Journal of Cellular Biochemistry 57:670–679 (1995)

DNA Methylation Is Not Involved in the Structural Alterations of *Ornithine Decarboxylase* or Total Chromatin of c-Ha-*ras*$^{Val\ 12}$ Oncogene-Transformed NIH-3T3 Fibroblasts

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Abstract The *ornithine decarboxylase* (*odc*) gene is an early response gene, whose increased expression and relaxed chromatin structure is closely coupled to neoplastic growth. In various tumour cells, the *odc* gene displays hypomethylation at the sequences CCGG. Hypomethylation of genes is believed to correlate with chromatin decondensation and gene expression. Since a given pattern of DNA methylation may not be preserved in neoplastic cells, we studied the methylation status of *odc* gene at the CCGG sequences in c-Ha-*ras*$^{Val\ 12}$ oncogene-transformed NIH-3T3 fibroblasts during the growth cycle and relative to their normal counterparts. We found that the methylation state of the *odc* gene and its promoter and mid-coding and 3′ regions remain unaltered during the cell cycle. We also found that in *ras* oncogene-transformed cells, which display a more decondensed nucleosomal organization of chromatin than the normal cells, the CCGG sequences in bulk DNA and at the *odc* gene were methylated to the same extent as in the nontransformed cells. These data suggest that DNA hypomethylation at the CCGG sequences is not a prerequisite for chromatin decondensation and cell transformation by the c-Ha-*ras*$^{Val\ 12}$ oncogene.

Key words: serum-stimulation, chromatin structure, gene expression and replication, cell cycle

The *ornithine decarboxylase* (*odc*) gene belongs to the class of immediate early genes [Stimac and Morris, 1987; Jähner and Hunter, 1991]. It encodes the enzyme ODC that catalyzes the formation of putrescine from ornithine [Pegg, 1986]. Several lines of evidence indicate that putrescine and its metabolic derivatives play an important role in the regulation of cell growth [Tabor and Tabor, 1984; Pegg, 1986]. Cell transformation by various oncogenes, like *ras*, v-*src*, and *neu*, is closely coupled to an increase in the levels of *odc* transcripts and enzymatic activity of ODC [Sistonen et al., 1987, 1989a,b; Hölttä et al., 1988]. Recent evidence further indicates that the rise in *odc* expression may be critical for cell transformation [Auvinen et al., 1992; Hölttä et al., 1993]. Moreover, DNA transfection-experiments have shown that normal *odc* sequences are capable of transforming NIH-3T3 and Rat 1 cells when overexpressed [Auvinen et al., 1992; Moshier et al., 1993].

Cell transformation appears also to be accompanied by DNA rearrangements and alterations in the structure of chromatin [Bremner and Balmain, 1990; Laitinen et al., 1990; and references therein]. For example, in c-Ha-*ras*$^{Val\ 12}$ oncogene-transformed NIH-3T3 fibroblasts the copy-number and expression level of *ras* oncogene correlate with the degree of transformation [Sistonen et al., 1987] and decondensation of the chromatin structure [Laitinen et al., 1990, 1994]. Transformation by *ras* oncogene is also associated with unfolding of the chromatin structure of the early genes *odc* and c-*myc* [Laitinen et al., 1990].

Several studies implicate a coupling between chromatin organization and DNA methylation [Solage and Cedar, 1978; Ball et al., 1983; Keshet et al., 1986; Holliday, 1987; Ferguson-Smith et al., 1993]. It should also be noted that relaxation of imprinted genes has recently been discovered as a novel epigenetic mutational mechanism in human cancer development [Rainier et al., 1993].

Received August 3, 1994; accepted September 19, 1994.

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In this context, it is also interesting to note that the Chinese hamster ovary cells appear to have only a single active *odc* allele, as a result of the other one being inactivated by hypomethylation. The inactive allele is, however, reactivatable by treatment with 5-azacytidine [Pilz et al., 1990]. In vitro-methylated genes, introduced into cells by transfection, become localized into the condensed, transcriptionally inactive chromatin domains, while the hypomethylated genes are localized into the active chromatin particles [Keshet et al., 1986]. Similarly, in vivo, a maternally inherited allele at the mouse *H19* locus that is expressed is accessible to nucleases and is hypomethylated in the CpG island promoter, while the transcriptionally inactive promoter of the parental-origin H19 allele is heavily methylated and has nuclease-resistant chromatin configuration [Ferguson-Smith et al., 1993].

