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ARTICLES

Methylation Status and Chromatin Structure of an Early Response Gene (*Ornithine Decarboxylase*) in Resting and Stimulated NIH-3T3 Fibroblasts

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Abstract The early response gene *ornithine decarboxylase* (*odc*) is indispensable for normal and malignant cell growth. Although DNA methylation is generally associated with chromatin condensation and gene inactivation, the *odc* gene is heavily methylated at CCGG-sequences in animal cell lines. In this work we analyzed the chromatin structure and the DNA methylation status at the CpG-rich promoter sequences at the *odc* locus in mouse 3T3 fibroblasts. We show that the proximal promoter region of the *odc* locus is not hypermethylated, while the distal promoter sequences appear to have a few methylated CCGG-sites and display methylation polymorphism. Furthermore, it was found that the 5′ promoter region of *odc* is constitutively more sensitive to micrococcal nuclease than the coding and 3′ regions of the *odc* gene. Stimulation of the cells with serum resulted in an appearance of a DNase I sensitive site at the promoter region. The chromatin structure of the mid-coding and 3′ regions of the *odc* gene also underwent structural changes that were accompanied by the rapid accumulation of *odc* mRNA. Such changes were not detected in the chromatin structure of *glyceraldehyde-3-phosphate dehydrogenase* (*gadph*) gene, whose expression remains invariant upon serum stimulation. These data suggest that the chromatin structure may play an important role in the rapid transcriptional activation of *odc* and other immediate early genes during serum stimulation. © 1994 Wiley-Liss, Inc.

Key words: chromatin structure, DNA methylation, serum stimulation, transcription, cell cycle

INTRODUCTION

Transcriptionally active chromatin displays two types of DNase I or micrococcal nuclease (MNase) sensitivity [Elgin, 1988; Gross and Garrard, 1988; Bonifer et al., 1991; Felsenfeld et al., 1992] called DNase I (MNase) domain sensitivity and DNase I hypersensitivity. An increase in these nuclease sensitivities has been reported to accompany ongoing transcription and replication and is perhaps an obligatory step in the initiation of transcription. Formation of the DNase I/MNase hypersensitive sites to gene promoters is known to correlate with the presence of chromatin bound transcription factors that together with RNA polymerase inititiate gene expression. Domain DNase I/MNase sensitivity, in turn, extends from few kilobases up to 100 kilobases of chromatin. It decreases, however, gradually towards the 5′ and 3′ end regions of various genes, like the chicken β-*globin* and the human *histone H4* and c-*fos* genes [Weintraub, 1984; Moreno et al., 1986; Feng and Villeponteau, 1990].

It is believed that DNA methylation of cytosine residues at the dinucleotide CpG is associated with the regulation of gene expression and chromatin condensation in animal cells [Solage and Cedar, 1978; Ball et al., 1983; Keshet et al., 1986; Antequera et al., 1990; Bird, 1992]. At the chromatin level, expression of genes may also be controlled by their assembly into the condensed higher-order histone-H1-dependent chromatin superstructures [Weintraub, 1984; Roche et al., 1985; Kamakaka and Thomas, 1990]. It is further possible that there exists a coupling between DNA methylation and the histone-H1-dependent chromatin condensation [Ball et al., 1983; Keshet et al., 1986]. It has been documented that the methylation per se is not sufficient for gene inactivation, but that it is the subsequent formation of the chromatin structure which is responsible for the block of transcription [Buschhausen et al., 1987; Weih et al.,

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1991]. Besides histones and transcription factors [Durrin et al., 1991], also some other chromatin-associated proteins, like certain sequence-specific [Huang et al., 1984; Wang et al., 1986] and nonsequence-specific methylated DNA-binding proteins [Bird, 1992], could play a regulatory role in chromatin organization. Bird and co-workers [for review see Bird, 1992] have presented evidence that the stabilization of the histone H1-dependent solenoidal structure of chromatin is brought about by cooperative and/or noncooperative DNA methylation of CpGs. In the promoter regions of genes this occurs indirectly via the sequence-nonspecific methyl-CpG binding protein 1 (MeCP1). The MeCP1 binds to the variously methylated CpGs with different affinities, while another protein, MeCP2, can bind to a single methylated CpG even at the noncoding and inactive chromatin regions.

Stimulation of serum-starved cells with growth factors or serum triggers the mitogenic signal from the plasma membrane to the nucleus and results in the rapid trancriptional activation of a set of cellular early response genes [Chen and Allfrey, 1987; Chen et al., 1990; Feng and Villeponteau, 1990; Jähner and Hunter, 1991]. This is also accompanied by rapid changes in the chromatin structure. For example, Chen and Allfrey [1987] have shown that rapid changes in nucleosome structure accompany the activation of c-*fos* and c-*myc* protooncogenes. Feng and Villeponteau [1990] have further documented that serum stimulation of the c-*fos* enhancer induces reversible changes in DNase I domain sensitivity at the c-*fos* chromatin.

In this work we addressed the question of whether the transcriptional activation of the early response gene, *ornithine decarboxylase* (*odc*) [Heby and Persson, 1990; Jähner and Hunter, 1991] is accompanied by modifications in its chromatin structure. The *odc* gene encodes the ODC enzyme that plays the key role in the synthesis of polyamines [Pegg, 1986] which are indispensable for normal cell growth [Heby et al., 1976; Pohjanpelto et al., 1985; Kaminska et al., 1990] and are important for cell transformation [Auvinen et al., 1992]. In various animal cell lines the transcriptionally active *odc* gene is, however, hypermethylated [Alhonen-Hongisto et al., 1987; Hölttä et al., 1989; Wahlfors, 1991]. For example, in human myleoma cells even the *odc* promoter is fully methylated at the CCGG sequences [Wahlfors, 1991].

Since very little is known about the chromatin structure and methylation of the *odc* locus the present study was also undertaken to examine the chromatin structure and methylation pattern of the endogenous *odc* gene in NIH 3T3 cells. We report that the entire promoter region of *odc* is not completely methylated at the CCGG sequences. It further displays a chromatin structure that is more open than that of the mid-coding and 3′ regions of *odc* gene. We also show that the internucleosomal interactions at the mid-coding and 3′ regions of the *odc* gene undergo structural changes in response to serum stimulation that correlate with the early accumulation of *odc* transcripts. To our knowledge this is the first report showing that the transcriptional activation of the *odc* gene by serum is associated with decondensation of *odc* chromatin.

