Journal of Cellular Biochemistry 63:221–228 (1996)

Requirement of Distal and Proximal Promoter Sequences for Chromatin Organization of the Osteocalcin Gene in Bone-Derived Cells

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Abstract The osteocalcin (OC) gene encodes a 10 Kda bone-specific protein which is expressed with the onset of mineralization during the differentiation of normal diploid osteoblasts. We have previously reported that transcriptional activation of this gene is accompanied by the presence of two DNase I hypersensitive sites, both located in the promoter region spanning key basal (proximal site, −170 to −70) and steroid-dependent enhancer (distal site, −600 to −400) elements. Here, we have examined stably transfected ROS 17/2.8 cell lines carrying OC promoter-reporter transgenes which contain a series of 5′-deletions and determined the effects of these truncations on the chromatin organization. It has been found that: (1) DNase I hypersensitivity at −600 is not a requirement for vitamin D-dependent transcriptional upregulation; (2) basal transcriptional activity and proximal nuclease hypersensitivity depend exclusively on protein-DNA interactions occurring within the proximal promoter region, and (3) within the chromatin context, the proximal 100 bp promoter fragment, containing essential elements such as the OC box (−99 to −76) and TATA box (−44 to −31), is insufficient to support formation of the proximal nuclease hypersensitive site and transcriptional activity. © 1996 Wiley-Liss, Inc.

Remodelling the chromatin organization of the osteocalcin (OC) gene promoter is a principal component of bone tissue-specific and steroid hormone-mediated transcriptional regulation. We have previously reported that in ROS 17/2.8 osteosarcoma cells as well as in normal diploid rat osteoblasts, which both express OC, key promoter regulatory elements reside in two DNase I hypersensitive sites [Montecino et al., 1994; in press]. These two domains can be further resolved into four sub-bands, each representing the limits of the hypersensitive regions [Montecino et al., 1994]. The proximal hypersensitive site (−170 to −70) includes sequence motifs that specifically interact with basal transcription factors such as Msx [Hoffmann et al., 1994; Towler et al., 1994a,b], Id [Tamamura and Noda, 1994], AP-1 [Banerjee et al., 1996b], and NMP-2 which was recently identified as a bone-specific nuclear matrix associated protein [Bidwell et al., 1993; Merriman et al., 1995], and a member of the AML family of transcription factors [Geoffroy et al., 1995; Banerjee et al., 1996a]. The distal hypersensitive domain (−600 to −400) contains the vitamin D responsive element (VDRE, −465 to −437), which has been shown to interact with the VDR-RXRα complex in a ligand-dependent manner [Markose et al., 1990; Demay et al., 1990; Terpening et al., 1991; Breen et al., 1994]. Two additional NMP-2 sites (Site A: −604 to −599, and Site B: −440 to −435) have been identified in the sequences flanking the distal DNase I hypersensitive domain that may support specific interactions between the nuclear matrix and the OC gene promoter [Bidwell et al., 1993; Merriman et al., 1995]. In addition we have identified a binding site for NMP-1 [Bidwell et al., 1993], a ubiquitous transcription factor which can partition between soluble nuclear proteins and the nuclear matrix fractions. NMP-1, which has been recently shown to be the YY-1 transcription factor [Guo et al., 1995], binds to a site that partially overlaps one of the VDRE half elements providing a potential mutual exclusion binding mechanism [Guo et al., in press].

Received April 15, 1996; accepted May 7, 1996.

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To begin dissecting the components involved in interrelationships between chromatin organization and transcriptional regulation of the OC gene, ROS 17/2.8 cells were stably transfected with a series of rat OC gene promoter-reporter constructs containing progressive 5′-deletions [Frenkel et al., 1996]. These deletions sequentially truncate the limits of the distal and proximal promoter DNase I hypersensitive domains. In addition, they eliminate sequences where key regulatory protein-DNA interactions have been shown to reside.

