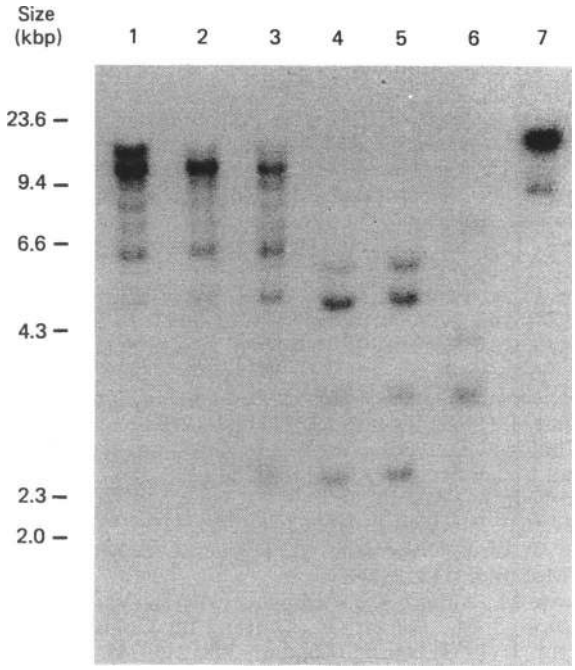

Fig. 6. Restriction-enzyme analysis of ODC sequences in genomic DNA isolated from normal human mononuclear leucocytes and from leucocytes of leukaemia patients

Isolated DNA (10 μg) from mononuclear leucocytes of healthy volunteers (lanes 1 and 2), of a myeloma patient (lane 3), of two patients with chronic lymphocytic leukaemia (lanes 5 and 6) or with chronic myelocytic leukaemia (lane 7) or from the Ball cells (lane 4) was digested with *Hpa*II, fractionated by electrophoresis, blotted and hybridized with nick-translated pODC16. The size markers (kbp) are shown to the left.

do not have a CG sequence at their cleavage site, DNA from both cell lines gave an identical restriction pattern as regards both the size and number of the fragments. It is, however, clear that DNA from the Ball cells was digested by *Hpa*II and *Cfo*I more effectively than DNA from the Sultan cells. In fact, the average restriction (*Hpa*II or *Cfo*I) fragment size containing ODC hybridization signals was in Ball cells about half of that found in the Sultan cells, yet the *Msp*I digestion patterns were identical. These results obviously indicate that the observed polymorphism between these two human tumour-cell lines is due to a different methylation status.

Fig. 6 shows a further blot analysis of human genomic DNA digested with *Hpa*II. The genomic DNA was isolated from mononuclear leucocytes obtained from healthy volunteers (lanes 1 and 2), from a myeloma patient (lane 3), from patients with chronic lymphocytic leukaemia (lanes 5 and 6) or from a patient with chronic myelocytic leukaemia (lane 7). Lane 4 represents genomic DNA isolated from the human Ball (B-cell) cell line. As indicated in Fig. 6, the DNA obtained from the Ball cells or from the mononuclear leucocytes of the patients with chronic lymphocytic leukaemia was much more effectively digested with *Hpa*II than that of healthy volunteers or of the myeloma or chronic-myelocytic-leukaemia patients. This polymorphism apparently indicates that human lymphocytic-leukaemia cells are less methylated at their ODC genes (assuming that intact ODC genes are much larger than the approx. 2.4-kb ODC transcript). It must be mentioned that, on digestion with *Msp*I, the DNA from the different human cells yielded a similar double signal at the 2.3–2.4 kbp fragment. To ascertain whether this kind of hypomethylation is typical of human B-cell leukaemia, more samples must be analysed.

DISCUSSION

ODC is one of those few mammalian enzymes the expression of which appears to be strictly related to the growth potential of a given cell (Jänne *et al.*, 1978). This view is supported by mounting experimental evidence indicating that an inhibition of ODC, and hence the formation of putrescine and spermidine, invariably results in growth inhibition of animal cells (Heby & Jänne, 1981; Jänne *et al.*, 1983).

The elucidation of the molecular biology of ODC has related many additional interesting and even unusual features concerning the gene(s) of this enzyme. The genes of ODC easily undergo amplification when exposed to DFMO, a mechanism-based irreversible inhibitor of the enzyme (Metcalf *et al.*, 1978), in both mouse (McConlogue *et al.*, 1984; Kahana & Nathans, 1984; Alhonen-Hongisto *et al.*, 1985*a*,*c*) and human tumour cells (Leinonen *et al.*, 1987).

DNA methylation, which in animal cells almost exclusively occurs on cytosine in the dinucleotide sequence CpG, is potentially a chemical modification of DNA that may regulate gene activity during differentiation (Adams & Burdon, 1985) and even contribute to the tumorigenicity and metastatic properties of malignant cells (Trainer *et al.*, 1985). As shown in the present results, ODC genes in mouse and in human tumour cells are heavily methylated. However, as revealed by the use of *Hpa*II and *Cfo*I, two restriction enzymes the cleavage sites of which involve CpG sequences and are inhibited by cytosine methylation, there exists methylation polymorphism between different mouse and human cell lines. It is extremely difficult to judge whether hypomethylation of ODC genes is associated with an enhanced transcriptional activity and an overproduction of the enzyme, as the synthesis (and mRNA expression) of ODC greatly fluctuates during the cell cycle. It is, however, possible that in mouse Ehrlich ascites cells the amplification of ODC genes involved sequences that initially were less methylated.

In contrast with the mouse tumour cells (L1210 leukaemia and Ehrlich ascites carcinoma), the genomic DNA of which gave about 20 hybridization signals on digestion with *Msp*I, human tumour DNA when cleaved with the same enzyme yielded only one apparent double signal in Southern-blot analysis. Interestingly, the two human cell lines, a myeloma and acute lymphoblastic leukaemia, showed completely different cleavage patterns when digested with *Hpa*II or *Cfo*I, but displayed similar patterns when cleaved with *Eco*RI, *Bam*HI or *Msp*I, apparently indicating a methylation polymorphism. As judged by *Hpa*II or *Cfo*I fragment sizes, the lymphocytic-leukaemia cells were less methylated at their ODC sequences than the myeloma cells. Our preliminary hybridization analyses (Fig. 6) also indicated that the ODC sequences in lymphocytic-leukaemia cells obtained from two leukaemic patients were strikingly hypomethylated in comparison with those found in normal mononuclear cells (obtained from two healthy volun-

teers). It is clearly premature to draw any conclusions regarding the possible clinical significance of this observation, as many more samples have to be analysed.

A further piece of information about the amplification of human ODC genes is likewise included in the present results. We previously showed that human ODC sequences segregate with chromosomes 2 and 7 (Winqvist *et al.*, 1986). We now present evidence that the amplified ODC sequences in the Sultan cells exposed to DFMO were derived from chromosome 2. Interestingly, in spite of the same sub-chromosomal localization of the sequences for the oncogene *N-myc*, these sequences were not co-amplified with the ODC genes.

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