Methylation analyses, such as digestions of DNA with methylation-sensitive and -insensitive restriction enzymes, have revealed that specific genes in different animal tissues display a characteristic and inheritable methylation pattern [Razin and Riggs, 1980; Holliday 1987; Antequera et al., 1990]. Furthermore, transfection experiments with phage ΦK-174 and chicken *thymidine kinase* sequences into cells have provided evidence that methylation remains unchanged through several cycles of DNA replication [Wigler et al., 1981; Stein et al., 1982]. Likewise, Szyf et al. [1985] have reported that in normal mouse lymphocytes cell cycling is not accompanied by transient changes in methylation patterns of bulk DNA. However, methylation changes are known to occur during cell transformation and tumour progression [Razin and Riggs, 1980; Feinberg and Vogelstein, 1983; Harrison et al., 1983; Gama-Sosa et al., 1983; Young and Tilghman, 1984; Goelz et al., 1985; Razin et al., 1986; Holliday, 1987; de Bustros et al., 1988]. The Hpa II methylation analyses have provided information that during differentiation and tumour development several genes undergo progressive hypomethylation [Feinberg and Vogelstein, 1983; Harrison et al., 1983; Gama-Sosa et al., 1983; Young and Tilghman, 1984; Goelz et al., 1985]. Also the oncogene-like *odc* gene [Auvinen et al., 1992] appears to be hypomethylated in some neoplastic cells. For example, in human chronic lymphocytic leukemia cells the CCGG sequences of *odc* gene are hypomethylated, while the same sequences are highly methylated in normal human lymphocytes [Alhonen-Hongisto et al., 1987; Wahlfors, 1991, 1992].

However, despite intensive research, the significance of the methylation changes in cell transformation still remains elusive. Since c-Ha-*ras*$^{Val\ 12}$ oncogene-transformed mouse 3T3 fibroblasts display a more decondensed chromatin than their normal counterparts [Laitinen et al., 1990], we addressed the question of whether the destabilization of chromatin in *ras* oncogene-transformed cells is due to hypomethylation at the CCGG sequences in bulk DNA and the *odc* promoter. We also examined whether cell cycling in the transformed 3T3 fibroblasts might be associated with changes in the accessibility of Hpa II to the sequences CCGG at the *odc* locus.

EXPERIMENTAL PROCEDURES

Cell Culture and Synchronization

The cell lines used, the normal NIH-3T3 cells (N1), and the c-Ha-*ras*$^{Val\ 12}$ oncogene-transformed NIH-3T3 (E4) cells, have been described previously [Sistonen et al., 1987; Hölttä et al., 1988; Laitinen et al., 1990]. The cells were normally synchronized by serum starvation and their cell cycle analyzed by flow cytometry (FACScan; Becton-Dickinson, Mountain View, CA) [Laitinen et al., 1990]. For synchronization at G_1/S boundary, exponentially growing cells were treated with 5 μg/ml aphidicolin for 24 h as previously described [Laitinen and Hölttä, 1994].

Isolation of Nuclei and Cytoplasmic RNA

Nuclei from 5×10^7 to 10^8 cells were isolated as described earlier [Laitinen et al., 1990; Laitinen and Hölttä, 1994]. In brief, the cells were lysed in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, the nuclei pelleted by centrifugation in an Eppendorf centrifuge (at 14,000*g* for 10 s), washed, and resuspended in 400–1,000 μl of the lysis buffer without NP-40. Total RNA was isolated from the supernatant fraction of the cell lysates [Laitinen and Hölttä, 1994].

Micrococcal Nuclease Digestions

The MNase (Boehringer Mannheim, Germany) digestion was carried out at 37°C as described elsewhere [Moreno et al., 1986; Laitinen et al., 1990].

Isolation of Genomic DNA and Restriction Enzyme Digestions

DNA was isolated using the recently published protein salting-out method [Laitinen and Hölttä, 1994; Laitinen et al., 1994a]. In Southern blot analyses, 10 μg of the high-molecular mass DNA was digested with 100 units of Pst I, Hpa II, or Msp I (Boehringer Mannheim) for 6 h.

Preparation of Insert Probes for Southern and Northern Blot Analyses

odc gene-specific insert probes were prepared from the pODC16 [Hickok et al., 1986] and p*ODC*cat [Eisenberg and Jänne, 1989] plasmids. From pODC16, a 936 bp Hind III cDNA fragment spanning from the 8th to 12th exon of *odc* was isolated, and from pODCcat a 1.7 kb Kpn I-Sac I fragment (Pst I insert) that covers the 5′ flanking region of *odc* gene [Eisenberg and Jänne, 1989; Palvimo et al., 1991]. For detecting the coding *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) gene sequences, a 1.3 kb Pst I insert probe was prepared from the pRGAPDH-13 plasmid [Fort et al., 1985]. The inserts were isolated and purified from 1% low melting point agarose (LMP-Agarose, BRL) gels. The gel slice was melted at 65°C for 15 min in a microcentrifuge tube, extracted for 3 min with one volume of neutralized phenol, and frozen at −70°C. This was followed by centrifugation at 13,000*g* for 10 min at room temperature and the insert in the aqueous phase was precipitated with 2 volumes of ice cold ethanol.