EXPERIMENTAL PROCEDURES

Cell Culture

The normal (N1) and c-Ha-*ras*$^{Val\,12}$ oncogene transformed (E4) clones of NIH 3T3 cells were grown as described previously [Laitinen et al., 1990]. For G_1 synchronization, the cells were grown to semiconfluency in ordinary medium, starved for serum for 24 h, and then triggered to reenter the growth cycle by adding 10% dialyzed FCS (dFCS) [Laitinen et al., 1990]. For synchronization at G_1/S boundary, exponentially growing cells were treated with 5 μg/ml aphidicolin for 24 h. Thereafter the cells were allowed to enter the S phase by addition of fresh medium without aphidicolin. The rate of DNA synthesis was determined by measuring the incorporation of ^{3}H-methylthymidine [8 μCi] into the acid-insoluble DNA [Laitinen et al., 1990].

Isolation of Nuclei and Cytoplasmic RNA

Nuclei from 5×10^7 to 10^8 cells were isolated as described previously [Moreno et al., 1986; Laitinen et al., 1990]. In brief, the cells lysed in 0.5% NP-40, RSB (RSB; 10 mM Tris-HCl; pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) were centrifuged at maximal speed in an Eppendorf centrifuge for 10 s and the pelleted nuclei were resuspended in 400–1,000 μl of the lysis buffer without NP-40. The supernatant fraction was used for isolation of cytoplasmic RNA. The supernatant was treated with proteinase K (1 mg/ml)

for ½ h at +37°C, and then subjected to extractions with 1 vol of neutralized phenol-chloroform (1:1), and with chloroform. RNA in the aqueous phase was precipitated with 2.5 vol of ethanol overnight at −20°C.

Micrococcal Nuclease and DNase I Digestions

The MNase (Boehringer Mannheim) digestion was carried out as described elsewhere [Moreno et al., 1986; Laitinen et al., 1990]. For the MNase digestion the DNA content of isolated nuclei from each cell sample was carefully standardized by resuspending the isolated nuclei in RSB to absorbance of $A_{\lambda=260nm}$ = 10. Supranucleosomes were prepared essentially as described by Weintraub [1984] and analyzed on 2% agarose gels (20 × 20 × 0.6 cm). Electrophoresis was performed in 20 mM Tris, pH 7.5; 50 mM sodium acetate; 2 mM EDTA at 100 mA for 6 to 15 h.

The DNase I (Boehringer Mannheim) digestion was carried out in RSB buffer supplemented with 1 mM $MgCl_2$. The isolated nuclei were resuspended to a density of $A_{\lambda=260nm}$ = 20, and the enzyme was added to a final concentration of 10 units/$A_{\lambda=260nm}$.

Preparation of the Plasmids and Inserts

The pODC16 plasmid [Hickok et al., 1986] and pODCcat plasmid, with a 1.7 kb 5′ flanking region of the *odc* gene [Eisenberg and Jänne, 1989; Palvimo et al., 1991] used for the preparation of probes, were generous gifts from Olli A. Jänne (University of Helsinki). The cloned 5′ flanking region of the *odc* gene (1644 bp Pst I fragment) was isolated from the pODCcat plasmid by a cleavage with Kpn I and Sac I. A 279 bp Stu-Pst I insert (Stu I probe) was isolated from the pODCcat plasmid by digestions with Stu I and Bgl II. A 936 bp Hind III cDNA fragment was isolated from the pODC16 plasmid. 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].

Isolation of DNA and Restriction Enzyme Digestions

DNA was isolated using the method described by Miller et al. [1988] with the following modifications. After digestion with proteinase K and adding 5 M NaCl to a final concentration of 1.2 M, the tubes were vortexed for 30 sec, and the precipitated proteins were removed by two centrifugations at 400*g* for 15 min at +4°C. The resulting supernatant was transferred into new tubes, and DNA was precipitated by adding 2 vol of room-temperature ethanol. The purity of the DNA samples was checked by measuring the A ratio 260/280 nm and was found to be between 1.7 and 2. Ten micrograms of the bulk DNA (>20–50 kb in size) obtained by the conventional isolation procedure or by the above salting-out method were digested with 100 units of Pst I, Hpa II, or Msp I (Boehringer Mannheim) for 3 to 6 h.

Electrophoresis, Blotting and Hybridization Analyses of Nucleic Acids.

Aliquots of DNA (10 μg) or RNA (20 μg) were separated on 1.0–2.0% agarose or 1.6% agarose/formaldehyde gels, respectively [Maniatis et al., 1984]. The fractionated nucleic acids as well as the supranucleosomal DNAs were transferred by capillary blotting to Hybond-N nylon filters (Amersham), dried and baked at +80°C for 2 h.

^{32}P-dCTP labelled probes 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]. The degree of chromatin digestion and the levels of mRNA were quantified by gray densitometric scanning (Hewlett-Packard, ScanJet Plus, Greeley, CO; Scan Analysis, Biosoft, Cambridge, UK) of the autoradiograms. In case of supranucleosomes the original autoradiogram images were magnified 8-fold providing a good resolution for the quantification of the individual particles. The relative nuclease-sensitivity ratios of the *odc* gene from quiescent and stimulated cells were calculated by normalizing band intensities with respect to the intensities of equivalent bands in 1.6 μg/ml MNase samples run on the same gel. This and the individual matched background subtraction enabled us to take into account variations in autoradiogram quality, probe quality, or in DNA transfer.

RESULTS

The 5′ CpG-Rich Islands of the *Ornithine Decarboxylase* Gene Are not Hypermethylated

The mouse *odc* gene contains 12 exons, of which the exons third to twelfth are coding [Katz and Kahana, 1988] (Fig. 1). The 5′ flanking region of the *odc* gene contains several putative regulatory elements [Eisenberg and Jänne,