MATERIALS AND METHODS

Cell Culture

Rat osteosarcoma-derived ROS 17/2.8 cells were maintained as described [Majeska et al., 1980]. Cells were subjected to treatment with 1,25-dihydroxyvitamin D_3 (vitamin D) for 24 h as indicated. Stable transfection of ROS 17/2.8 cells, as well as determination of the average number of integrated transgenes, promoter (CAT) activity, and its induction by vitamin D have been described previously [Frenkel et al., 1996].

Nuclease Digestion Analysis

Nuclei isolation and nuclease digestion analysis were performed according to previously reported protocols [Montecino et al., 1994, 1996]. Briefly, the isolated nuclei were incubated with increasing amounts of DNase I or with the restriction endonucleases Hinc II and Pst I (500 units/ml) as indicated. The purified genomic DNA was then completely cleaved with several combinations of restriction enzymes (as indicated in each case), fractionated electrophoretically in agarose gels, and analyzed by Southern blotting. Hybridization was with probes directed against the coding region of the CAT [Frenkel et al., 1996] and the endogenous OC [Montecino et al., 1994] genes. The probes were labeled by the random primer method [Feinberg and Vogelstein, 1983] using reagents from Stratagene (La Jolla, CA). Hind III-digested bacteriophage Lambda DNA and HinfI-digested pUC19 DNA, labeled with T4 Polynucleotide kinase and (^{32}P-γ) ATP, were used as DNA size markers. Accessibility to the different restriction enzymes was determined by densitometric scanning of the Southern blot autoradiogram.

RESULTS

Analyses of chromatin structure indicate that nuclease hypersensitive domains are generally associated with active genes or genes that are poised to be expressed [Elgin, 1988; Gross and Garrard, 1988]. We have previously reported that bone-derived cells expressing OC exhibit two nuclease hypersensitive promoter domains. We defined regions in the OC gene promoter involved both in formation of the proximal and distal hypersensitive sites and in transcriptional activation within the chromatin context. ROS 17/2.8 cells were stably transfected with rat OC gene promoter-reporter (CAT) constructs containing progressive 5′-deletions [Frenkel et al., 1996]. Here, the consequences of promoter deletions on chromatin organization of these transgenes were determined by nuclease digestion analysis using the indirect end-labeling method [Wu, 1980].

The first stably transfected cells to be analyzed, designated ROSZc [Frenkel et al., 1996] contained an average of 15 copies (Table I) of the −1097OCCAT construct (see diagram in Fig. 1). These cells expressed high levels of CAT protein and showed a seven fold induction following treatment with vitamin D (Table 1). Similar to the endogenous gene promoter, this transgene promoter exhibited two DNase I hypersensitive domains (Fig. 1A). A strong, broad proximal hypersensitive site (nt −170/−70) spans important promoter elements implicated in the regulation of basal levels of transcription, and a less intense distal hypersensitive site, represented by a single sub-band, is located at −400. This is in contrast to what was previously observed for the endogenous OC gene promoter, where the distal hypersensitive site encompasses the extended −600/−400 domain, with two intense sub-bands at −600 and −400 [Montecino et al., 1994; Frenkel et al., 1996]. This difference was further confirmed by comparing accessibility of the GTTGAC sequence at −529 to the restriction endonuclease Hinc II. This site in the endogenous OC gene is highly accessible for cleavage by Hinc II, both in the parental ROS 17/2.8 cells [Montecino et al., 1996] and in the transfected ROSZc cells (Fig. 1B). However, we observe resistance to cleavage in the transgene promoter (Fig. 1A). Because it is well established that DNA sequences packaged into nucleosomes show reduction in accessibility to restriction endonuclease activity [Archer et al., 1991; Simpson, 1991], these results suggest that in the −1097OCCAT transgene the DNA segment located upstream of −400 can be organized as a nucleosome. These results also indicate that hy-

TABLE I. Summary of Expression Levels and Restriction Nuclease Accessibility of OCCAT Stably Transfected Constructs*