Electrophoresis, Blotting, and Hybridization Analyses of DNA

Aliquots of DNA (10 μg) and RNA (20 μg) were separated on 1.0–1.8% agarose gels and 1.6% agarose/formaldehyde gels, respectively [Maniatis et al., 1984]. The fractionated DNA fragments and RNAs were transferred by capillary blotting to Hybond-N nylon filters (Amersham). The ^{32}P-dCTP labeled probes for hybridizations were prepared by standard random priming reactions according to the directions of the manufacturer (DuPont or Amersham) and hybridizations were carried out as described earlier [Laitinen et al., 1990].

End-Labeling of DNA

One microgram of Hpa II or Msp I-digested DNA or 50 ng of marker DNA (λ DNA cut with Hind III and ΦK174 DNA cut with Hae III, respectively) (Pharmacia) was incubated with 5 units of the Klenow-fragment of *E. coli* DNA polymerase I at 37°C for 5 min. This was followed by an additional incubation at 37° for 15 min in the presence of 2 mM of dATP, dGTP, and dTTP and 5 μCi of [^{32}P]-dCTP. Both incubations were carried out in 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$. The end-labeling reaction was stopped by the addition of 250 mM EDTA (pH 8.0) to a final concentration of 40 mM. Nonincorporated nucleotides were removed by gel filtration through Bio-Spin 30 colums (Bio-Rad).

Determination of the CCGG-Repeat and Nucleosomal Repeat Length (NRL)

The photographs (positives) of the Hpa II/Msp I and micrococcal nuclease digestion patterns [Laitinen et al., 1990, 1994b] were scanned with a grey-scale scanner coupled to a computer (Apple Computer, USA; Hewlett-Packard, ScanJet Plus, Greeley, CO; Scan Analysis, Biosoft, Cambridge, UK). For calculating nucleosomal repeat length (NRL) or sizes of the DNA fragments obtained by Hpa II and Msp I digestions, the DNA fragment migration was calculated from the mobilities of the resulting computerized images of the scans [Annunziato and Seale, 1982; Moreno et al., 1986; D'Anna and Tobey, 1989; Laitinen et al., 1994b].

RESULTS

The mouse *odc* gene measures about 6 kb in length, containing 12 exons flanked by 11 intervening sequences. The protein coding information is found within the 3–12 exons [see Katz and Kahana, 1988] (Fig. 1). The promoter region of *odc* gene [Eisenberg and Jänne, 1989] has several CCGG tetranucleotide sequences that are recognized by the restriction endonucleases Hpa II and Msp I, respectively. Our Hpa II and Msp I digestion analyses of the *odc* promoter have shown that the *odc* locus displays methylation polymorphism in mouse NIH-3T3 fibroblasts [Laitinen and Hölttä, 1994] (Fig. 1).

Although a given pattern of DNA methylation is usually preserved during cellular proliferation, there is also evidence indicating that segments of DNA may become demethylated under certain conditions, even in the absence of DNA synthesis [Razin et al., 1986; Paroush et al., 1990], but the mechanisms involved are not known [Paroush et al., 1990]. Here, we exam-

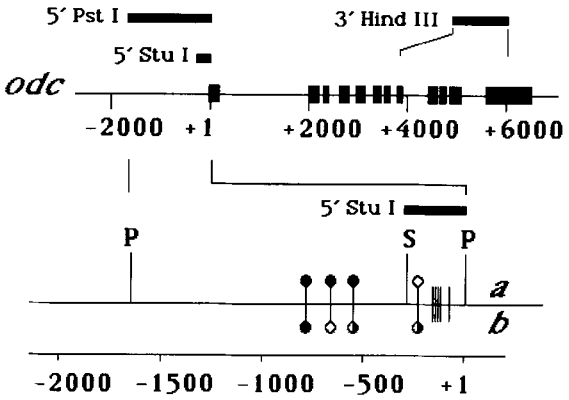

Fig. 1. Probes for the structure of mouse ornithine decarboxylase gene and methylation status of its promoter region. The exon-intron structure of the *odc* gene is shown on top. Exons are denoted by grey boxes. The probes used in this study are shown above the gene structure. Beneath are indicated the numbers and positions of base pairs; +1 indicates the RNA synthesis start site. The 936 bp 3′ Hind III probe spans from the 8th exon to the 12th exon. The 5′ Stu I probe is 279 bp long. Below the exon-intron-structure are shown two differentially methylated alleles (a and b) at the 5′ region of *odc* locus. The open, solid, and half solid circles symbolize unmethylated (Hpa II-sensitive), methylated (Hpa II-resistant), and partially methylated sites along the *odc* promoter, respectively. The tiny vertical cross-lines denote CCGG-sites whose methylation state is unknown. P and S indicate the recognition sites for the Pst I and Stu I restriction enzymes, respectively.