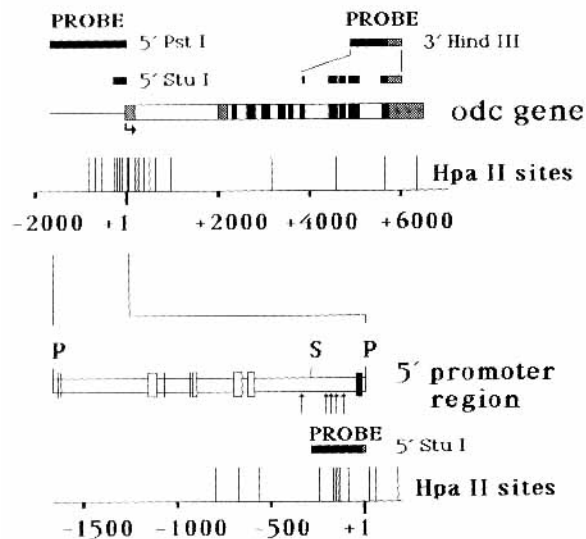

Fig. 1. A computerized illustration of the mouse *ornithine decarboxylase* (*odc*) gene. The exon-intron structure and Hpa II recognition sites of the 8.2 kb *odc* gene. The gene has 12 exons (grey and black boxes), of which the exons third to twelfth are coding (black boxes). Introns are shown as open boxes. The probes used in this study are shown above the gene structure. The 936 bp 3′ Hind III probe spans from the 8th exon to the 12th exon. The angled arrow displays the transcription start site. The diagrams below the exon-intron-structure show the Hpa II sites along the *odc* gene. Numbers at the horizontal line indicate positions of base pairs, +1 indicates the transcription start site. Below the exon-intron-structure are shown the putative 5′ regulatory elements covered by the 5′ Pst I probe (−1658 to +13): TATA box (black box), four steroid hormone response elements (open boxes), four putative TRE-sites (vertical cross-lines), and four Sp-1 recognition sites (long vertical arrows). The 5′ Stu I probe (−266 to +13) (black horizontal bar) detects the TATA box and the five Sp-1 sites and spans over six Hpa II sites. P and S indicate the recognition sites for the Pst I and Stu I restriction enzymes, respectively.

1989; Palvimo et al., 1991] (Fig. 1). The 8.2 kb odc gene with its 1.7 kb 5′ flanking region is very rich in CCGG tetranucleotide sequences ("CpG island"). Altogether there are 26 Hpa II-sites, most of which are clustered to the promoter region (Fig. 1).

It is believed that DNA methylation together with methylated DNA-binding proteins and transcription factors regulate the expression of genes. Since the CpG islands of gene promoters function as potential targets for DNA methylation we wanted to evaluate the symmetrical methylation patterns at the CCGG sequences at the *odc* locus. For this, genomic DNA was isolated from the 3T3 (E4) fibroblasts, 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 (see Fig. 1). The analysis of the *odc* promoter region using the GC-rich Pst I insert probe (see Fig. 1) revealed both Hpa II and Msp I-specific fragments (Fig. 2A; left panel). The 1.5 kb Msp I fragment obtained by probing with the 5′ Pst probe most likely represents the 1.5 kb region without Hpa II sites in the 1.7 kb region shown in the map in Figures 1 and 2B. The intense 1.8 kb and 2.2 kb fragments in the Hpa II digests (Fig. 2A; left panel) can be inferred to be produced if one or three of the restriction sites were methylated at positions −802, −667, and −564 of the distal *odc* promoter region (see Fig. 2A,B).

For a verification of these restriction sites, genomic DNA was double digested with Pst I and Hpa II or Msp I, and the resulting DNA fragments were hybridized to the 5′ Pst probe. As shown in Figure 2A, the Pst I digestion liberates the predicted 1.7 kb fragment corresponding to the 5′ flanking region of the *odc* gene. The major fragments are the Hpa II-specific 1.0 kb and 1.4 kb fragments and the Msp I-specific 0.9 kb fragment (Fig. 2A; middle panel). The 1.0 kb and 1.4 kb Hpa II-fragments are likely to be derived from the 1.8 kb and 2.2 kb fragments in the Hpa II digests (see above). Generation of the 1.0 kb Hpa II-specific fragment indicates that the CCGG-site at the position −802 of the *odc* enhancer is methylated. The presence of the 1.4 kb fragment suggests the existence of a more methylated *odc* allele. Anyhow, the absence of the full-length 1.7 kb, 667 bp or 574 bp Pst-Hpa fragments in the Hpa digest indicates that the promoter region of the *odc* locus cannot be completely methylated (Fig. 2A). Indeed, the analysis of the proximal promoter region using the Stu I insert probe excluded the possibility that the very proximal promoter region is completely methylated as the Pst I-Hpa II digest lacks fragments that are slightly smaller than the 325 bp Msp I fragment (Fig. 2A). The replacement of the 325 bp fragment in the Msp I digest by the 0.4 kb Hpa II specific band suggests that the sites at the positions −667/−235 or −564/−167 are hypomethylated. Our results that are summarized in figure 2B indicate that the *odc* promoter is not completely methylated but also contains a considerable amount of unmethylated CCGGs.

For analysis of the *odc* coding region the filters were hybridized with the 936 bp Hind III cDNA fragment (3′ Hind III probe). That the fragments in the Pst I-Hpa II double digests were larger than the corresponding Pst I-Msp I

