Identification and Characterization of Two Proximal Elements in the Rat Osteocalcin Gene Promoter That May Confer Species-Specific Regulation

A.A.J. Heinrichs, C. Banerjee, R. Bortell, T.A. Owen, J.L. Stein, G.S. Stein, and J.B. Lian

Department of Cell Biology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655

Abstract   The rat osteocalcin gene encodes a 6-kD osteoblast-specific protein that is expressed postproliferatively. The developmental and steroid hormone responsive expression of the osteocalcin gene is transcriptionally regulated by a promoter with multiple basal and enhancer elements that exhibit activity controlled by a series of physiological mediators (e.g., $1,25(OH)_2D_3$, glucocorticoids). In this study, we established the contribution of the rat osteocalcin (OC) box domain (−99 to −76), a proximal basal element with a CCAAT motif as a central core, to transcriptional activity of the rat osteocalcin gene with in vivo co-transfection assays. By this same assay, however, the highly homologous (22 of 24 nt) human OC box element was unable to compete for transcription factor binding with the rat OC promoter. In vitro protein/DNA interaction studies confirm the presence of two protein binding sites in the OC box region, one of which overlaps the CCAAT motif and, at least in part, accounts for species-specific expression. Competition analysis established that the single nucleotide substitution of adenine for thymine, which converts the core motif of the rat OC box (CCAAT) to the core motif of the human OC box (CCAAA), accounts for observed species differences in transcription factor interactions. The CCAAT-specific protein/DNA interactions are heat stable and insensitive to phosphatase treatment. A second protein/DNA interaction located upstream of the CCAAT motif includes two steroid-like half-elements. These interactions are heat labile and sensitive to phosphatase treatment in contrast to the CCAAT-specific interactions. The human OC promoter contains only a single steroid-like half-element, while two steroid half-elements with an 11 nucleotide spacer are present in the rat OC promoter. These observed variations in sequence organization and transactivation factor binding in analogous proximal basal regulatory regions of the OC gene promoter may provide a basis for species-restricted variations in responsiveness to physiological mediators of OC gene expression at the transcriptional level.   © 1993 Wiley-Liss, Inc.

Osteocalcin (OC) is a vitamin K-dependent, 6 kD bone-specific extracellular matrix protein, which binds to hydroxyapatite [reviewed by Lian and Gundberg, 1988; Hauschka et al., 1989]. Numerous studies have established expression of OC as a marker of the mature bone cell phenotype. For example, in primary cultures of fetal rat calvarial osteoblasts which undergo a developmental sequence of gene expression associated with bone cell differentiation, osteocalcin transcription is initiated post-proliferatively and further upregulated at the onset of extracellular matrix mineralization in vitro [Owen et al., 1990a; Aronow et al., 1990]. In vivo studies [Yoon et al., 1988] and in situ hybridization of bone tissue [Weinreb et al., 1990; Pockwinse et

al., 1992] show osteocalcin expression in mature osteoblasts at the bone surface.

The developmental and bone tissue-specific expression of the OC gene is transcriptionally regulated [Owen et al., 1993; Bortell et al., 1993] by a promoter with a modular organization of multiple regulatory elements that mediate both steroid hormone responsive and basal transcriptional control, e.g., vitamin D responsive element [Markose et al., 1990; Terpening et al., 1991; Demay et al., 1990], glucocorticoid responsive element [Morrison et al., 1989; Heinrichs et al., in press] and a CCAAT containing OC box [Lian et al., 1989]. A silencer element that contributes to repression of OC gene transcriptional activity has additionally been described within the mRNA coding region of the rat OC gene [Frenkel et al., 1993].

TABLE I.