Cell pool	Construct	Copy no.	Relative basal expression[a]	Vitamin D induction (fold)	Restriction enzyme accessibility[b] Hinc II (−529)	Pst I (−145)
ROSZc	−1,097 OCCAT	15	84	7	<0.045	0.49
ROSHc	−529 OCCAT	14	100	2	NA	0.71
ROSBb	−343 OCCAT	9	37	1	NA	0.73
ROSAc	−108 OCCAT	4	13	1	NA	NA
ROSGd	CAT	3	7	1	NA	NA

*ROS 17/2.8 cells were stably transfected as previously described [Frenkel et al., 1996]. Selected cell pools, carrying the indicated OC promoter-CAT constructs, were assayed for steady-state CAT level, inducibility by 10^{-8} M $1,25(OH)_2$ vitamin D_3, and accessibility of promoter sequences to the restriction endonucleases Hinc II and Pst I, as described in Materials and Methods.

[a]Activity in ROSHc cells was arbitrarily defined as 100.

[b]Restriction enzyme accessibility to the Hinc II and to the Pst I sites (at −529 and −145 relative to the transcription start site) is based on the data shown in Figures 1–3 and is expressed as the ratio between the accessibility measured in the stably transfected promoter and that measured in the endogenous OC promoter. Results are presented for non-treated cells. Vitamin D treatment had no effect on these ratios. NA, restriction site deleted.