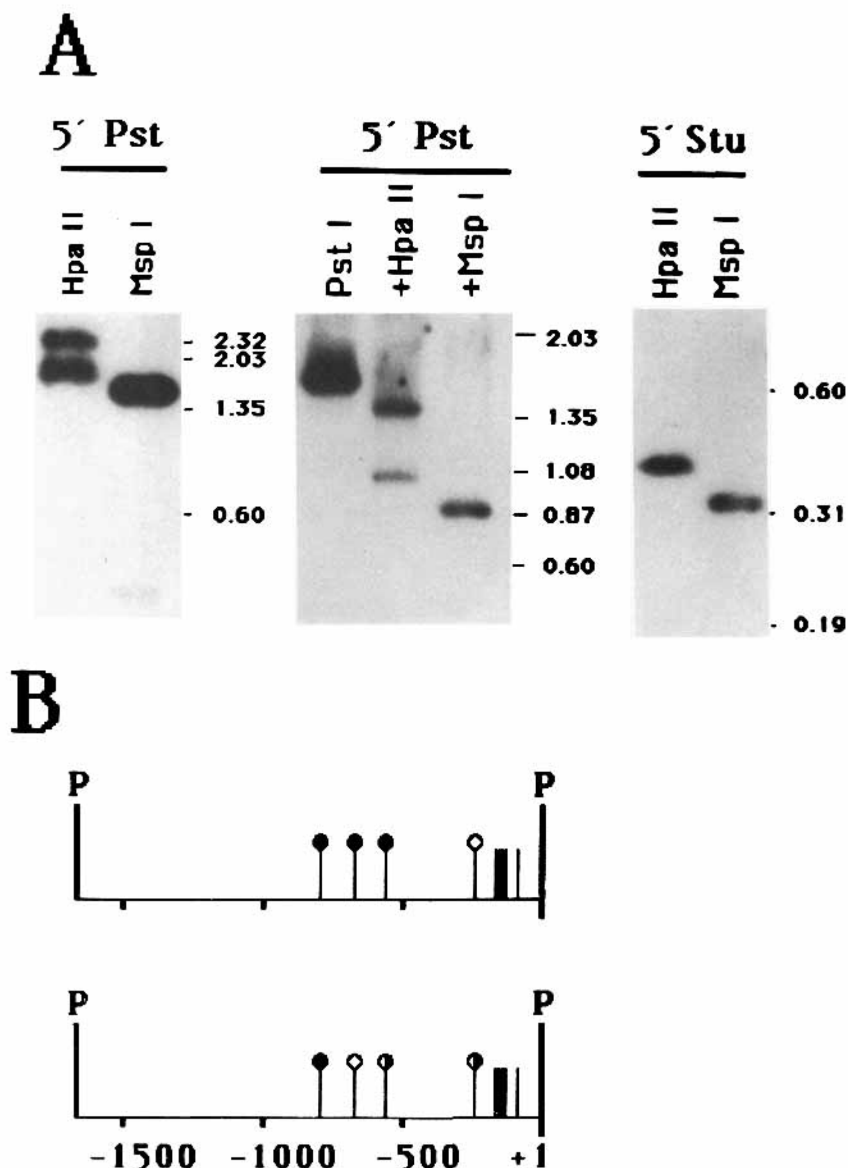

Fig. 2. Methylation state of *odc* gene in 3T3 cells. **A:** Genomic DNA (10 μg) isolated from the 3T3 (E4) fibroblasts was digested with Hpa II and Msp I or with Pst I, Hpa II, and Msp I (the middle panel). The resulting DNA fragments were separated by 1.6% agarose gel electrophoresis and transferred by capillary blotting to nylon filters. The filters were then hybridized to random-primed probes specific to the whole 1.7 kb flanking region (5′ Pst) or to the 279 bp promoter region of the *odc* gene (5′ Stu). **B:** The methylation status of the *odc* promoter at the *odc* locus. In this figure the two putative alleles are shown; the upper and lower panels refer to the 1.4 kb and 1.0 kb Hpa-fragments, respectively. The open, solid, and half solid circles symbolize unmethylated (Hpa II-sensitive), methylated (Hpa II-resistant), and partially methylated sites, respectively. The tiny vertical cross-lines denote CCGG-sites whose methylation state remained uncharacterized. P indicates the recognition sites for the Pst I restriction enzyme.

fragments suggests methylation of some CCGGs in this locus (data not shown).

The *odc* Gene Is Localized Into Nuclease Sensitive Chromatin Structure

Transcriptional activity of genes appears to correlate with their sensitivity to micrococcal nuclease digestion [Smith et al., 1983; Yu and Smith, 1985; Moreno et al., 1986] and localization into active or inactive supranucleosomes [Weintraub, 1984]. Supranucleosomes obtained by MNase digestion migrate in agarose gels as three distinct fractions, designated A, B, or C particles. The most slowly migrating particles, the A particles, contain condensed chromatin and are rich in the histone H5/H1° [Weintraub, 1984; Kamakaka and Thomas, 1990]. The B particles, in turn, contain transcribed genes.

Since the *odc* gene belongs to the immediate early genes [Jähner and Hunter, 1991] we wanted to know what kind of chromatin configuration it has in the quiescent and growth-stimulated cells. To obtain quiescent and synchronized E4 fibroblasts 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 nuclei were isolated for supranucleosomal analysis. It is evident from Figure 3 that both in the quiescent and stimulated cells the 5′ flanking region of the *odc* gene is predominantly localized to the active B particles (Fig. 3). The hybridization intensity of the 5′ *odc* probe to the A and B particles decreases with increasing nuclease concentrations suggesting increased nuclease-sensitivity [Weintraub and Groudine, 1976; Smith et al., 1983; Yu and Smith, 1985] that is characterized as a reduction of the long 1.7 kb Pst I probe to hybridize to small chromatin fragments, like mononucleosomes or smaller fragments (Fig. 3). In this context, it should be noted that there is no decrease in the DNA content of the chromatin particles as a function of nuclease concentration as revealed by the EtBr staining (Fig. 3) or by probing the filter with bulk DNA labeled with ^{32}P-dCTP (data not shown) or by probing the

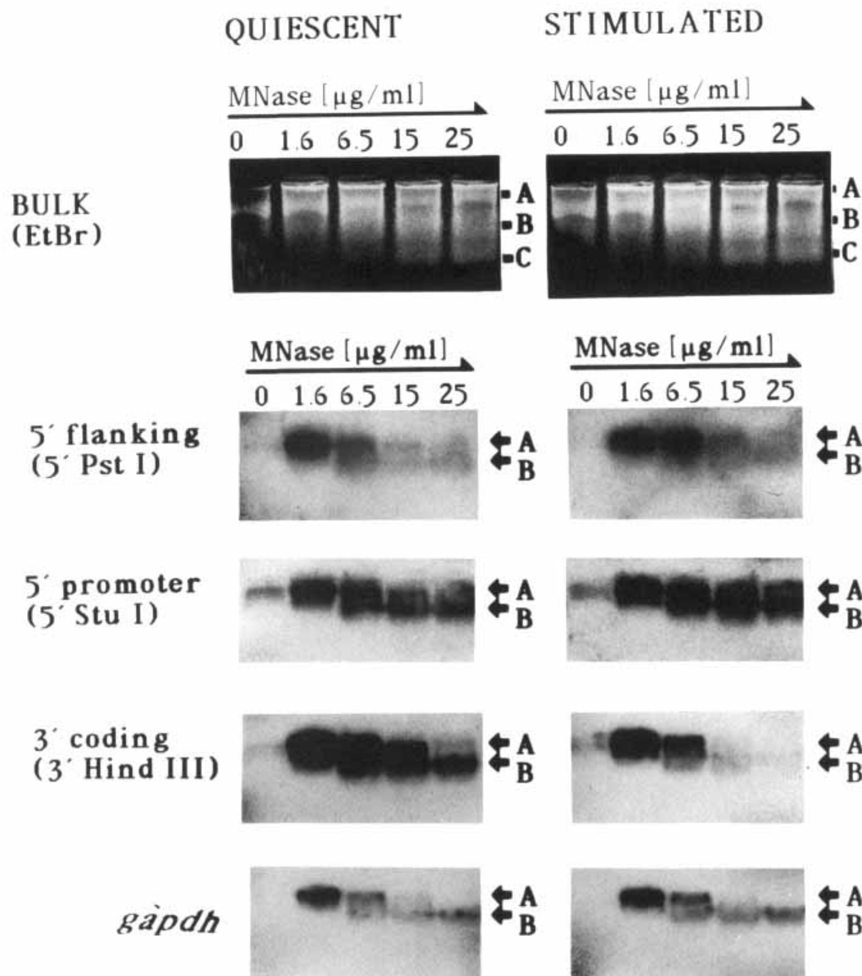

Fig. 3. Supranucleosomal localization of the *odc* gene sequences in quiescent and stimulated cells. Chromatin was prepared from the nuclei of the quiescent cells (left panel) and cells stimulated with serum for 1 h (right panel), subjected to micrococcal nuclease (MNase) digestion and electrophoresed on 2% agarose gels. The supranucleosomal particles were visualized by ethidium bromide (EtBr) staining and UV illumination (first panel). For localization of the *odc* gene sequences the supranucleosomal particles were transferred onto filters and their DNA was hybridized to probes specific to the 5′ enhancer/promoter region (5′ Pst I, second panel), promoter region (5′ Stu I, third panel), to the mid-coding and 3′ regions (3′ Hind III, fourth panel) of the *odc* gene, or to the control probe (*gapdh*, fifth panel). The concentrations of MNase are indicated on top. The letters A, B, and C indicate the positions of the supranucleosomal A, B, and C particles, respectively.

