Journal of Cellular Biochemistry 57:90–100 (1995)

Proximal Promoter Binding Protein Contributes to Developmental, Tissue-Restricted Expression of the Rat Osteocalcin Gene

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Abstract Osteocalcin is a 6 kD tissue-specific calcium binding protein associated with the bone extracellular matrix. The osteocalcin gene is developmentally expressed in postproliferative rat osteoblasts with regulation at least in part at the transcriptional level. Multiple, basal promoter and enhancer elements which control transcriptional activity in response to physiological mediators, including steroid hormones, have been identified in the modularly organized osteocalcin gene promoter. The osteocalcin box (OC box) is a highly conserved basal regulatory element residing between nucleotides −99 and −76 of the proximal promoter. We recently established by in vivo competition analysis that protein interactions at the CCAAT motif, which is the central core of the rat OC box, are required for support of basal transcription [Heinrichs et al. J Cell Biochem 53:240–250, 1993]. In this study, by the combined utilization of electrophoretic mobility shift analysis, UV cross linking, and DNA affinity chromatography, we have identified a protein that binds to the rat OC box. Results are presented that support involvement of the OC box-binding protein in regulating selective expression of the osteocalcin gene during differentiation of the rat osteoblast phenotype and suggest that this protein is tissue restricted.

Key words: osteocalcin, homeodomain protein, osteoblasts, transcriptional regulation, bone specific, developmental

The rat osteocalcin (OC) gene encodes a 10 kD bone-specific protein, osteocalcin (bone gla protein), which is processed to a 49 amino acid polypeptide that is one of the most abundant noncollagenous calcium binding proteins in the bone extracellular matrix [reviewed by Hauschka et al., 1989]. The expression of the OC gene is stringently regulated during bone formation in vivo [Yoon et al., 1987] and bone tissue-like development in vitro [Owen et al., 1990; Aronow et al., 1990; Lian et al., 1989]. Primary cultures of fetal rat calvarial osteoblasts undergo a developmental sequence with respect to the temporal expression of genes encoding osteoblast phenotypic markers [reviewed in Stein et al., 1990; Stein and Lian, 1993]. Following the decline in proliferation and down-regulation of cell cycle and cell growth related genes, genes encoding alkaline phosphatase and other osteoblast synthesized proteins are expressed with formation of the bone-like extracellular matrix. During extracellular matrix mineralization the osteopontin and osteocalcin genes are further upregulated. The developmental regulation of osteocalcin during osteoblast growth and differentiation is reflected by modifications in protein-DNA interactions at both steroid hormone and basal regulatory elements within the proximal promoter [Bortell et al., 1993; Owen et al., 1993].

The post-proliferative regulation of OC gene expression is under the control of several steroid hormones that include vitamin D [Markose et al., 1990; Yoon et al., 1988; Demay et al., 1990; Terpening et al., 1991; Owen et al., 1991] and glucocorticoids [Morrison et al., 1989; Schepmoes et al., 1991; Shalhoub et al., 1992]. The rat OC promoter has a modular organization comprised of multiple independent and overlapping cis acting elements. Our previous studies [Mar-

Received September 15, 1993; accepted May 28, 1994.

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kose et al., 1990] and those of others [Terpening et al., 1991; Demay et al., 1990] have established a vitamin D responsive element between −462 and −437 flanked by bone-specific nuclear matrix protein binding sites [Bidwell et al., 1993] and a glucocorticoid responsive element adjacent to the TATA box (−16 to −1) [Heinrichs et al., 1993a]. Recently a cis acting silencer element which is located in the first intron, a transcribed inhibitory element (TIS), was identified [Frenkel et al., 1993]. The proximal promoter element designated the OC box (−99 to −76) [Lian et al., 1989; Markose et al., 1990] is highly conserved between rat and human OC gene promoters (22 out of 24 nt homology). This 24 nt promoter element contains a central CCAAT motif but the canonical CCAAT consensus is represented in a modified form in the human OC box (CCAAA). We recently established the role of the rat OC box as a functional promoter element which is involved in control of basal transcriptional activity [Heinrichs et al., 1993b].

The CCAAT sequence is a common cis acting promoter element in many eukaryotic protein coding genes. It usually resides 80–100 bp upstream of the RNA initiation site [Bucher and Trifonov, 1988], may function in either orientation [Knight et al., 1987; Graves et al., 1986; McKnight et al., 1984; Sierra et al., 1983; Busslinger et al., 1980; Harvey et al., 1982; Bucher and Trifonov, 1988] and may be present in one or more copies [Klenova et al., 1989; Knight et al., 1987; Barberis et al., 1987]. In the rat OC promoter, a single CCAAT motif is located between −92 and −88 in the direct orientation. Although the pentanucleotide is conserved among CCAAT binding sites, there is not a single, ubiquitous CCAAT binding protein. Rather, there are several proteins. A basis for recognition of CCAAT motifs associated with expression of specific genes by promoter binding factors may reside in unique flanking sequences. This may explain why distinct CCAAT type proteins can coexist in the same cell type [Chodosh et al., 1988; Raymondjean et al., 1988] and participate in both independent and coordinate control of gene promoter activities. In this way, CCAAT binding proteins can contribute to transient and long term transcriptional requirements of cells and tissues during differentiation and maintenance of phenotypic properties.