	-120	-99	-76
rOCbox45	5'TGGGTT<u>TGACC</u>TATTGCGCACA<u>TGACCCCC</u>AATTAGTCCTGGCAG 3'		
rOCbox32	5'TGGGTTT<u>TGACC</u>TATTGCGCACA<u>TGACCCCC</u>AA 3'		
rOCbox24		5' A<u>TGACCCCC</u>AATTAGTCCTGGCAG 3'	
rOCbox Mu 1		5' A<u>TGACCCC</u>**gtg**TTAGTCCTGGCAG 3'	
rOCbox Mu 2		5' A<u>TGACCCCC</u>AA**a**TAGTCCTGGCAG 3'	
rOCbox Mu 3		5' A<u>TGACCCCC</u>AATTAG**c**CCTGGCAG 3'	
hOCbox		5' A<u>TGACCCCC</u>AAATAGCCCTGGCAG 3'	
Histone H3II	5'GATCTCACAGAGATGGA<u>CCAAT</u>CCAAGAGGG 3'		

Steroid half-elements (TGACC) and CCAAT and CCAAA motifs are underlined; mutations are shown in bold, lower-case letters.

In this study, we addressed the contribution of the OC box (−99 to −76) to basal promoter activity and characterized the protein/DNA interactions that occur at this regulatory element and within flanking sequences. The concept of the OC box (−99 to −76 in the rat OC gene and −121 to −98 in the human OC gene) as a putative promoter element was initially based on a striking homology (conservation of 22 out of 24 nts [Lian et al., 1989]) between this promoter domain in the human and rat osteocalcin genes. Sequences within the OC box include consensus for AP-1 and steroid responsive elements [Owen et al., 1990b; Theofan et al., 1989]. In the present experiments, in vitro protein/DNA interactions analysis established that there are two distinct protein binding sites in the OC box domain. One site is located primarily outside the OC box in a region that contains two perfect, identical steroid-like half elements. The other site, which resides within the OC box, overlaps the CCAAT motif and appears to account for the difference in protein/DNA interactions and in vivo co-transfection competitions between the homologous rat and human OC box elements.

MATERIALS AND METHODS

Tissue Culture and Preparation of Nuclear Extracts

Ros 17/2.8 rat osteosarcoma cells, a gift from Drs. Gideon and Sevgi Rodan (Merck, Sharp & Dohme), or MG63 human osteosarcoma cells (ATCC, CRL 1427)) were grown to confluence in F12 medium supplemented with 5% FCS (fetal calf serum). Nuclear extracts were prepared according to the method of Dignam et al. [1983], modified by Holthuis et al. [1990].

Gel Mobility Shift Assays

[32]P end-labeled probes were prepared using T4 poly nucleotide kinase (New England Bio-

labs, Beverly, MA). The oligonucleotides that were used as a probe or as competitor are described in Table I. Gel mobility shift assays were performed as described previously [Markose et al., 1990] under the following conditions for these studies: 2 μg polydGdC-polydGdC and a final concentration of 50 mM KCl were used with rOCbox24 and hOCbox probes; 2 μg polydGdC-polydGdC and 1 μg of polydAdT-polydAdT and a final concentration of 90 mM KCl were used in binding reactions with the rOCbox45 as a probe. Specific competitor oligonucleotides were used in 200 fold molar excess. Sweet potato phosphatase (Sigma Chemical Co., St. Louis, MO) incubations were carried out at 37°C for 15 min.

Methylation Interference

Methylation interference was performed as described [Ausubel et al., 1989], and the chemical DNA sequencing method was described by Maxam and Gilbert [1977].

Plasmids and Constructs

The target plasmid for in vivo competition studies consisted of rat osteocalcin 5' promoter fragment (−1,097 to +23) placed upstream of the CAT gene and cloned into pGEM 7zf(+). Two or three copies of the oligonucleotides (in direct repeats) (Table I) were cloned into pUC19 to produce the competitor plasmids. rOCbox24 had 3 copies each, and hOCbox had 2 copies.