filter with the very short 5′ Stu I insert (see Figs. 1 and 3). Hybridization of the filter to the 936 bp Hind III fragment shows that the mid-coding and 3′ regions of the *odc* gene from the serum-stimulated cells, in contrast to the quiescent cells, displayed again a decrease in the hybridization signal when the nuclease concentration was increased (Fig. 3). As a reference, we also analyzed the chromatin structure of the house-keeping *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) gene. As is evident from the hybridization results in Figure 3 the hybridization signal of the *gapdh* gene does not vary upon serum stimulation.

To show that the decrease in the hybridization signal of the mid-coding and 3′ regions of the *odc* gene is, in fact, different for the quiescent and stimulated cells the autoradiograms shown in Figure 3 were magnified, and scanned with a densitometer. This and the individual matched background subtraction enabled us to take into account variations in autoradiogram quality and provided an improved resolution for the quantification of the individual particles (data not shown). As compared to the resting cells, the decrease in the hybridization signal using the coding probe of the *odc* gene (Hind III) was decreased 14-fold after the serum stimulation for 1 h (Fig. 4). These changes correlated with the early accumulation of *odc* mRNA following serum stimulation (Fig. 5). Such changes were not seen in the chromatin structure of *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) gene, whose expression remains unaltered during growth stimulation (Figs. 3–5).

Altogether, our results indicate that in quiescent cells the entire *odc* promoter has a more loosened chromatin structure than that of the mid-coding and 3′ regions of the gene. Further, stimulation of the E4 cells with serum resulted in structural alterations of the *odc* chromatin at the mid-coding and 3′ regions of the gene. These changes were accompanied by the rapid accumulation of *odc* transcripts.

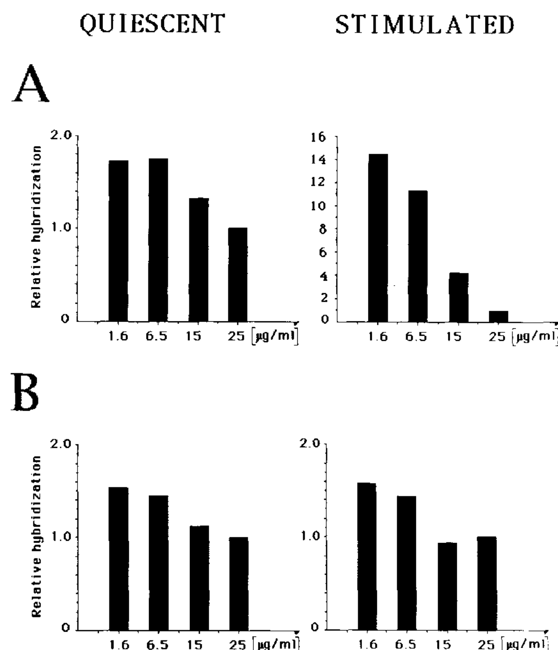

Fig. 4. Densitometric scans of the supranucleosomal particles. The densitometric scans of the supranucleosomal particles shown in Figure 3 were generated from autoradiograms. The left and right panels indicate the relative level of MNase-sensitivity at the coding regions of the *odc* (panel A) and *gapdh* (panel B) genes in the quiescent and stimulated cells, respectively. For each gene the level of micrococcal nuclease sensitivity in the last lane (25 µg/ml) was used as a reference. The level of sensitivity was quantitated in arbitrary units. The concentrations of MNase are indicated on the x-axis. The relative MNase-sensitivity is indicated on the y-axis.

The Promoter Region of *odc* Displays an Altered Nuclease-Sensitivity

Next we studied the nucleosomal organization of the *odc* gene during the growth stimulation. Nuclei from the cells at 0, 1, and 3 h after serum stimulation were isolated and subjected to the MNase digestion, DNA purification, and Southern hybridization analyses. As shown in Figure 6A, the 5′ flanking region (Pst I fragment) of the *odc* gene displayed a smeared nucleosomal organization both in quiescent and stimulated cells, suggesting a constitutively loosened chromatin folding (Fig. 6). Compared to this, the promoter region displayed a more distinct nucleosomal ladder (Fig. 6C). The 3′ region of the *odc* gene in turn exhibited a distinct nucleosomal ladder both in the serum-starved and stimulated cells (Fig. 6B). These results show that the decrease in the hybridization signal to the supranucleosomal particles (Fig. 3) does not indicate a disappearance of the nucleosomal structure as the mid-coding and 3′ sequences of *odc* are wrapped around nucleosomes. Further, control hybridizations with the coding and 3′ end regions of the *gapdh* gene revealed a very clear nucleosomal ladder (Fig. 6D).

As the smeared nucleosomal organization could also represent different nucleosomal phas-

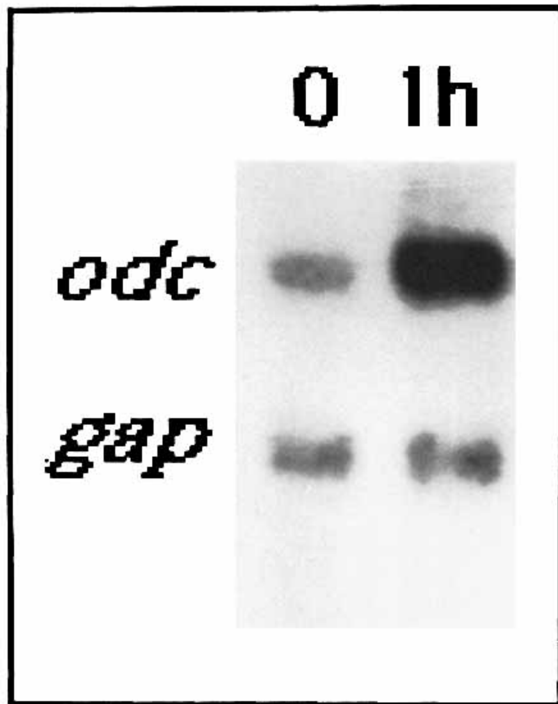

Fig. 5. Expression of *ornithine decarboxylase* and *gapdh* genes in serum stimulated NIH 3T3 cells. E4 cells were synchronized by serum starvation for 16 h, whereafter 10% FCS was added to allow the cells to enter the cell cycle. Northern blots were sequentially hybridized to insert probes specific to *odc* (pODC16), and *gapdh* (pRGAPDH-13) genes.