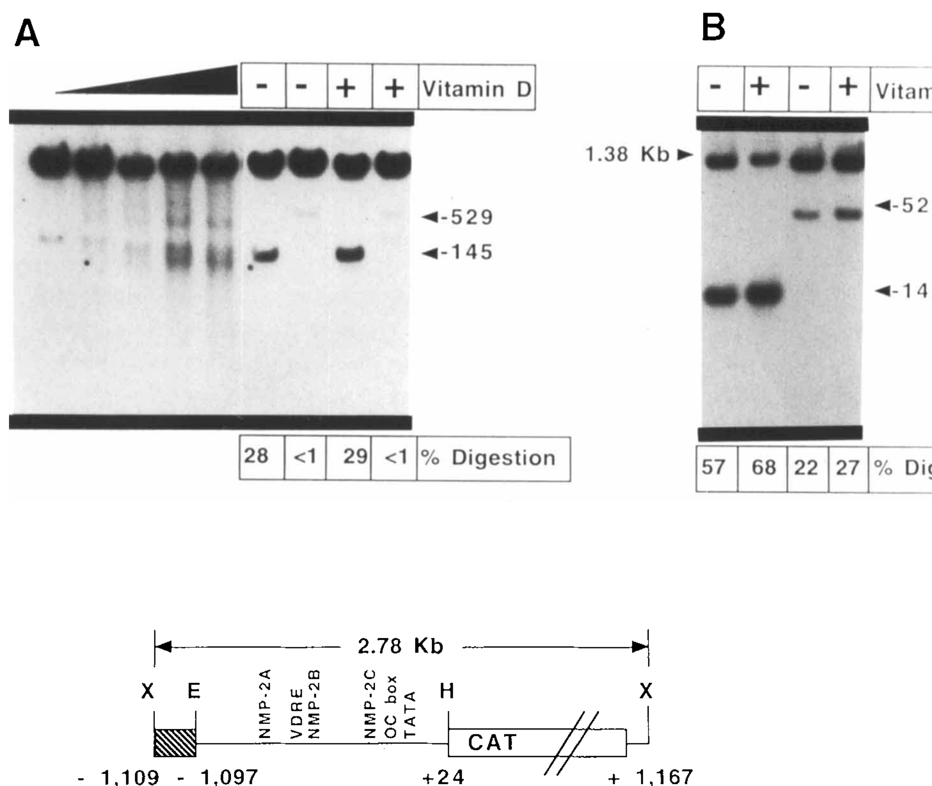

Fig. 1. Chromatin structure of the −1097OCCAT transgene promoter. Nuclei isolated from ROS 17/2.8 cells stably transfected with the −1097OCCAT construct were digested with increasing concentrations of DNase I for 10 min or with the restriction endonucleases Hinc II or Pst I (500 units/ml) for 30 min, as indicated. **A:** The purified DNA samples were completely cleaved with Xba I, electrophoresed in a 1.2% agarose gel, blotted, and hybridized with a CAT gene probe. The proximal and distal DNase I hypersensitive sites (pDHS, dDHS) are indicated. Fragments resulting from Hinc II or Pst I digestion are indicated by the position of the restriction site relative to the transcription start site (−529 and −145, respectively). Restriction endonuclease accessibility expressed as percent digestion is shown below. **B:** Hinc II and Pst I cleavage activity on the endogenous osteocalcin gene promoter was determined after digesting the samples with Apa I and hybridizing with an OC gene probe [Montecino et al., 1994]. The lower diagram depicts important OC promoter cis-acting elements. The filled box represents vector sequences and the open box indicates the CAT gene. X = Xba I; H = Hind III; E = EcoR I.

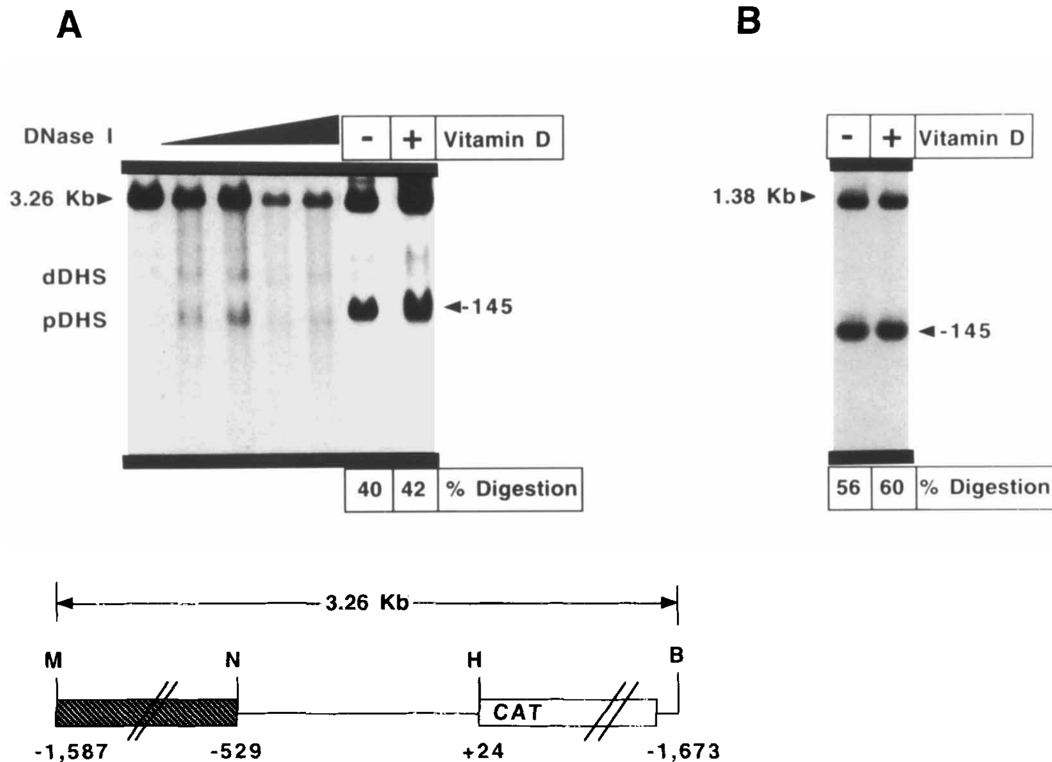

Fig. 2. Chromatin organization in the −529OCCAT transgene promoter. Nuclei isolated from ROS 17/2.8 cells stably transfected with the −529OCCAT construct, were digested with DNase I or with Pst I (restriction site at position −145) as described in Figure 1. **A:** The DNA samples were completely cleaved with BamH I (B) and Xmn I (M), electrophoresed in a 1.2% agarose gel, blotted, and hybridized with a CAT gene probe. **B:** Cleavage of the endogenous OC gene promoter by Pst I. DNA samples were completely digested with Apa I and hybridized with an OC gene probe [Montecino et al., 1994]. See Figure 1 for an explanation of the symbols.

persensitivity at −600 may not be a requirement for vitamin D-dependent transcriptional enhancement (Table I).