Co-Transfection Experiments

ROS 17/2.8 cells plated at a density of 4×10^5 cells/100 mm plate were used for transient co-transfection experiments by the DEAE-Dextran method [Ausubel et al., 1989]. Co-transfections were performed with pOCZ-CAT (1 μg) as the target plasmid and the cloned oligonucleotides (12–14 μg) as the competitors. The total DNA was maintained at 15 μg/plate and pUC19 was

used to make up that amount when required. Control cells were co-transfected with pOCZ-CAT (1 μg) and 14 μg of pUC19 (because it constitutes the backbone of cloned oligonucleotides).

Cells were subjected to glycerol shock 3 h post-transfection, then incubated in F12 supplemented with 5% fetal calf serum and 10^{-8} M $1,25(OH)_2D_3$. Cells were harvested after 60 h and CAT activity determined as an indicator of in vivo competition.

CAT Assays

Chloramphenicol acetyltransferase (CAT) activity was determined as previously described by Ausubel et al. [1989]. The samples were incubated with 0.25 μCi of ^{14}C-chloramphenicol (Dupont, Boston) for 10–12 h. After ethyl acetate extraction, the samples were subjected to chromatography on TLC plates (Whatman Labsales, Hillsboro, OR). The plates were exposed to Kodak X-OMAT.AR film. CAT activity was quantified by photodensitometry.

RESULTS

Proximal OC Gene Promoter Elements Influence Basal Transcription In Vivo

Promoter deletion studies involving transfection of a series of OC gene promoter–chloramphenicol acetyl transferase gene constructs into ROS 17/2.8 osteosarcoma cells revealed that the initial 103 nts of the promoter, which includes the OC box, supports basal transcriptional activity (Fig. 1A). The OC box containing construct (−103 to +23) consistantly showed greater transcriptional activity than the full length promoter (−1,097 to +23). To determine the contribution of the rat OC box to basal promoter activity in vivo, a series of competition-cotransfection experiments were carried out. pOCZ-CAT (−1,097 to +23) [Schepmoes et al., 1991] was transfected into ROS 17/2.8 cells with either the native rat OC box oligonucleotide (rOCbox24) or the human OC box (hOCbox) oligonucleotide cloned in pUC19 as competitors (Table I). Figure 1 shows that a 28-fold molar excess of rOCbox24 abolished basal transcription (Fig. 1B,D). Interestingly, the human OC box oligonucleotide [Kerner et al., 1989] did not compete at the same molar excess (Figs. 1C,D). This result indicates that the CCAAT motif contributes significantly to activity of this regulatory element in the rat OC box, since the human OC box does not contain an intact CCAAT motif. Furthermore, co-transfection of the OC promoter-reporter construct with equal molar excess of rOCbox Mu1 containing a mutated CCAAT motif did not compete basal promoter activity (Fig. 1E). Thus, the CCAAT motif of the rat OC box is involved in regulation of basal OC transcription.

### Species Specificity of the CCAAT(A) OC Box Binding Proteins

The in vivo competition studies showed that a construct containing the oligonucleotide hOCbox did not compete for transcriptional activity of the rOC gene promoter construct pOCZCAT (Fig. 1C). Thus, the specificity of the protein/DNA interactions with either the rat OC box or the human OC box was studied by gel mobility shift assays using appropriate competitors. [^{32}P]-end-labeled rOCbox24 with ROS 17/2.8 cell nuclear extracts revealed a CCAAT specific protein/DNA interaction pattern that cannot be competed by high molar excess of hOCbox (Fig. 2A, left). The hOCbox probe with ROS 17/2.8 cell nuclear extracts gives rise to a very different diffuse protein/DNA interaction pattern (Fig. 2A, right). The protein/DNA interactions appear to be rather nonspecific since rOCbox24 and hOCbox compete equally effectively. As a comparison, the protein/DNA interactions that were formed with MG 63 human osteosarcoma cell nuclear extracts, using either [^{32}P]-end-labeled rOCbox or hOCbox, are distinct from protein/DNA complexes formed with nuclear proteins from ROS 17/2.8 cells. However, the major protein/DNA complexes are similar with respect to the two probes (Fig. 2B). Interestingly, a minor complex is observed with the rOCbox24 probe that is specifically competed by itself (but not by mutant rat or human OC box oligonucleotides) suggesting sequence-specific interactions with the CCAAT motif by nuclear proteins from the human MG 63 cells.