The CCAAT motif together with its flanking regions in the rat OC box does not resemble previously identified CCAAT-containing cis elements, suggesting that this element may exhibit recognition for a novel binding protein. Indeed, we have recently identified the nucleotide contact sites for binding of rat osteosarcoma nuclear extracts to the rat OC box [Heinrichs et al., 1993b]. We find protein-DNA contacts for the sequence AATTAG, which spans the proximal domain of the CCAAT motif as well as several nucleotides downstream. The lack of nucleotide contacts consistent with a classical CCAAT motif and the stringent homology of the AATTAG sequence to reported homeobox elements [Catron et al., 1993] suggest the possibility that a homeobox protein binds the rat OC box promoter element.

In this study we identified a protein which binds to the rat OC box. Changes in OC box protein-DNA interactions accompany differential expression of the OC gene during development of the osteoblast phenotype. Abundance of the OC box specific binding protein in nuclear extracts from ROB cells also varies at different stages of osteoblast differentiation. OC box specific protein/DNA interactions were detected in bone-derived and fibroblast nuclear extracts but were not present using nuclear extracts from other rat tissues or cell types, based on migration of protein-DNA complexes and competition studies in gel shift assays. In addition, the protein is predominantly represented in ROS 17/2.8 cells and in differentiated ROB cells, whereas in other rat tissues and in other cell types this protein is less abundant or not detectable. These findings suggest that the OC box binding protein may play a role in tissue restricted regulation of OC gene expression.

MATERIALS AND METHODS

Cell Culture and Tissues

Ros 17/2.8 cells, a gift from Drs. S. and G. Rodan (Merck, Sharp and Dohme) were grown to confluence in F12 medium supplemented with 5% FCS (fetal calf serum). All media and serum were purchased from Gibco, Grand Island, NY.

Rat osteoblasts (ROB) were isolated from the calvaria of 21 day fetal rats and subjected to sequential digestions in 2 mg/ml collagenase A, 0.25% trypsin at 37°C. Cells released from the third digest were plated in MEM (minimal essential medium) supplemented with 10% FBS (fetal bovine serum) in 100 mm dishes at a density of 5×10^5 cells/dish and were fed with the same medium on day 4 after plating. Beginning at 7

days after plating (confluence) and on every second day throughout the experiment, the cells were fed with BGJ_b medium supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerol phosphate (Sigma Chemical Co., St Louis, MO), and 10% FCS [Owen et al., 1990].

HeLa S3 cells were seeded in spinner flasks at a density of 3×10^5 cells/ml in Joklik-modified S-MEM supplemented with 7% calf serum. MG63 Human osteosarcoma cells were grown in MEM + penicillin + streptomycin and 5% FCS and they were passaged every 7 days in 100 mm dishes at a density of 5×10^5 cells. H4 Rat hepatoma cells were grown in MEM and 5% FCS and 5% horse serum. They were passaged every 7 days at a density of 5×10^5 per 100 mm dish. Rat tissues were isolated for preparation of nuclear extracts from the pregnant mother rats which were sacrificed to isolate calvarial osteoblasts.

Nuclear extracts from ROS 17/2.8 cells, rat osteoblasts and MG63 cells were prepared according to the Dignam protocol [Dignam et al., 1983] modified by Holthuis et al. [1990] by using 0.5% NP40 for the cell lysis. Nuclear extracts from rat tissues, HeLa cells and H4 cells were prepared according to the original Dignam protocol [Dignam et al., 1983] without the use of detergent.

Gel Mobility Shift Assays

^{32}P end labeled probes were prepared using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The oligonucleotides which were used as a probe or as a competitor are listed in Table I. Gel mobility shift assays were performed as described before [Markose et al., 1990]; 2 μg poly dGdC-polydGdC and a final concentration of 50 mM KCl were used with the rOCbox24 and hOCbox probes; 2 μg poly dGdC-polydGdC and 1 μg of poly dAdT-polydAdT and a final concentration of 90 mM KCl were used in binding reactions with rOCbox45 as a probe. Specific competitor oligonucleotides were used in 200 molar fold excess.

UV Cross Linking

BrdUTP substituted probe was prepared using 2 pmoles of rOCbox45 as a template and 10 pmoles of a 9 nt primer which is complementary to the 3′ end of the template; 20 μl [α-^{32}P] dCTP (3,000 Ci/mmol), 5 μl 10 × dNTP/BrdUTP mix (10 mM dATP, 10 mM dGTP, and 10 mM BrdUTP:dTTP = 9:1), 5 μl 10 × Klenow buffer (New England Biolabs), and 2 μl Klenow fragment were added to the hybridized mixture and incubated for 1 h at 4°C. Then we chased with cold dCTP (5 mM final concentration) and incubated for another hour at 15°C. After heat inactivation of the Klenow fragment, a chloroform extraction and subsequent ethanol precipitations were performed to remove unincorporated nucleotides.