A similar DNase I hypersensitivity pattern was observed with the −529OCCAT deletion construct in ROSHc cells (Fig. 2 and Table I). These cells contain an average of 14 copies of the transgene and show high levels of basal transcription and a twofold transcriptional enhancement following vitamin D treatment (Table I). These results confirm that the hypersensitive site at −600 is not a pre-requisite for vitamin D inducibility, but suggest that sequences located in the region upstream of −529 contribute to maximal vitamin D responsiveness. However, these upstream promoter sequences do not influence the chromatin structure of the proximal OC promoter as reflected by nuclease hypersensitivity at −400 and at −170/−70 in the stably integrated −529OCCAT compared with the −1097OCCAT construct. Furthermore, the promoter sequences contained in the −529OCCAT construct are sufficient to render the CTGCAG sequence at −145 accessible to the restriction enzyme PstI, similar to the accessibility observed with the −1097OCCAT construct (compare Fig. 2A to 1A).

When the same analysis was performed in the cell line carrying the −343OCCAT transgene [ROSBb cells, Frenkel et al., 1996] (Fig. 3A), we observed a broad proximal DNase I hypersensitive site with similar location and intensity to those previously observed in the −1097OCCAT and the −529OCCAT transgenes. As expected, the transcriptional response to vitamin D is lost completely when the segment −529 to −343, containing the well-established VDRE [Markose et al., 1990; Demay et al., 1990; Terpening et al., 1991] is deleted. This result indicates that formation of the proximal nuclease hypersensitive domain in the OC gene promoter is independent of the distal hypersensitive domain, and implies that both basal transcriptional activity and proximal nuclease hypersensitivity are attributable to protein-DNA interactions occurring within the −343/+24 sequence. However, when the