It therefore appears that despite the striking homology, the rat OC box and the human OC box are cis elements with unique factor recognition specificities. The 2-nt difference between the rat OC box and the human OC box appears to be responsible for the protein/DNA interactions in this promoter domain. Two rat OC box mutant oligonucleotides were therefore designed, each carrying only one mutation that corresponds with either of the two differences between the rat and the human OC boxes;
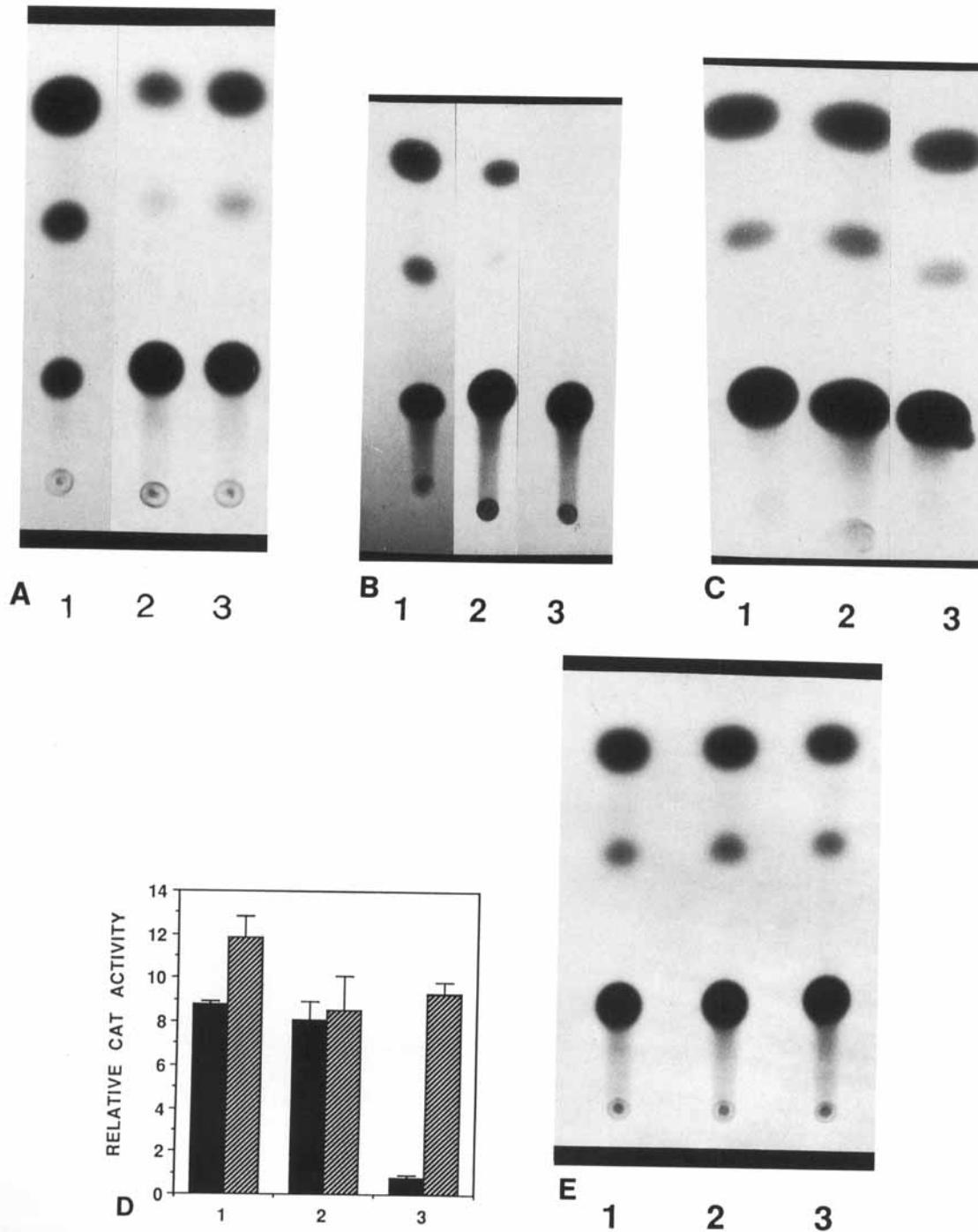
Fig. 1. In vivo assessment of the contribution of OC box sequences to transcriptional activity of the OC gene. **A:** ROS 17/2.8 osteosarcoma cells were transfected with 10 µg of chimeric gene constructs consisting of three different promoter segments fused to a CAT reporter: the SV40 promoter **(lane 1)**, the −1,097 to +23 segment of the OC promoter **(lane 2)** and the −103 to +23 segment of the OC promoter **(lane 3)**. **B–E:** ROS 17/2.8 cells were co-transfected with 1 µg of a chimeric gene construct consisting of the −1,097 to +23 segment of this OC gene fused to a CAT reporter **(lane 1)** and 12 µg **(lane 2)** or 14 µg **(lane 3)** of a specific competitor oligonucleotide cloned in pUC19 and analyzed by CAT assays. **B:** Representative autoradiogram of CAT assays where co-transfection was with rOCbox 24 DNA (see Table I). **C:** Representative CAT assay where co-transfection was with hOCbox DNA (see Table