The 3× binding reaction for UV crosslink, DNaseI/Micrococcal nuclease digestions, and electrophoresis on SDS-PAGE gels were performed as previously described [Ausubel et al., 1989].

Protein Purification

The rOCbox24 was cloned in pUC19 (SmaI) and amplified by polymerase chain reaction. Flanking regions of pUC19 were removed by digestion with HindIII and the resulting fragment was filled in with biotin-14-dATP (Gibco/BRL), [α-^{32}P] dCTP, dTTP, and dGTP. Biotin-cellulose resin preparation and the biotin precipitation were performed as previously described [Ausubel et al., 1989]. The resulting biotin fractions were run on a 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue or the gel was silver stained (Daiichi Pure Chemicals, Ltd., Tokyo, Japan).

RESULTS

Characterization of Protein/DNA Interactions at the OC Box

We have previously compared the protein/DNA interactions which were formed using nuclear extracts from subconfluent (day 3) and

TABLE I. OC Box Oligonucleotides*

	−120	−99	−76
rOCbox45	5′TGGGTTTGACCTATTGCGCACATGACCCCCAATTAGTCCTGGCAG3′		
rOCbox24		5′ATGACCCCCAATTAGTCCTGGCAG3′	
rOCbox Mu1		5′ATGACCCC**gtg**TTAGTCCTGGCAG3′	

*The CCAAT motif is underlined; mutations are shown in bold, lower-case letters.

confluent (day 8) ROS 17/2.8 cells [Bortell et al., 1993; Owen et al., 1993]. Their respective protein/DNA interactions look very similar and, interestingly, several complexes are qualitatively comparable to those obtained with proliferating normal diploid osteoblast nuclear extracts (Fig. 1).

When we used ^{32}P end labeled rOCbox24 oligonucleotide with ROS 17/2.8 cell nuclear extracts in gel mobility shift assays (Fig. 1), protein/DNA interactions were formed which could all be competed by 200-fold molar excess of rOCbox24 oligonucleotide and not by the same fold molar excess of a mutated rOCbox (rOCbox Mu1) (see Table I). Thus all the protein/DNA interactions are specific for the CAA nucleotides within the rat OC box.

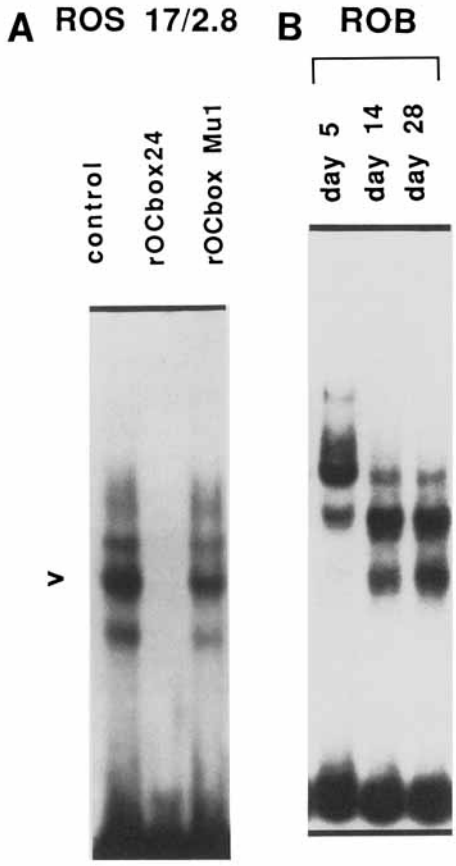

Fig. 1. OC box-specific protein/DNA interactions in the 24 bp rat OC box. **A:** ^{32}P end labeled rOCbox24 oligonucleotide and 5 μg ROS 17/2.8 cell nuclear extracts were used in a 20 min protein/DNA binding reaction. No competitor oligonucleotide was included in the control assay. A 200-fold molar excess of rOCbox24 and rOCbox Mu1 oligonucleotide were included as specific competitors in the binding reactions shown in middle and right lanes. **B:** Binding reactions contained ^{32}P end labeled rOCbox24 and 5 μg of nuclear extracts from primary rat osteoblasts (ROB) in different stages of their development (day 5, 14, and 28).

Previously we have shown that the rat OC box is involved in basal transcriptional activity [Heinrichs, 1993b]. To investigate the possibility that this site also plays a role in the regulation of stage-specific OC gene expression during the development of the osteoblast phenotype, we compared the protein-DNA interactions at the rat OC box which were formed using nuclear extracts from proliferating (day 5) and differentiating (days 14 and 28) rat osteoblasts. The protein/DNA interaction pattern changed dramatically when we compared these developmentally staged nuclear extracts from primary rat osteoblasts (Fig. 1). All the protein/DNA complexes which are formed with the different nuclear extracts from the diploid cells during development of the osteoblast phenotype compete specifically with the rat OC box but not the mutated OC box (rOCbox Mu1) (data not shown). Slow mobility complexes which are dominant in extracts from day 5, proliferating normal diploid rat osteoblasts, exhibit decreased representation on days 14 and 28 when the differentiating rat osteoblasts expressed the OC gene at moderate and maximal levels respectively [Owen et al., 1990]. Nuclear proteins from the postproliferative, differentiating rat osteoblasts form protein/DNA complexes with the rOCbox24 probe with increased electrophoretic mobility. The representation of specific bands change in addition to the appearance or loss of protein/DNA complexes.