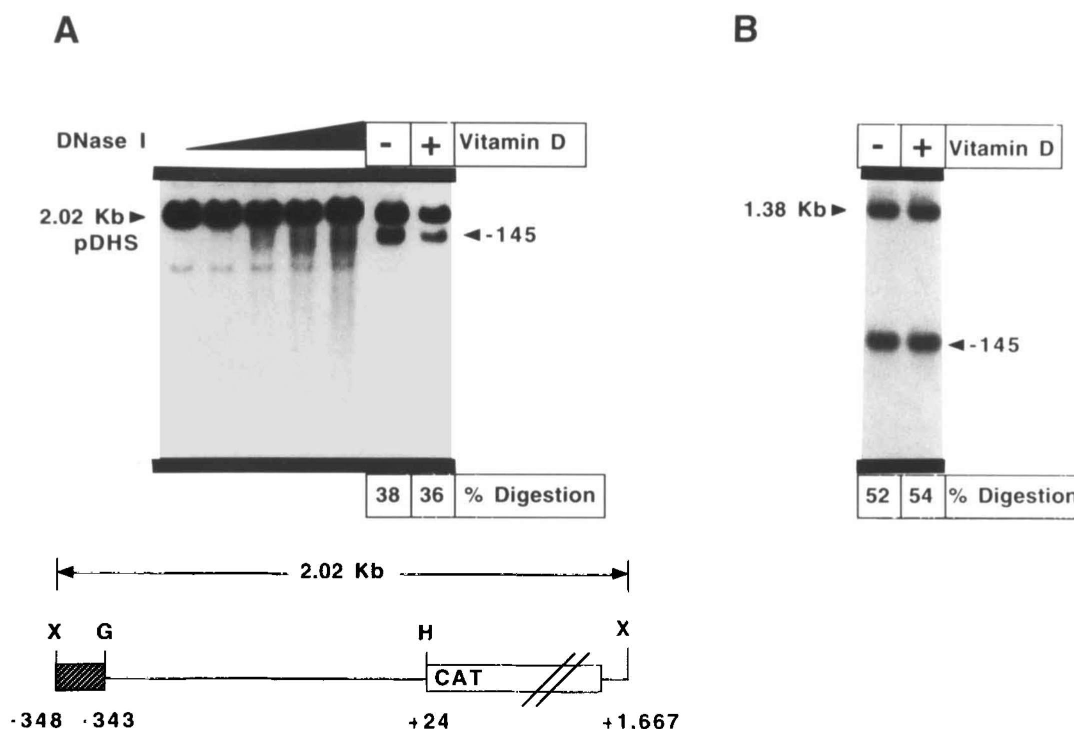

Fig. 3. Formation of the proximal DNase I hypersensitive domain is independent of the distal promoter region. Nuclei isolated from ROS 17/2.8 cells stably transfected with the −343OCCAT construct were digested with DNase I or with Pst I as described in Figure 1. **A:** The purified DNA samples were completely digested with Xba I and hybridized with a CAT gene probe. **B:** Cleavage of the endogenous OC gene proximal promoter region by Pst I. In the lower diagram: X = Xba I; G = Bgl II; H = Hind III.

DNA fragment −343 to −108 was eliminated [−108OCCAT in ROSAc cells] no detectable hypersensitivity or significant basal transcriptional activity was observed (Fig. 4 and Table I). Interestingly, the −108OCCAT construct contains important basal regulatory elements, such as the OC box (−99 to −76), a glucocorticoid response element [Aslam et al., 1995], and the TATA box (−44 to −31), which have been shown to suffice for expression in transiently transfected ROS 17/2.8 cells [Hoffmann et al., 1996; Towler et al., 1994a].

Within the range of 3 to 20, the number of integrated copies did not have a significant effect on the chromatin organization or transcriptional activity of the different transgenes. Thus, cells containing 3 or 15 copies of the −1097OC-CAT construct showed the same hypersensitivity pattern and similar transcriptional activity (not shown).

DISCUSSION

Specific arrangements of chromatin structure have been associated with the regulated expression of eukaryotic genes [Felsenfeld, 1992]. These structural features have been studied principally by analyzing the accessibility of regulatory regions to nucleases such as DNase I and restriction endonucleases [Simpson, 1991]. To experimentally address the contribution of the different regions of the rat OC gene promoter to chromatin organization and transcriptional activity, ROS 17/2.8 cells were stably transfected with constructs carrying progressive 5′ deletions of the OC gene promoter [Frenkel et al., 1996]. The resulting chromatin structure was then determined by the indirect end-labeling method [Wu, 1980].

We had previously found that the transgene containing 1.1 kb of the rat OC gene promoter [in ROSZa cells; Frenkel et al., 1996] exhibits two DNase I hypersensitive sites with reduced hypersensitivity in the distal region (−400/−600), when compared to the endogenous gene. Here, we confirm this result in independent transfectants (ROSZc cells) and further demonstrate inaccessibility of a Hinc II site located at this region (−529) to this restriction enzyme.