I). **D:** Quantitation of the effect of the competitor plasmids on rat osteocalcin promoter expressed as CAT activity relative to the control. Solid bar is for rOCbox and shaded bar is for hOCbox. The values represent mean + S.D. of relative CAT activities from 5 (for rOCbox) or 2 (for hOCbox) independent experiments, each performed in duplicate. Panel E shows a representative CAT assay where co-transfection was with rOCbox Mul DNA (see Table I).
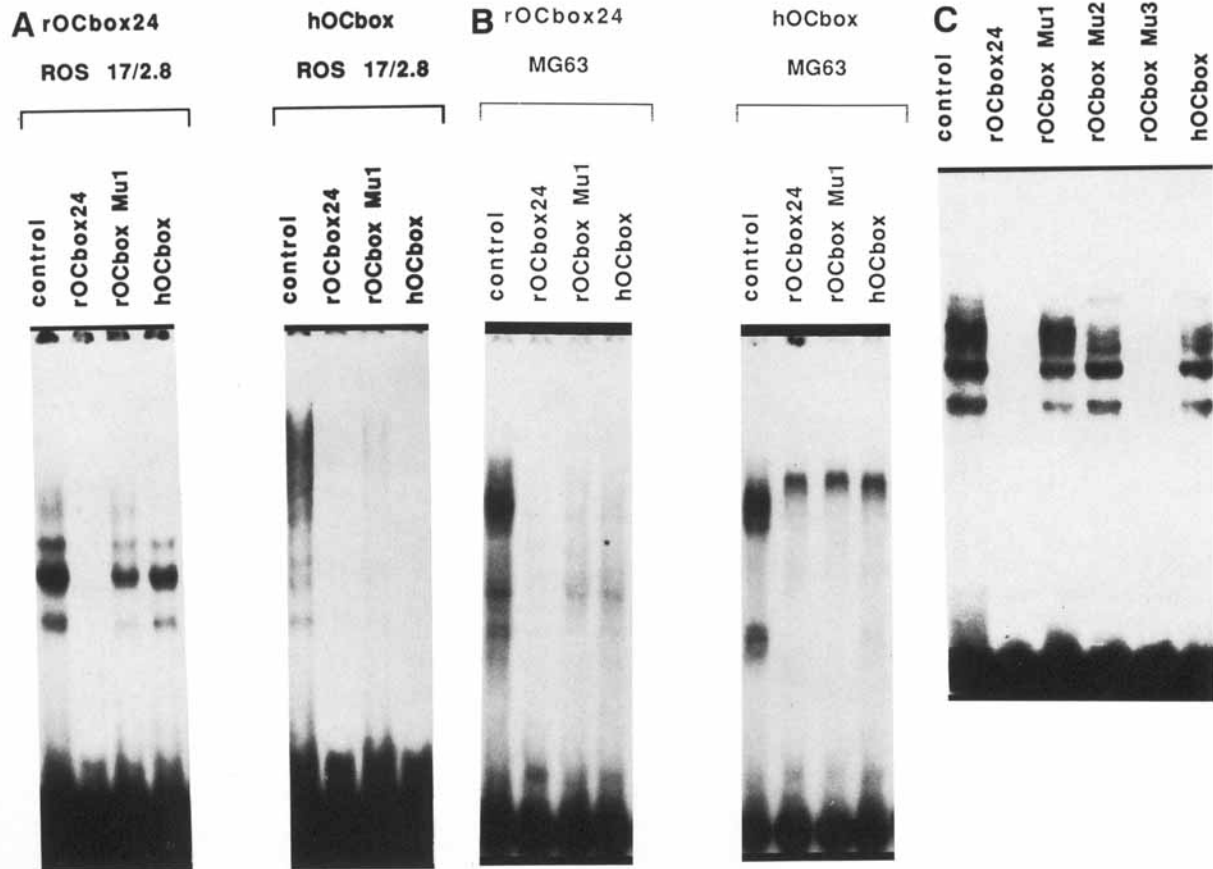
Fig. 2. Comparison of protein/DNA interactions using either [32P]-end-labeled rOCbox24 or hOCbox DNA with ROS 17/2.8 or MG63 cell nuclear extracts. **A:** Gel mobility shift assays with either [32P]-end-labeled rOCbox24 or hOCbox DNA and 5 μg ROS 17/2.8 rat osteosarcoma nuclear extracts were performed. Control assays did not contain competitor DNA. A 200-fold molar excess of rOCbox24, rOCbox Mu1 and hOCbox oligonucleotides was included in protein/DNA binding reactions. **B:** Gel mobility shift assays with either [32P]-end-labeled rOCbox24 or hOCbox DNA and 5 μg MG63 human osteosarcoma nuclear extracts were performed. Competitions were done as in A. **C:** Competition studies were done using 200-fold molar excess of several oligonucleotides containing the mutated rat OC box: rOCbox Mu1, rOCbox Mu2, rOCbox Mu3 and also hOCbox. rOCbox24 DNA was used as a probe with 5 μg of ROS 17/2.8 cell nuclear extracts. Control assays did not contain competitor DNA.