Tissue-Restricted Specificity of OC Box Binding Proteins

Since osteocalcin is a bone-specific protein, we also examined the possible involvement of the rat OC box promoter element in tissue-restricted expression of the rat OC gene. Gel mobility shift assays were performed with the ^{32}P end labeled rOCbox24 using cell nuclear extracts from several rat tissues (liver, brain, and thymus) and cell types (H4 rat hepatoma, HeLa, and MG63 human osteosarcoma cells). In Figure 2 the protein/DNA interaction patterns are shown and compared to the bands which were formed with ROS 17/2.8 cell nuclear extracts. Similarities as well as differences in the protein/DNA interaction patterns are evident. Some of the coincident bands appear to be specific based on competition with native OC box oligonucleotides but not with mutated sequences. However, although other protein-DNA complexes from non

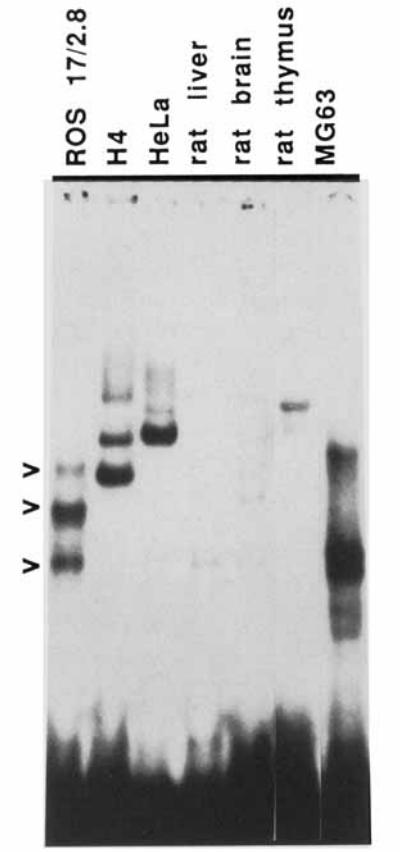

Fig. 2. Tissue- and species-restricted protein/DNA interactions at the rat OC box. Gel mobility shift assays were performed using ^{32}P end labeled rOCbox24 and 5 μg of nuclear extracts obtained from rat tissues and cells (H4, brain, thymus, and liver) and human cervical carcinoma (HeLa) or osteosarcoma (MG63) cells. The electrophoretic migrations of the protein/DNA complexes were compared to those with ROS 17/2.8 cell nuclear extracts.

osseus cells and tissues exhibit similar electrophoretic mobilities and therefore appear to comigrate with one of the ROS 17/2.8 protein/DNA interactions, these bands are competed equally well with the rat OC box or the mutated rat OC box oligonucleotides (data not shown), suggesting they are non-specific. Additionally, we have observed some OC box specific binding of nuclear extracts from rat R2 fibroblasts (data not shown) indicating that the protein-DNA interactions at the OC box are not strictly confined to bone cells. Interestingly, when we assayed nuclear extracts from MG63 human osteosarcoma cells, we detected only minor bands which were specifically competed or comigrated with any of the bands formed with rat osteosarcoma cell (ROS 17/2.8) nuclear extracts. Based on electrophoretic migration and oligonucleotide competition of protein/DNA interactions using nuclear proteins from several different rat tissues and cell types, binding of proteins to the rat OC box may be tissue and species restricted.

Characterization of the OC Box Binding Protein

To initiate identification and characterization of the proteins which bind to the rat OC box, we fractionated nuclear extracts from ROS 17/2.8 cells by a biotin affinity precipitation protocol. A biotinylated DNA fragment which contains rOCbox24 was prepared to use in a protein/DNA binding reaction. The biotinylated DNA fragment complexed with nuclear factors binds tightly to streptavidin and this "sandwich complex" was adsorbed to a biotin cellulose resin. Proteins which did not bind to the rat OC box were eliminated by low salt washes and the DNA binding protein(s) were eluted with high salt. An equivalent of 25 binding reactions was fractionated electrophoretically in a 10% SDS-PAGE gel adjacent to 0.25 μg of unfractionated nuclear extract for comparison (Fig. 3a). In the eluted fraction a single major band was found, whereas multiple bands with comparable intensities were seen in the crude nuclear extract.

Gel mobility shift assays with ^{32}P end labeled rOCbox45 (see Table I) and crude nuclear extracts or the biotin affinity fraction showed that the major protein/DNA complex which was defined and characterized by oligonucleotide competition in crude nuclear extract is also present in the purified fraction based on the electrophoretic mobility of the complex (Fig. 3b). This band was also specifically competed by the rOCbox24, but not by the OC box mutant, rOCbox Mu1.