ing frames or could be due to the intrinsic sequence-specificity of MNase we wanted to ensure that the entire 1.7 kb promoter region at the *odc* locus is DNase I sensitive. To study this, nuclei from the cells at 0, 1, and 3 h after serum stimulation were subjected to the DNase I digestion, extracted for DNA, digested with Pst I, and finally subjected to Southern hybridization analyses. It was found that the 5′ flanking region of *odc* gene is markedly more sensitive to the DNase I digestion than control bulk chromatin. Moreover, we found and tentatively identified a DNase I sensitive site at the 5′ flanking region of *odc* after serum stimulation (data not shown).

The 5′ Flanking Region of *odc* Displays a Chromatin Structure That Is More Open Than That of the 3′ Region

Our data suggest that the methylated CCGGs at the *odc* locus cannot inhibit the formation of the active and nuclease-sensitive chromatin structure. It is possible that these sites are not critical for chromatin formation. Thus, we wished to study whether the highly clustered CpGs at the *odc* promoter region could function as nucleation targets in triggering the known changes in the chromatin structure during the S phase of cell cycle [Riley and Weintraub, 1979; Annunziato and Seale, 1982; Moreno et al., 1986; Laitinen et al., 1990].

To study the possible correlation between CpG islands and chromatin condensation changes the cells were synchronized at the G_1/S boundary by treatment with aphidicolin (APC) and then released to the S phase by replacing the old medium by fresh medium (Fig. 7A). The chromatin structure of *odc* gene in the aphidicolin-treated cells during their progress through the S phase was studied by MNase digestion analyses. Figure 7 also illustrates that the 5′ and 3′ regions of the *odc* gene underwent condensation when the cells traversed from the S phase to the S-G_2 phase as judged from the increased MNase resistancy (Fig. 7B,C). Bulk chromatin and the chromatin of the coding regions of *gapdh* gene were found to display similar cell cycle-dependent changes (data not shown). The chromatin of the 3′ region of *odc* shown is clearly less susceptible to MNase than the 5′ flanking region. It can also be seen that the dynamic changes in chromatin structure of the mid-coding region of the *odc* gene are smaller than those of the promoter region of *odc*. The CCGG-rich 5′ flanking region undergoes a greater condensation than the mid-coding region of the *odc* gene.

These results show that there is a correlation between CpG islands and chromatin condensation changes in the late S phase. Thus, our data suggest that the CCGG-islands of the *odc* gene could play a role during the condensation of chromatin.

DISCUSSION

In this work we examined the methylation status of CpGs at CCGG-sequences and the chromatin structure of the *odc* gene. It was found that in 3T3 cells the proximal promoter region of the *odc* locus is not completely hypermethylated. One methylated site was localized to the distal promoter region. It was also shown that the promoter region of *odc* gene displayed altered MNase sensitivity and it was more accessible to MNase than the mid-coding and 3′ regions of this gene. Further, the analyses on the supranucleosomes showed that both in the quiescent and serum stimulated 3T3 cells the nucle-