rOCbox Mu2 has an adenine residue instead of a thymidine residue at −88, whereas rOCbox Mu3 has a cytosine residue instead of a thymidine residue at −84 (Table I). Gel mobility shift competition experiments with the rOCbox24 probe and ROS 17/2.8 cell nuclear extracts were performed (Fig. 2C). rOCbox Mu2 did not compete, as observed for hOCbox and rOCbox Mu1. However, rOCbox Mu3 competes as effectively as rOCbox24. Apparently, the dramatic differences in protein/DNA interactions between the highly homologous human and rat OC boxes are determined by a single base pair difference at −88; the thymidine residue of the CCAAT motif.

Distinct Protein/DNA Interactions in the OC Box Domain

Further examination of sequences in the OC box region indicated the presence of potential steroid hormone receptor binding domains. The rOCbox32 construct includes a 32-bp oligonucleotide corresponding to the 21-bp sequence immediately upstream of the rat OC box and 11 bp of the distal segment of the rat OC box (Table I). Each of these regions contains an identical steroid-like half-element motif (TGACC). The potential role of these steroid-like elements in modulating OC expression is consistent with a previous report of a DNase I footprint spanning and immediately upstream of the OC box (−113 to −85) [Markose et al., 1990] when the osteocalcin gene is actively transcribed.