UV crosslinking analysis was carried out on crude nuclear and DNA affinity purified proteins complexed with the ^{32}P end labeled rOCbox45 oligonucleotide (Fig. 4, left panel). The DNA affinity purified fraction retains DNA binding properties since three bands representing cross linked proteins were observed (Fig. 4, right panel). The three bands comigrate with bands which are also found when we subjected unfractionated nuclear extract to UV crosslink analysis. These bands were UV irradiation-dependent and proteinase K sensitive. The upper band is competed to a greater extent by rOCbox than by the OC box mutant, rOCboxMu1. The two lower bands are apparently non-specific, as they are competed equally well by the wild type or mutated OC box.

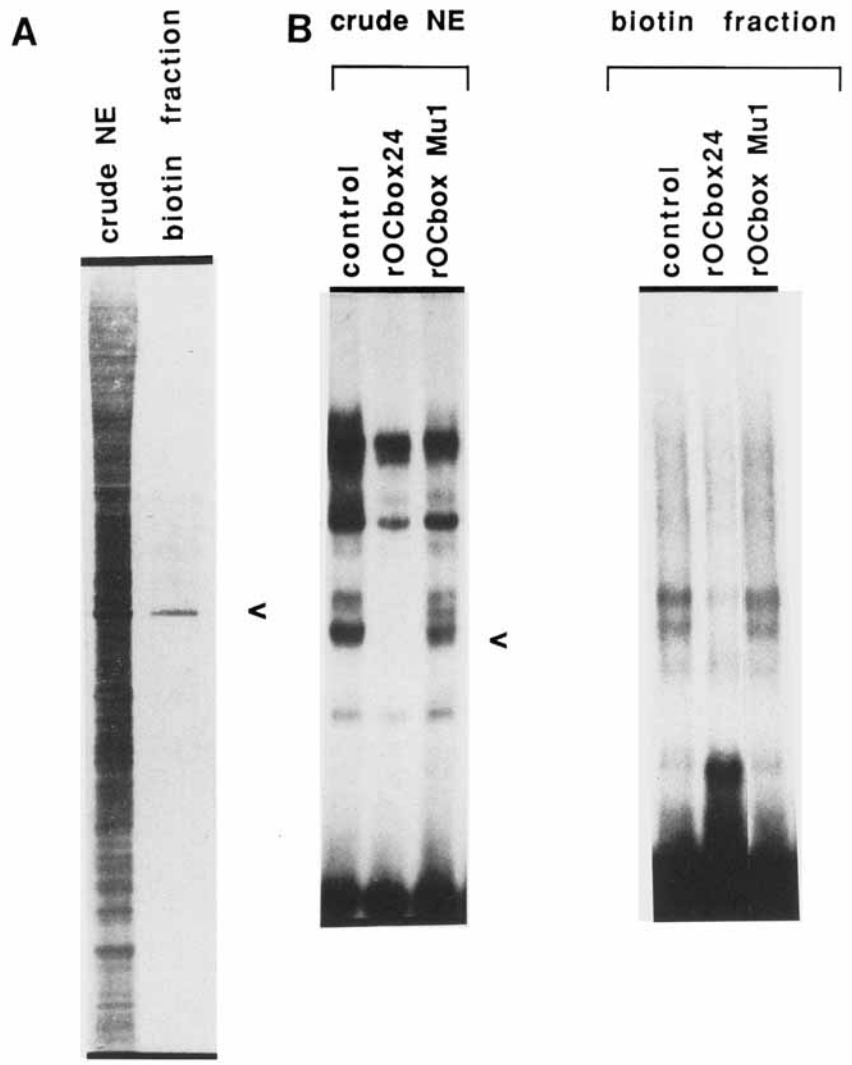

Fig. 3. A DNA affinity fractionated protein exhibits sequence-specific recognition for the rat OC box. **A:** A biotinylated rOCbox24 probe was used to purify an equivalent of 750 DNA binding reactions (b.r.) (=3,750 μg) nuclear extracts from ROS 17/2.8 cells with a single precipitation step. An equivalent of 25 b.r. of the eluate (biotin fraction) was fractionated electrophoretically in a 10% SDS-PAGE gel and compared to 0.25 μg (equivalent to 0.05 b.r.) of unfractionated ROS 17/2.8 cell nuclear extracts (crude NE). **B:** A gel shift assay containing 15 μg of unfractionated nuclear extract (crude NE) (left panel) or an equivalent of 22 b.r. of DNA affinity purified fraction (biotin fraction) (right panel) and rOCbox45 probe. The binding reaction mixture was loaded on a 4% native poly acrylamide gel (20:1). No specific competitor DNA was included in the "control" binding reaction. A 200-fold excess of rOCbox24 and rOCbox Mu1 oligonucleotides was present in the binding reactions, shown in the middle and right lanes. The arrow indicates specific competition.