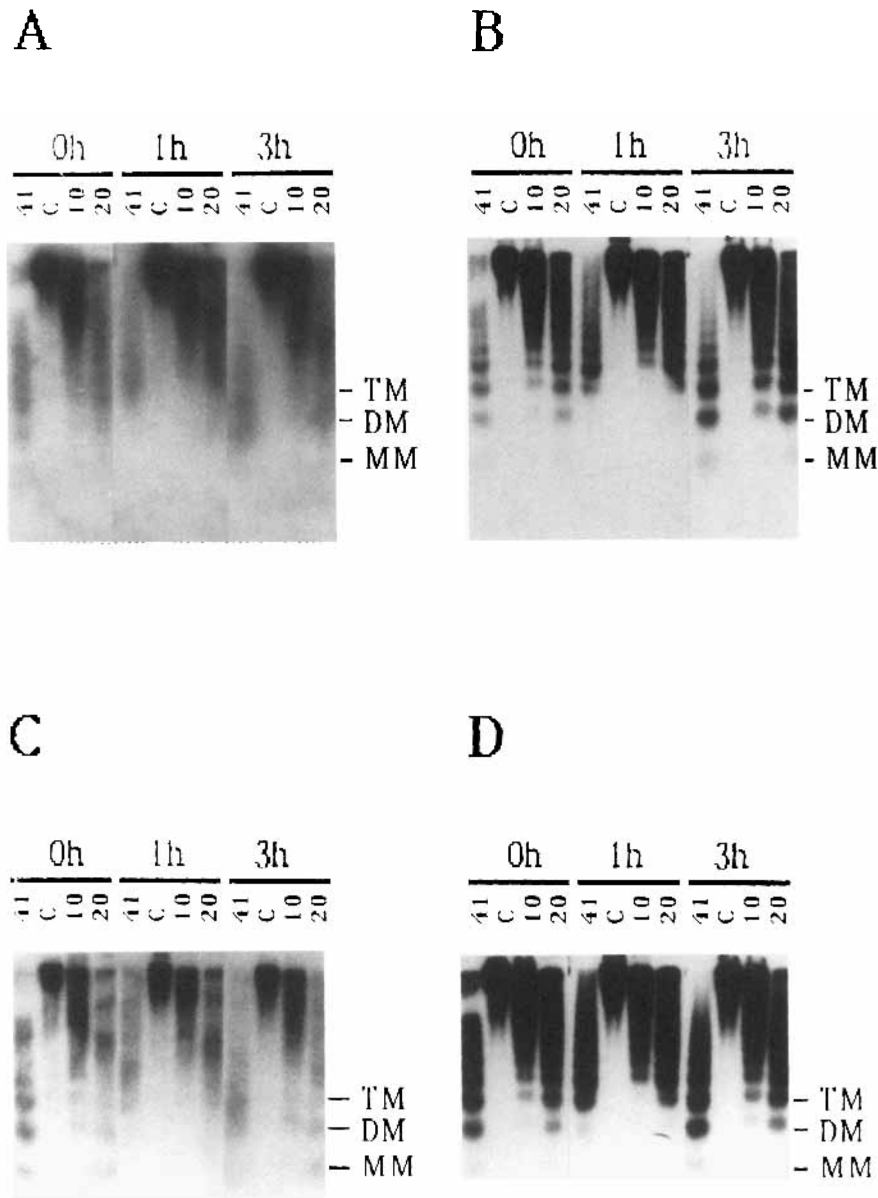

Fig. 6. Micrococcal nuclease digestion patterns of the 5′ and 3′ regions of the *odc* gene during serum stimulation. Following serum starvation, the synchronized cells were stimulated with 10% foetal calf serum, and nuclei were isolated at 0, 1, and 3 h of stimulation. Nuclei were digested with micrococcal nuclease (75 units/ml), extracted for DNA and electrophoresed on 1.6% agarose gels, and subjected to Southern blot analysis. **A:** Hybridization with the 5′ Pst I probe specific to the 5′ flanking region of the *odc* gene. **B:** Hybridization with the 3′ Hind III probe specific to the mid-coding and 3′ region of the *odc* gene. **C:** Hybridization with the promoter-specific Stu I probe. **D:** Hybridization with the *gapdh* insert probe (pRGAPDH-13). The numbers underlined indicate the duration of serum stimulation. Oh stands for the quiescent cells. The vertical numbers on top indicate digestion time with nuclease in minutes. C indicates DNA from undigested nuclei. Abbreviations MM, DM, and TM stand for monomer, dimer, and trimer, respectively.

ase-sensitive promoter sequences of the *odc* gene predominantly adopted the nuclease-sensitive and transcriptionally active chromatin structure.

The exact determination of the methylation status of the *odc* gene promoter/enhancer region in 3T3 cells and in other rodent cells by methylation analyses is, however, difficult due to a very large number of densely clustered CpG enzyme-specific sites and the presence of differentially methylated alleles [Berger et al., 1984; Kahana and Nathans, 1984; McConlogue et al., 1984; Pilz et al., 1990]. Indeed, the presence of the 1.4/2.2 kb fragment in Hpa II digests could

suggest the existence of a methylated *odc* allele. Interestingly enough, this fragment was never seen in the Msp I digests and it was always present in Hpa II digests from four different mouse 3T3 cell lines (J.L. and E.H., unpublished data). In contrast to our results, in human myeloma cells the CCGG sequences at the CpG-islands of the *odc* gene appear to be fully methylated [Wahlfors, 1991]. Therefore, it is possible that methylated CCGGs at the promoter region are not critical for the chromatin structure and gene expression. For example, it is possible that certain chromatin stabilizing methylated DNA binding proteins are more abundant in some cells and less abundant in some other cell lines or tissues. Indeed, Meehan et al. [1989] have shown that the MeCP1 protein is less abundant in self-renewing than in differentiated cells.

It is suggested that disruption of nucleosomal structure is triggered by binding of a transcription factor/RNA polymerase complex and movement of the RNA polymerase along the chromatin fiber [Gross and Garrard, 1988; Feng and Villeponteau, 1990; Lee and Garrard, 1991]. In this work we studied the supranucleosomal organization of the *odc* sequences. Interestingly, the CpG-rich promoter region of the *odc* gene has both in quiescent and stimulated NIH 3T3 cells a constitutively MNase-sensitive chromatin configuration. This suggests that the entire promoter region and its flanking sequences are at least partially nuclesome-free and/or they contain altered nucleosomes (see discussion below). In this study it was also found that the coding and the 3′ regions of *odc* in the supranucleosomes revealed increased MNase-sensitivity of their structure in response to serum stimulation of the cells. The changes at the mid-coding and 3′ regions of *odc* correlated well with the

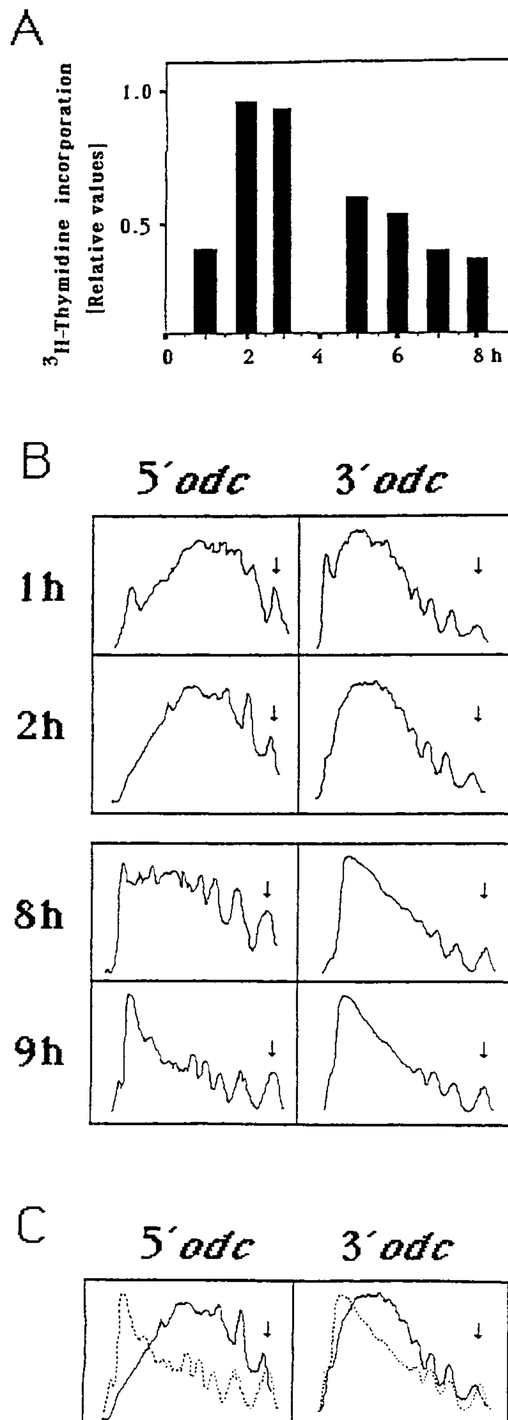

Fig. 7. Densitometric scans of the nucleosomal organization of the 5′ and 3′ chromatin domains of the *odc* gene during S phase. The cells were synchronized by aphidicolin treatment for 24 h and allowed to enter the cell cycle by adding fresh medium. **A:** The rate of DNA synthesis. The x-axis indicates 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:** Nuclei were isolated from the aphidicolin synchronized cells at the different times and subjected to micrococcal nuclease digestion (50 units/ml). The scans representing 10 min digestion patterns from the nuclei isolated at 1, 2, 8, and 9 h are shown. They were generated from autoradiograms of the Southern blots hybridized with probes specific to the 5′ flanking region (left panel) or the mid-coding and the 3′ region (right panel) of the *odc* gene. The numbers on left indicate progression through the S phase. The arrows indicate the positions of DNA from the nucleosome monomer. **C:** The scans representing the chromatin structures of the 5′ and 3′ regions of the *odc* gene at 2 and 9 h after the aphidicolin block are superimposed. The solid and dashed lines indicate the nucleosomal organization of *odc* in the mid S phase (2 h) and in the S-G_2 phase (9 h), respectively.