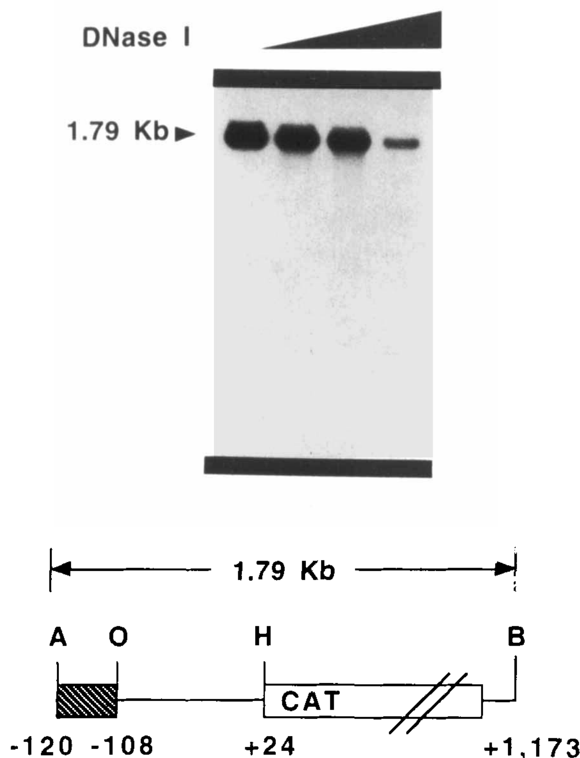

Fig. 4. The promoter segment −343 to −108 is required for the formation of the proximal DNase I hypersensitive domain. Nuclei isolated from ROS 17/2.8 cells stably transfected with the −108OCCAT construct were digested with increasing amounts of DNase I. After purification, the DNA samples were completely cleaved with BamH I and Apa I, electrophoresed in a 2% agarose gel, blotted, and hybridized with a CAT gene probe. In the lower diagram: A = apa I; O = Aos I; H = Hind III; B = BamH I.

We therefore propose that sequences upstream of −400 may be organized as a nucleosome-like structure [Archer et al., 1991; Simpson, 1991]. The −1097OCCAT stably integrated construct responded to vitamin D with a 7-fold transcriptional enhancement suggesting that this chromatin organization supports functionality of the VDRE. We have recently observed that the distal region of the OC gene promoter retains nucleosomal organization under conditions of histone hyperacetylation [Montecino et al., unpublished]. These results indicate that the nucleosomal transition which results in the formation of the distal hypersensitive site may not involve complete nucleosomal loss.

Nucleosomal retention at the OC-VDRE is consistent with recent chromatin analyses of transcriptional elements in other model systems. Beato and colleagues [Truss et al., 1995] have described the simultaneous interaction of the progesterone receptor and the transcription factors NF-1 and OCT-1 with cognate motifs located on the surface of a rotationally phased nucleosome in the stably integrated MMTV LTR sequence. The authors indicated that this nucleosome is neither removed nor shifted following hormone induction, which appears to contradict previous reports [Archer et al., 1992]. In addition, Zaret and colleagues [McPherson et al., 1993] have reported that in hepatocytes, the serum albumin enhancer is organized as an array of 3 positioned nucleosomes, which is absent in non-expressing tissues. This array appears to allow the binding of the HNF-3 transcription factor to a site located on the surface of one of the arrayed nucleosomes.

Formation of the hypersensitive site at −400 is solely dependent on protein-DNA interactions occurring downstream of −529 because it was found in cells carrying the −529OCCAT construct. This transgene exhibited only twofold vitamin D-mediated transcriptional enhancement. Consequently, we suggest that within the chromatin context sequences located around or upstream of −529 are required for maximal vitamin D stimulation. Importance of these sequences is further supported by in vivo LMPCR footprints demonstrating extensive footprinting which not only covers the VDRE [Breen et al., 1994], but extends farther upstream beyond −530 [Montecino et al., unpublished]. The nature of the DNA-binding proteins that interact with this region is currently being investigated.

The promoter segment −343 to −108 was found to be critical for inducing both proximal nuclease hypersensitivity and basal transcriptional activity. The transgene −108OCCAT contains regulatory elements (e.g., OC box, −99 to −76 and TATA box, −44 to −31) sufficient for expression in transiently transfected ROS 17/2.8 cells [Hoffmann et al., 1996; Towler et al., 1994a]. Furthermore, it has been recently reported that the NMP-2C element (−138 to −130), which recognizes an AML-related, tissue-specific transcription factor [Merriman et al., 1995] contributes to basal transcriptional activity of the OC gene promoter [Banerjee et al., 1996a; Geoffroy et al., 1995]. Absence of DNase hypersensitivity in the −108OCCAT transgene suggests this element may be required to induce nucleosomal remodeling which supports transcriptional activity of the OC gene within a chromatin context.

ACKNOWLEDGMENTS

These studies were supported by grant AR42262 from the National Institutes of Health. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health. The authors are appreciative of the editorial assistance from Ms. Elizabeth Bronstein in the preparation of this manuscript. The authors thank S. Pockwinse for the photography, and J. Green, E. Buffone, and R. Mastrototaro for technical support. We also thank Drs. A. van Wijnen and M. Birnbaum for the critical reading of this paper.

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