DNA affinity fractionation of nuclear extracts from ROB cells at different stages of bone cell phenotype development (days 5 and 28; proliferating and post proliferative) were performed using the same approach as for fractionation of ROS 17/2.8 cell nuclear extracts. The developmental representation of the eluted DNA binding proteins and the abundance of the previously described OC box-specific protein were determined by SDS-PAGE gel fractionation of proteins from 5, 10, or 20 protein/DNA binding reactions (Fig. 5). There is significantly more of the OC box protein present in the affinity purified fraction from day 28 ROB cells, compared to the affinity purified fraction from day 5 ROB cells. Qualitative and quantitative changes in OC box related protein/DNA interactions (Fig. 1) and the OC box during differentiation of rat osteoblast cells may indicate that the OC box element and its corresponding trans acting binding factors may contribute to the developmental regulation of OC gene transcription during pro-

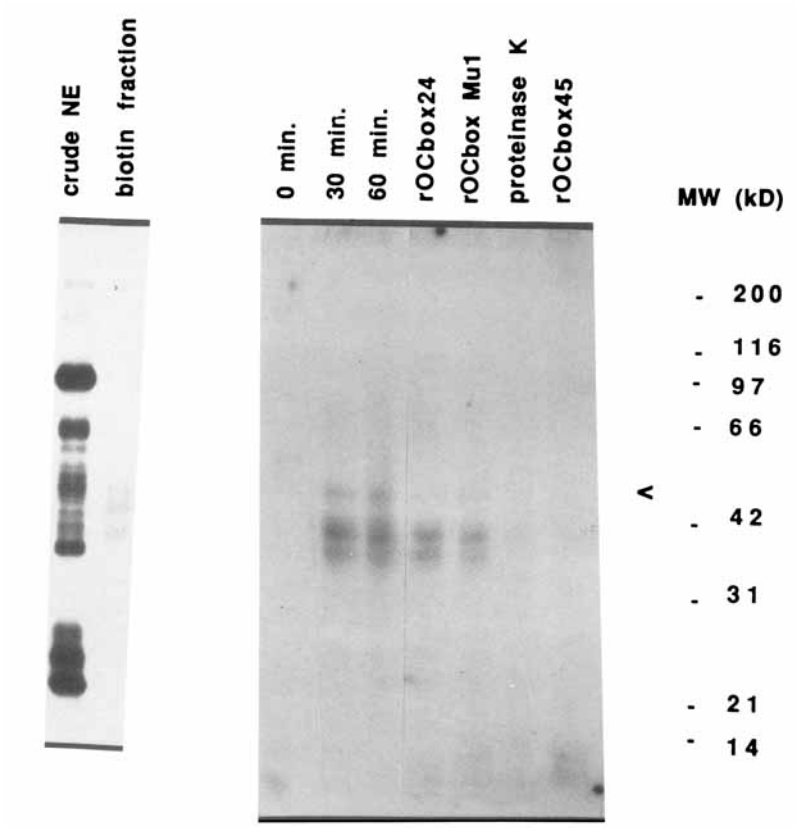

Fig. 4. UV crosslinking of proteins in both unfractionated ROS 17/2.8 cell nuclear extracts and a DNA affinity purified fraction to rOCbox45. A comparison of UV-crosslinked proteins in non-fractionated nuclear extract (crude NE) (3 b.r.) with DNA affinity purified (biotin) fraction (22 b.r.) is shown in the left panel. The samples which were described in Figure 3B (45 μl of the binding reaction mixture) were treated with 5 units DNaseI and 1 unit micrococcal nuclease for 30 min at 37°C and samples were fractionated electrophoretically in a 10% SDS-PAGE gel, stained, dried, and exposed to X-ray film (right panel). Lane 1: no UV irradiation; lane 2: 30 min UV irradiation; lanes 3–7: 60 min UV irradiation. Lane 4: 200-fold molar excess rOCbox24; lane 5: 200-fold molar excess of rOCbox Mu1; lane 6: incubation with 10 μg proteinase K for 10 min.; lane 7: 200-fold molar excess rOCbox45. The arrow indicates a band which is competed specifically by the native but not the mutated OC box sequence.

gressive expression of the rat osteoblast phenotype. DNA-affinity fractionation using the rOCbox24 sequence was also performed using nuclear extracts from non-bone cell types and rat tissues (Fig. 6). The representation of OC box proteins was significantly higher in ROS 17/2.8 cells (Fig. 6) and differentiated ROB cell nuclear extracts (Fig. 5), compared to any of the other cell types and rat tissues examined (Fig. 6).

DISCUSSION

Taken together, these results suggest that the OC box-binding protein in the DNA affinity fraction may play a role in regulating developmental and tissue-restricted expression of the bone specific protein, osteocalcin. Results which are consistent with a tissue-restricted regulatory role for the OC box-binding protein are differences in OC box-specific protein/DNA interactions and differences in the abundance of the OC box binding protein in DNA affinity fractions. These differences were observed when comparing nuclear extracts from the osteocalcin-expressing rat bone cell line ROS 17/2.8 with nuclear extracts from other rat tissues and cell types which do not express osteocalcin. Support for involvement of the OC box binding protein in developmental control of osteocalcin expression is the change in protein/DNA interactions and the increase in representation of the OC box binding protein with the upregulation of osteocalcin expression during osteoblast differentiation.