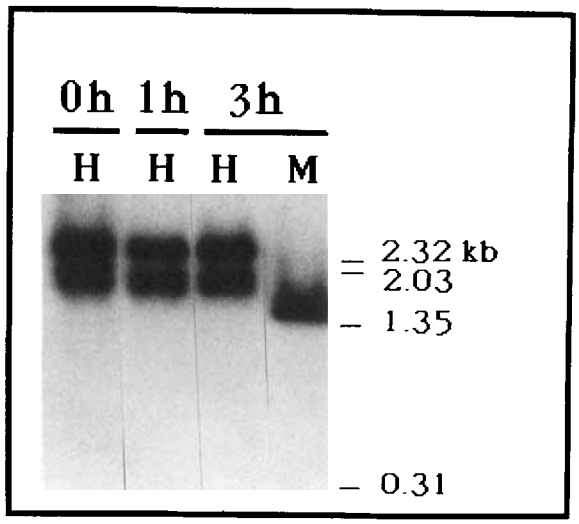

Fig. 2. Methylation analysis of *ornithine decarboxylase* gene promoter in c-Ha-*ras*$^{Val\ 12}$ oncogene-transformed NIH-3T3 cells after serum stimulation. E4 cells were synchronized by serum starvation for 16 h, whereafter 10% FCS was added to allow the cells to enter the growth cycle. Nuclei were isolated from the quiescent and stimulated cells (1 and 3 h), extracted for DNA and digested with Hpa II (H) and Msp I (M). The resulting fragments were electrophoresed on 1.6% agarose gels, and transferred by capillary blotting to nylon filters. The filters were then hybridized to a random-primed probe specific to the 1.7 kb *odc* promoter region (5′ Pst). Relative molecular mass markers (λ DNA cut with Hind III and ΦK174 DNA cut with Hae III, respectively) are indicated on the right side of the figure.

ined the methylation status of the mouse *odc* gene during the serum stimulation of quiescent *ras* oncogene-transformed NIH-3T3 (E4 cells) fibroblasts at a time when changes in chromatin structure and expression of *odc* are known to occur [Hölttä et al., 1988; Laitinen et al., 1990; Laitinen and Hölttä, 1994]. To obtain quiescent and synchronized E4 cells, we starved subconfluent cultures for serum for 24 h. A part of the synchronized cells was then stimulated with 10% foetal calf serum for 1–3 h, and their DNA was isolated. Genomic DNA was digested with Hpa II and Msp I, and the resulting DNA fragments were separated by agarose gel electrophoresis, blotted onto nylon filters, and hybridized to the different random-primed probes of *odc* gene (Fig. 1). The analysis of the *odc* promoter region using the 5′-flanking region probe revealed in the quiescent E4 cells two intense 1.8 kb and 2.2 kb Hpa II-specific fragments and a 1.5 kb Msp I-specific fragment (Figs. 1 and 2). The 2.2 kb fragment reflects the presence of a highly methylated *odc* allele (allele a) shown in Figure 1 [Laitinen and Hölttä, 1994]. Following serum stimulation of the cells there appeared to be no changes in the Hpa II digestibility of the *odc* promoter/enhancer as analyzed with the 5′ Pst I probe. Hybridization of the filter with the 3′ Hind III probe likewise revealed no changes in the Hpa II digestibility of DNA at the 3′ region of the *odc* gene during growth stimulation (data not shown). These results show that the methylation state of the CCGG sequences along the studied regions of *odc* locus remain unaltered during the first 3 h of serum stimulation.

During DNA replication the chromatin structure of many genes, including *odc*, undergoes well-known modifications [Moreno et al., 1986; D'Anna and Tobey, 1989; Laitinen et al., 1990; Laitinen and Hölttä, 1994]. Thus, we examined whether the known modifications in the chromatin structure of the *odc* gene during the S phase of cell cycle could be coupled to changes in the symmetrical methylation at Hpa II restriction sites of the *odc* gene. The E4 cells were synchronized by aphidicolin treatment for 24 h (at G_1/S boundary), whereafter the drug was removed and fresh medium was added to allow the cells to enter the S phase. To follow the cell cycle progression, the rate of DNA synthesis was determined