To characterize mechanisms contributing to activity of the OC box domain and sequences immediately upstream of the OC box, protein/DNA interactions were studied. We synthesized an oligonucleotide designated rOCbox45 that includes the 24-bp rat OC box and the contigu-
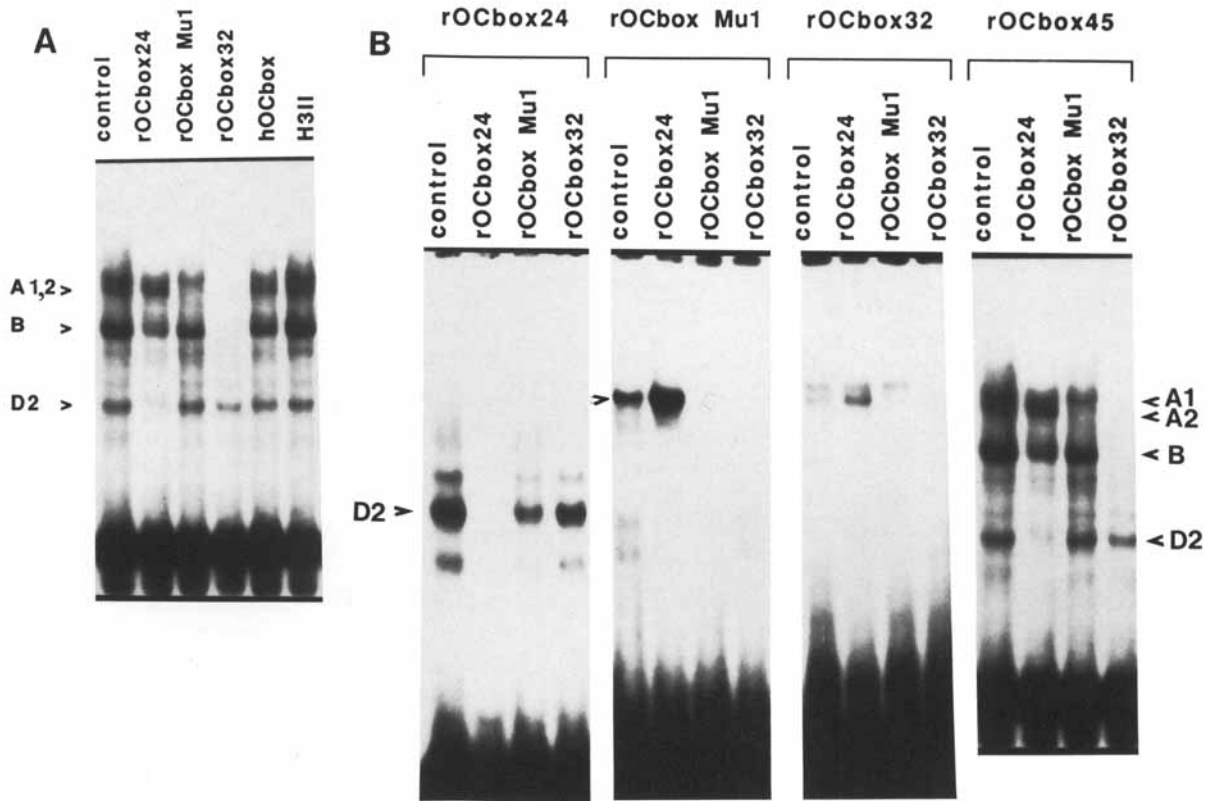
Fig. 3. Identification of protein/DNA interactions in the OC box region of the OC gene promoter by electrophoretic mobility shift analysis in the presence of wild-type or mutated OC box competitor oligonucleotides. A: Gel mobility shift assays were performed with 5 μg of ROS 17/2.8 cell nuclear extract and [^{32}P]-end-labeled rOCbox45 DNA, using a 200-fold molar excess of the following nucleotides: rOCbox24, rOCbox Mu1, rOCbox32, hOCbox and H3II. Sequences of the oligonucleotides are listed in Table I. The control assay was carried out in the absence of competitor. Bands A$_{1,2}$ and B represent protein/DNA interactions which can be competed by rOCbox32. D$_2$ represents a CCAAT-specific protein/DNA interaction that is competed by a 200-fold molar excess of rOCbox 24 and not by the same excess of rOCbox Mu1. B: In cross-competition experiments, we used [^{32}P]-end-labeled rOCbox24, rOCbox Mu1, rOCbox32, and rOCbox45, and the same unlabeled oligonucleotides were used as competitors in 200-fold molar excess. Bands A$_{1,2}$, B, and D$_2$ are as in A.