The DNA binding sequences for proximal promoter regulatory proteins such as CREB protein [Landschultz et al., 1988; Hoeffler et al.,

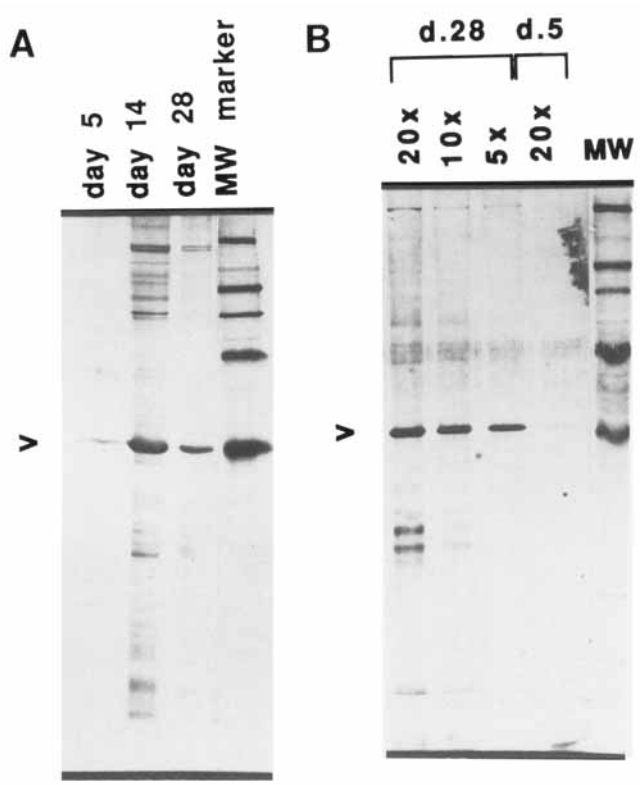

Fig. 5. Representation of the OC-box protein in DNA affinity purified (biotin) fractions from ROB cell nuclear extracts in different stages of osteoblast differentiation. **A:** Biotin fractions were prepared from 250 μg (=50 b.r.) of nuclear extracts from primary cultures of normal diploid rat osteoblasts (day 5 and 28). An equivalent of 5, 10, or 20 b.r. was fractionated electrophoretically in a 10% SDS-PAGE gel and silver stained. **B:** The quantitative difference in developmental regulation of this factor in proliferating (day 5) and differentiated (day 28) osteoblasts is shown by the signals observed when 20x, 10x, and 5x aliquots of day 28 samples are compared with a 20x aliquot of the day 5 sample.

1988; Gonzalez et al., 1989] and NF-Y/CP1/CBF subunit B [Hooft van Huijsduijnen et al., 1990; Chodosh et al., 1988; Maity et al., 1992; Celada and Maki, 1989] are significantly different from the sequences flanking the CCAAT motif in the rat OC box. There is however significant sequence homology of the OC box domain, the core motif, and the flanking regions, with reported homeobox elements [Catron et al., 1993] which may relate to embryonic origin and developmental expression of the osteocalcin gene in bone cells. It therefore appears that we have identified a novel OC box binding protein with a unique DNA binding site (AATTAG) [Heinrichs et al., 1993b]. It remains to be established how the protein/DNA interactions we have identified at the OC box and the manner in which differences in the representation of the OC box-specific protein between proliferating and differentiating rat osteoblasts can contribute to support of basal transcriptional activity and/or participate in steroid hormone-mediated enhancement of osteocalcin gene transcription. However, while such mechanisms remain to be defined, our observations suggest that the OC box-binding protein may be involved in regulation of differential expression of the rat osteocalcin gene during the development of the rat osteoblast phenotype. Indeed, our findings are consistent with recent studies demonstrating that several different factors which bind proximal regulatory elements, such as CCAAT-related proteins, play a role in developmental regulation of transcriptional activity [Ron and Habener, 1992; Flamant et al., 1987; Christy et al., 1989].

We investigated the possibility that the OC box plays a role in tissue-specific expression of the rat osteocalcin gene. The rationale was that osteocalcin is a bone-specific protein and several CCAAT-related proteins are known to be involved in regulation of tissue-specific gene expression [Ron and Habener, 1992; Falb and Maniatis, 1992; Abel et al., 1992; Lopez-Cabrera et al., 1991; Morabita et al., 1991; Uzan et al., 1991; Becker et al., 1991; Zhang et al., 1990;