by addition of ^{3}H-methylthymidine at 1 h or 2 h intervals (Fig. 3A). To see the symmetrical methylation patterns of the *odc* gene, genomic DNA of the cells was digested with the restriction endonucleases Hpa II and Msp I at 1, 2, 3, 6, 8, and 9 h after removal of the aphidicolin block and subjected to Southern blot analysis. When the blots were hybridized with the probe specific to the 5′ flanking region of the *odc* gene no changes in the Hpa II digestibility were observed (Fig. 3B). Analysis of the promoter region of the *odc* gene with the Stu I insert revealed in agreement with our previous studies a 0.4 kb Hpa II specific band [Laitinen and Hölttä, 1994] indicating that the sites at the *odc* promoter/enhancer positions −667/−235 or −564/−167 are hypomethylated throughout the S phase (Figs. 1 and 3C). Since the Hpa II fragment was not replaced by the 325 bp Msp I band (Fig. 3C), or any other sequences, it appears that the methylation status at the CCGG sequences of *odc* proximal promoter region remained invariant during DNA replication. Analysis of the mid-coding and 3′ regions of the *odc* gene with the 3′ Hind III probe did not either reveal changes in methylation at the CCGG sequences (data not shown). These results indicate that even transient changes in methylation do not take place at the CCGG sequences of the *odc* promoter during the growth cycle of the c-Ha-*ras*$^{Val\ 12}$ oncogene-transformed E4 cells.

Several lines of evidence suggest, however, that methylation patterns at CCGG sequences are not the same in normal and transformed cells [Razin and Riggs, 1980; Feinberg and Vogelstein, 1983; Alhonen-Hongisto et al., 1987; Holliday, 1987]. Since we have earlier shown that bulk chromatin is significantly more sensitive to MNase digestion in the *ras*-oncogene transformed NIH-3T3 fibroblasts than in the normal fibroblast [Laitinen et al., 1990, 1994b], we wished to study whether the methylation patterns at the sequences CCGG are different in these two cell lines. We compared first the methylation patterns of bulk DNA in the normal (N1) and *ras*-transformed E4 cells. Genomic DNA was isolated from the cell lines and digested with Hpa II and Msp I, whereafter the resulting DNA fragments were end-labeled with [^{32}P]-dCTP by using the *E. coli* DNA polymerase I [Antequera et al., 1989, 1990], and analyzed in 8% polyacrylamide gels. Surprisingly, both in the normal and *ras*-transformed cells the CCGG sequences appeared to be methylated to an equal extent (Fig. 4). In agreement with earlier studies by Bird and coworkers [Antequera et al., 1989, 1990; Tazi and Bird, 1990] the digestion with Msp I generated a nonuniform cleavage pattern, a ladder with preferential cleavage sites in the genomic DNA (Fig. 4). Upon a longer exposure of the autoradiogram film, a faint ladder was also seen in the Hpa II-digested samples. This ladder resembles very much the nucleosomal ladder of chromatin produced by the micrococcal nuclease digestion as the average spacing between the fragments forming the "CCGG-repeat" is about 145 bp (data not shown). These data seem thus to support the notion that nucleosomes are preferentially associated with methylated CpGs [Solage and Cedar, 1978; Ball et al., 1983; Bird, 1992].

Altogether, no apparent differences between the cell lines were revealed by the analysis of the methylation of bulk DNA. Then, we decided to study the methylation patterns of *odc* gene in the normal and *ras*-transformed cells. We first evaluated the symmetrical methylation patterns at CCGG sequences of the promoter region of the *odc* gene. Genomic DNA from the N1 and E4 cells was digested with Pst I, Hpa II, and Msp I, and the resulting DNA fragments were hybridized to the 1.7 kb 5′ flanking fragment of the *odc* gene (the 5′ Pst I probe). Figure 5A shows that in both cells single digestions with Hpa II and Msp I produced the two 1.8 kb and 2.2 kb Hpa II-specific fragments and the 1.5 kb Msp I-specific fragment (Fig. 5A). As shown in Figure 5B, the Pst I digestion generated the expected 1.7 kb band corresponding to the promoter/enhancer region of the *odc* gene. Double digestions with Pst I and Hpa II or Msp I decreased the size of Hpa II and Msp I-specific fragments to 1.0 kb, 1.4 kb, and 0.9 kb, respectively. Consequently, in both N1 and E4 cells, the CCGG at the position −802 of the enhancer region of the *odc* gene appears to be methylated as the Hpa II digests lack a 0.9 kb fragment that is present in the Msp I digests (Fig. 5B). Like the enhancer/promoter region, the mid-coding and 3′ untranslated regions of the *odc* gene displayed no cell transformation-specific changes at the sequence CCGG (data not shown).