increase in the levels of *odc* mRNA following serum stimulation. In addition to the *odc,* the chromatin structures of the c-*myc* and c-*fos* protooncogenes undergo structural changes during serum stimulation [Chen and Allfrey, 1987; Chen et al., 1990; Feng and Villeponteau, 1990]. Allfrey and co-workers [Chen and Allfrey, 1987; Chen et al., 1990] have used mercury-affinity chromatography columns and nuclear run on analyses in studies on the transcriptional activation and repression of the c-*myc* and c-*fos* genes during serum and growth factor stimulation. Hg-chromatography columns separate nucleosomes which have the sulfhydryl-reactive histone H3 of transcriptionally active nucleosomes from the tight and non-reactive nucleosomes of transcriptionally inactive genes. In quiescent 3T3 cells, when c-*myc* transcription was low, the corresponding c-*myc* sequences were in the Hg-unbound fraction, while the signal was recovered in the Hg-bound fraction when c-*myc* RNA accumulated 1h after serum stimulation. The nucleosome unfolding kinetics of the c-*fos* gene were more rapid than those of the c-*myc* sequences. Further, Feng and Villeponteau [1990] have shown in HeLa nuclei that the inducible DNase I domain sensitivity of c-*fos* chromatin appeared to correlate with enhancer activity during serum induction and repression of the c-*fos* gene. After 90 s of serum stimulation the proximal DNA regions on both sides of the c-*fos* promoter displayed increased DNase I sensitivity. These changes translated to other regions of the c-*fos* gene as 15 min after serum stimulation the chromatin structure of promoter, mid-coding, and 3′ regions along the c-*fos* gene became more sensitive to DNase I than the corresponding regions in the nonstimulated cells. Altogether, under the conditions of serum stimulation described here and in the other studies [Chen and Allfrey, 1987; Chen et al., 1990; Feng and Villeponteau, 1990; Jähner and Hunter, 1991], our results show that in G_1 phase of the growth cycle the structure of *odc* chromatin undergoes modifications which correlate with the transcriptional activation of the *odc* gene.

In this work, we have found that the entire enhancer/promoter region of the *odc* gene displays domain DNase I sensitivity. This is in agreement with the findings on the supranucleosomal structures and with the analyses on the nucleosomal organization of *odc* chromatin. The formation of such regions as well as hypersensitive sites are likely to be associated with a binding of trans-acting factors to chromatin [Elgin, 1988; Gross and Garrard, 1988; Bonifer et al., 1991; Felsenfeld, 1992]. Although little is still known about the transcriptional regulation of *odc,* the DNase I footprinting and gel retardation assays of the *odc* promoter/enhancer region have already revealed some transcription factors (e.g. CREB-like factor) that may be involved in its activation [Palvimo et al., 1991].

Our data further indicate that the entire promoter region of *odc* displays a nucleosomal organization that is markedly more open than that of the mid-coding and 3′ regions. For example, the mid-coding and 3′ regions of the *odc* gene are always incorporated into moderately MNase-resistant nucleosomes (Fig. 6), although the supranucleosomal organization changes upon serum stimulation (Figs. 3 and 4). It also appears that the internucleosomal interactions at the mid-coding and 3′ regions of the *odc* gene undergo structural changes in response to serum stimulation in a way that the *odc* chromatin is not held together by bonds between the nucleosomes. Yet the promoter region may have nucleosomes present all the time as the proximal promoter region of *odc* detected by the very short Stu I probe displayed distinct hybridization signals to the supranucleosomal particles (Fig. 3). This somewhat surprising finding of intact or modified nucleosomes at the proximal *odc* promoter and its flanking regions is nonetheless compatible with the notion that certain active promoters have nucleosomes [Durrin et al., 1991]. The "smeared MNase digestion pattern" of the *odc* promoter, however, suggests a less well ordered chromatin structure than that of the mid-coding and 3′ regions of *odc* chromatin. The enhancer/promoter region of *odc* thus seems to have a constitutively loosened and transcriptionally active chromatin structure due to a loss or modification of nucleosomes [Gross and Garrard, 1988]. However, the smeared MNase ladder could also be due to different phasing frames of nucleosomes [Gross and Garrard, 1988]. Thus, the localization of nucleosomes at the *odc* promoter remains an interesting object for further study.

We found that the *odc* locus, like the human c-*fos* gene [Feng and Villeponteau, 1990], displays domain nuclease sensitivity that decreases towards the 3′ end region. The CpG-rich chromatin of the 5′ flanking region of mouse *odc* ap-

pears to undergo more marked structural changes upon replication than the coding and 3′ regions of the genes. Therefore, a possibility arises that the CpG islands of genes function as nucleation targets in triggering the changes in chromatin structure. It is tempting to speculate that the binding of certain methylated DNA-binding proteins to the methylated cytosines is cell cycle dependent and controlled by phosphorylation. For example, the methyl-CpG binding protein, MeCP2, has a consensus recognition site for p^{34CDC2}, the main controller of the cell cycle [Lewis et al., 1992]. However, in contrast to the *odc* and c-*fos* genes that have CpG islands in their promoter regions [van Beveren et al., 1983; Eisenberg and Jänne, 1989], the *histone H4* gene lacks the corresponding CpGs [Seiler-Tuyns and Birnstiel, 1981] and still undergoes similar chromatin changes [Moreno et al., 1986]. Thus, this indicates that there must still be other factors, like transcription factors, replicating, and transcribing polymerases [Riley and Weintraub, 1979; Annunziato and Seale, 1982; Feng and Villeponteau, 1990; Lee and Garrard, 1991], besides the MeCPs that contribute to the universal dynamic changes of different genes during the cell cycle. This is especially true for organisms that lack DNA methylation. For example, *Drosophila* cells have a demethylated genome but are still capable of condensing chromatin [Urieli-Shoval et al., 1982].

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