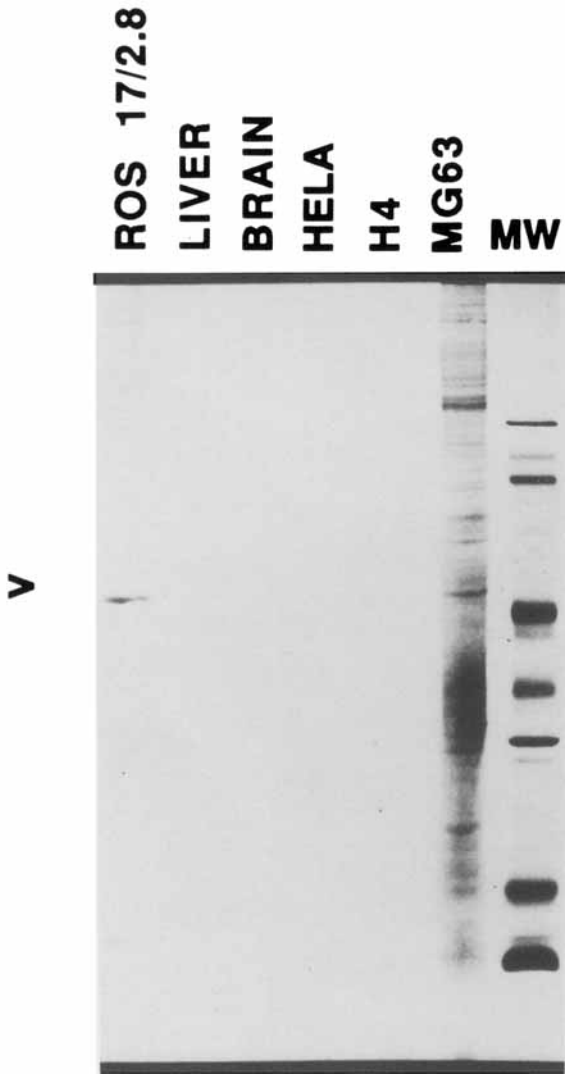

Fig. 6. Tissue- and species-restricted representation of the OC box binding protein in DNA affinity purified (biotin) fractions from rat tissue and other cell types. DNA affinity purified fractions were prepared starting from 250 μg (=50 b.r.) of nuclear extracts from rat tissues and cells (liver, brain, H4), human cervical carcinoma (HeLa), or osteosarcoma (MG63) cells. An equivalent of 10 b.r. was loaded on a 10% SDS-PAGE gel.

Maniatis et al., 1987; Raymondjean et al., 1991; Skalnik et al., 1991]. Representation of the OC box binding protein in rat bone cells (ROS 17/2.8 and differentiating normal diploid osteoblasts) and decreased or non-detectable levels in other rat tissues (brain, liver, and thymus) or other rat derived, non-osseous cells (e.g., H4) support tissue-specific control at least in part residing in the OC box and cognate binding proteins. The absence or low abundance of the OC box protein in bone and non-osseous cells of human origin (e.g., MG63 and HeLa) suggests that this protein may not only be tissue-restricted, but also species restricted. Since the human OC promoter has been shown to be transcriptionally active in ROS 17/2.8 cells [Morrison et al., 1989], it appears that functional interactions of both rat and human OC box factors occur with the human OC gene promoter, yet such cross-species compatibility of OC factors with the rat OC gene promoter are not observed. At the rat OC box there is unidirectional species compatibility of transcription factor-OC box interactions that are paralleled by the relationship of transcription factor binding and promoter activity. That is, the human OC box oligonucleotide does not compete for protein/DNA interactions at the rat OC box with ROS 17/2.8 cell nuclear extracts, whereas rOCbox and hOCbox oligonucleotides compete equally well for the major protein/DNA interactions at the human OC box [Heinrichs et al., 1993b].

Characterization of OC gene promoter sequences and cognate factors, both upstream, downstream, and within the OC box, together with the OC box binding protein, is required to fully define the contribution to tissue-specific expression. Indeed, preliminary data indicate that the CCAAT motif within the OC box is part of a complex, multi-component regulatory system, which in present studies appears to be involved in regulation of expression of the rat OC gene [Heinrichs et al., 1993a]. The requirement for integration of the activities of multiple factors for tissue-specific gene expression has recently been shown for several genes. CHOP-10 (C/EBP homologous *p*rotein) is induced upon differentiation of 3T3-L1 fibroblasts and inhibits its DNA binding activity of C/EBP by formation of a heterodimer [Ron and Habener, 1992]. In the adult fat body a negative regulatory element AEF1 and the positive regulatory factor C/EBP form a tissue-specific regulatory unit which operates by mutually exclusive binding [Falb and Maniatis, 1992]. Our observation of several OC box-specific protein/DNA interactions is consistent with a multi-subunit protein complex which includes an OC box binding protein.

In summary, previously we characterized the rat OC box as a cis acting positive regulating element of the rat OC gene promoter and we showed that the integrity of the central AATTAG motif is crucial for its function. Here we identified an OC box binding protein which may contribute to basal promoter activity and additionally play a role in developmentally regulated and tissue-restricted expression of the rat OC gene.

ACKNOWLEDGMENTS

These studies were supported by NIH grants (AR33920, AR35166, AR42262, and AR39588). We thank A.J. van Wijnen and S. Rahman for helpful discussions during the course of these studies. We are indebted to Ms. Elizabeth Bronstein and Ms. Patricia Jamieson for editorial assistance with the preparation of this manuscript.

NOTE ADDED IN PROOF

One of the OC box binding factors has been identified as an MSX homeodomain protein [Towler et al., 1994].

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