DISCUSSION

DNA methylation has long been known to be closely associated with chromatin condensation and long-term repression of genes, but there are only a few studies on the possible role of DNA

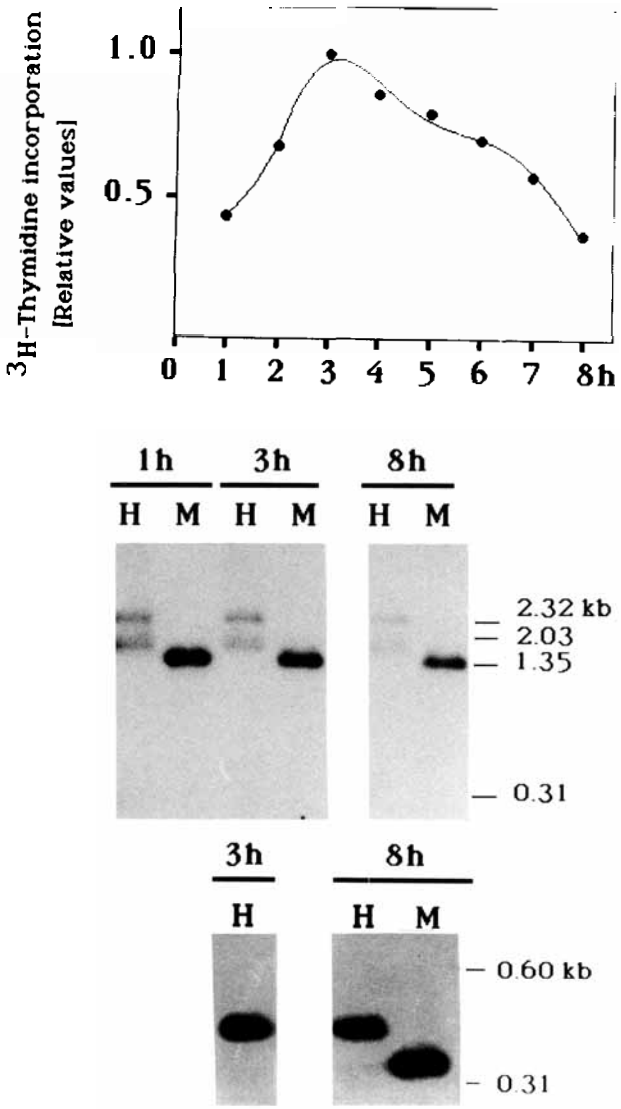

Fig. 3. Methylation state of the *odc* promoter during the S phase progression in *ras*-transformed cells. E4 cells were synchronized at G_1/S boundary by treatment with aphidicolin (5 μg/ml) for 24 h, and allowed to enter the cell cycle by adding fresh medium. **A:** The rate of DNA synthesis. 8 μCi ^{3}H-methylthymidine was added at 1 h or 2 h intervals after serum addition and the incorporation of radioactivity into the acid-insoluble fraction was measured during a 30 min pulse. The x-axis indicates the time in hours after release from the aphidicolin block. The y-axis indicates relative values for the ^{3}H-methylthymidine incorporation into DNA. The values are means of duplicate cultures. **B,C:** Methylation patterns of the 5′ chromatin domains of the *odc* gene. After release from the aphidicolin block, genomic DNA was extracted from the cells at the indicated times, digested with Hpa II and Msp I and subjected to Southern blot analysis. The filters were hybridized to the Pst I probe covering the 1.7 kb 5′ region of the *odc* gene (B), and to the 5′ Stu I probe specific to the proximal promoter region of *odc* gene (C). The symbols and relative molecular mass markers are as in Figure 2.

methylation in the short-term regulation of chromatin structure or gene expression. It is also worth noting that methylation can occur at all phases of the cell cycle, although the activity of DNA methylase(s) seems to be highest during DNA replication [for refs see Razin, 1984]. In this study we investigated whether the methylation pattern of the growth- and transformation-associated *odc* gene [Stimac and Morris, 1987; Jähner and Hunter, 1991; Auvinen et al., 1992; Hölttä et al., 1993; Moshier et al., 1993] at the sequences CCGG is strictly inherited during the growth cycle of c-Ha-*ras*$^{Val\ 12}$ oncogene-transformed cells. We recently documented that serum-stimulation of these cells results in an appearance of a DNase I sensitive site at the *odc*

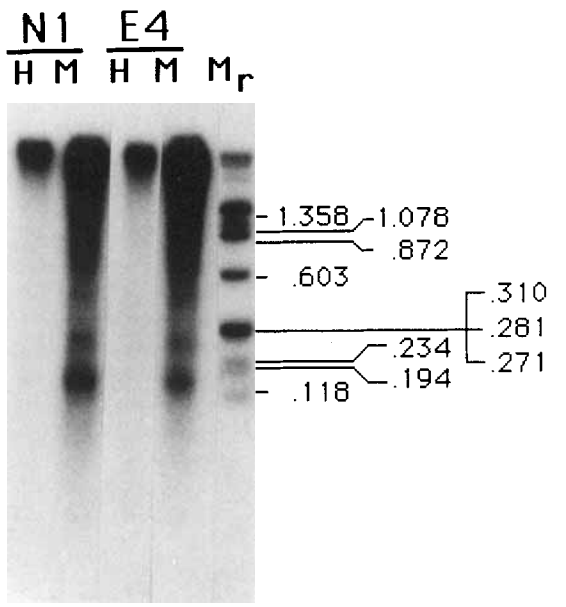

Fig. 4. The Hpa II target sequences are methylated to an equal extent in the normal and *ras*-transformed cells. Genomic DNA (10 μg) isolated from the normal N1 cells and transformed E4 cells was digested with Hpa II (H) and Msp I (M), and the resulting fragments were end-labeled and electrophoresed in a 8% polyacrylamide gel. The ^{32}P-dCTP labeled relative molecular mass markers (λ DNA cut with Hind III and ΦK174 DNA cut with Hae III) are indicated on the right side of the figure (M_r).

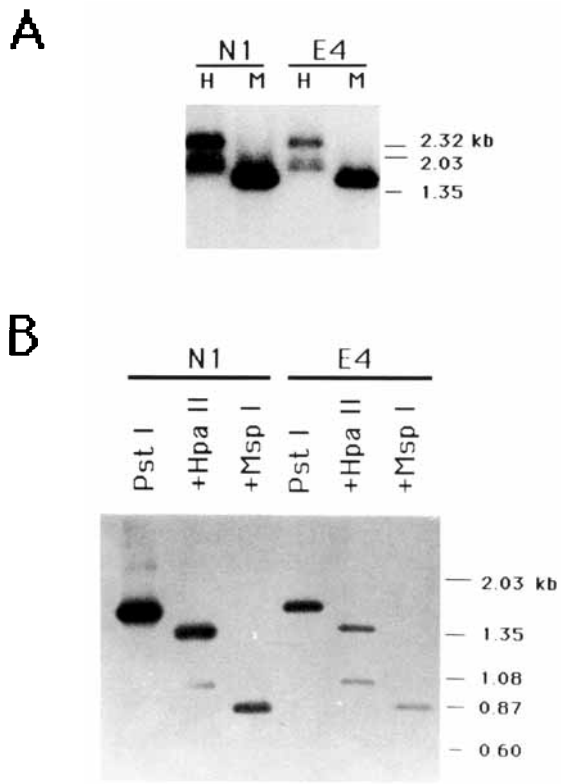

Fig. 5. Methylation status of the enhancer/promoter region of *odc* in normal and *ras*-transformed cells. Genomic DNA (10 μg) isolated from the normal N1 and *ras* transformed E4 cells was digested with Hpa II and Msp I (**A**) and with Pst I alone or with Pst I + Hpa II and Pst I + Msp I (**B**). The resulting DNA fragments were electrophoresed on 1.6% agarose gels, and transferred by capillary blotting to nylon filters. The filters were hybridized to a random-primed probe specific to the 1.7 kb 5′ flanking region of *odc* (5′ Pst). The relative molecular mass markers are as in Figure 2.

promoter region [Laitinen and Hölttä, 1994; J.L. and E.H., unpublished data], and that the nucleosome-interactions at the mid-coding and 3′ regions of *odc* gene undergo structural changes in correlation with the accumulation of *odc* mRNA [Laitinen and Hölttä, 1994]. Moreover, we have found that the CpG-rich chromatin of the 5′ flanking region of *odc* gene undergoes significant structural changes upon replication [Laitinen and Hölttä, 1994]. Despite these conformational changes in the *odc* chromatin structure, the DNA methylation analyses in this study revealed no concomitant alterations in the methylation patterns at the CCGG sequences in the 5′ or 3′ regions of the *odc* gene during the cell cycle. In the same way Hsieh and Verma [1989] have found no differences in DNA methylation patterns of *odc* in T24 cells upon treatment with the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate that results in a manifold increase in *odc* transcription. However, that study did not include the methylation analysis of the most important part, the 5′ promoter/enhancer region of *odc*. We also failed to see methylation changes in bulk chromatin after serum-stimulation. Thus, DNA methylation is clearly not the mechanism regulating the dynamic changes in chromatin during the cell cycle. Similarly, Razin and coworkers [Szyf et al., 1985; Razin et al., 1986] have found that the methylation pattern of bulk DNA remains unaltered in response to lymphocyte stimulation. That methylation changes are not found during cell cycle of normal cells is quite understandable, as deamination of 5-methylcytidines could lead to transition mutations with potentially hazardous consequences [Selker, 1990; Rideout et al., 1990]. For example, Rideout and coworkers [1990] have documented that in the human *p53* gene cytosine residues that have undergone a germ-line transition mutation to thymidine were originally methylated in all normal tissues investigated. Interestingly, the data presented here show that also in the highly transformed E4 cells the methylation pattern of a specific gene,

odc is strictly inherited during the cell cycle. Even after several months of subculturing we could not detect any methylation changes in the *odc* gene promoter or its coding region (J.L. and E.H., unpublished data) suggesting that the transformation ("dedifferentiation") by *ras* oncogene does not alter the CCGG methylation at the *odc* locus. Similar to the *odc* gene, also the transcriptionally active U2-snRNP-specific *b''* gene was found to be methylated to an equal extent in the normal and *ras* transformed cells (J.L. and E.H., unpublished data).

Nevertheless, several studies clearly indicate that the either hypo- or hypermethylation [Holliday, 1987; Feinberg and Vogelstein, 1983; Harrison et al., 1983; Young and Tilghman, 1984; Alhonen-Hongisto et al., 1987; Wu et al., 1993] could play a role in cell transformation. The aberrations in DNA methylation in transformed cells are assumed to reflect alterations in the transcriptional activity and chromatin structure. In *ras*-transformed cells, the transcriptional activity of many genes including *odc* [Hölttä et al., 1988] is known to be increased, but here we could not, however, detect any alterations in the methylation status of the *odc* gene at CCGG sequences. Whether methylation changes might have occurred at other CpG sites remains to be seen. However, since HpaII/Msp I digestions have been successfully used in various tumour cells for detection of genomic hypomethylation, including the *odc* locus [Wahlfors, 1992], it seems more likely that factors other than methylation primarily regulate the *odc* gene activity. One possibility is, of course, that the methylated cytosines contribute to or interfere with the binding of certain transacting proteins. The nonrandom distribution of methylated cytosines ("CCGG ladder") observed in our and other studies [Antequera et al., 1989, 1990; Tazi and Bird, 1990] seems to be compatible with an idea that these residues reside close to nucleosomes [Ball et al., 1983; Bird, 1992], but evidently at their linker regions recognizable by specific regulatory proteins of transcription/chromatin organization. In support of this idea, we have earlier documented that the nucleosomal core as well as oligonucleosomal DNA from N1 and E4 cells is trimmed by Msp I into subnucleosomal or oligonucleosomal DNA smear [Laitinen et al., 1994a; J.L. and E.H., unpublished data].

Our earlier studies show that the nucleosomal organization of bulk chromatin is more decondensed in the *ras*-oncogene transformed E4 fibroblasts than in the normal N1 cells [Laitinen et al., 1990]. Here we found that there are no differences in the methylation patterns of the CCGG sequences in bulk DNA between the normal and *ras*-transformed cells. These data thus suggest that the methylation changes at CCGG sequences are not a prerequisite for the nucleosomal organization changes and transformation induced by the *ras* oncogene.

Altogether, our data indicate that the changes in the chromatin structure induced by cell transformation by the c-Ha-*ras*$^{Val\ 12}$ oncogene do not appear to be accompanied by changes in DNA methylation at the CCGG sequences. Consequently, it appears that factors other than methylation per se are responsible for the *odc* chromatin changes during the normal cell growth and transformation [Laitinen et al., 1990, 1994b; Laitinen and Hölttä, 1994]. It is, however, possible that there is an indirect coupling between DNA methylation and chromatin decondensation in transformed cells. Recent studies show that the methylation at DNA alone is not sufficient for gene inactivation, but it has to be converted to chromatin to inhibit or decrease transcription [de Bustros et al., 1988; Bird, 1992]. A number of chromatin-associated and methylated CpG-binding proteins (MeCPs), like sequence-specific [Huang et al., 1984; Wang et al., 1986] and nonsequence-specific proteins [Bird, 1992], have been isolated and characterized. Comparisons in MeCP binding activities in different cell lines have revealed that the rapidly dividing cells are a less abundant source of MeCP than slowly growing cells [Meehan et al., 1989]. It remains interesting to see whether the same is true for the transformed cells, and whether the quantitative or qualitative changes in chromatin-associated proteins or transcription factors could be responsible for the structural alterations and activity of the chromatin.

ACKNOWLEDGMENTS

We thank Philip Fort for the pRGAPDH-13 plasmid, Olli A. Jänne for the pODC16 and pODCat plasmids, and Lea Sistonen and Kari Alitalo for the N1 and E4 cell lines. Anna Maria Siivonen and Anne Aronta are acknowledged for their technical assistance in cell culturing. Monica Schoultz is acknowledged for the FACS analyses and Björn Lindroos for the photography. This work was supported by the University of Helsinki, the Finnish Cancer Organizations,

the Sigrid Jusélius Foundation, the Magnus Ehrnrooth Stiftelsen, the Emil Aaltonen Foundation, the Finnish Academy of Sciences, and the Finnish Life and Pension Insurance Companies.

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