ous 21 bp upstream of the OC box (Table I). When we assayed binding of ^{32}P-end-labeled rOCbox45 to factors in nuclear extracts from ROS 17/2.8 osteosarcoma cells by gel mobility shift analysis, we identified two sets of bands (Fig. 3A); CCAAT specific bands and steroid-like bands. Three protein/DNA complexes, two minor bands and a major band designated D$_2$ are competed with 200-fold excess of rOCbox24 and not with the same amount of the mutated CCAAT box sequence rOCbox Mu1, which is the basis for concluding that these bands are specific for the OC box CCAAT motif. However, H3II, which is a CCAAT containing oligonucleotide (from a histone H3 promoter) with a binding site for a CCAAT binding protein that is thought to be related to the CCAAT binding protein CP1/NF-Y [van Wijnen et al., 1991], does not compete for any of the CCAAT specific protein/DNA interactions in the OC gene proximal promoter (Fig. 3A).

In contrast, the slower migrating, upper bands A$_1$, A$_2$, B and the lower minor band are not competed by rOCbox24, indicating that additional sequences are required for their formation. Indeed, when we used rOCbox32 (Table I) as a competitor (not containing an intact CCAAT element), the bands A$_1$, A$_2$, B, and the lower, minor band were competed and the three CCAAT-specific bands were unaffected. Since rOCbox32 includes the two steroid responsive half elements, it appears that the bands which were competed by rOCbox32 are "steroid related." Interestingly, A$_2$ was competed by rOCbox Mu1. Apparently, with the CAA → GTG substitution in rOCbox Mu1, we created a steroid-related binding site. Immediately upstream of the OC box we find the sequence GTG (-102 to -100), which is included in rOCbox32 (Table I).

The protein/DNA interactions within the rOCbox45 region were further investigated by

gel mobility shift analysis, comparing several smaller OC box probes (Fig. 3B). [32P]-End-labeled rOCbox24 revealed a protein/DNA interaction pattern that represented only D2 and its two flanking, minor bands, which competed the CCAAT motif specifically. The steroid related bands were absent, indicating that all protein/DNA interactions at the rat OC box are related to binding at the CCAAT element. [32P]-End-labeled rOCbox32 DNA gave rise to protein/DNA interactions that were all competed by rOCbox32 and none of the bands were competed by rOCbox24. rOCbox Mu1 as a probe does not show any CCAAT specific bands, but yields a rOCbox32 competable band that co-migrates with the A_2 band. rOCbox45 as a probe yields both CCAAT-specific and steroid-related protein/DNA interactions. Again, A_2 was competed by rOCbox Mu1 as in Figure 3A. These results indicate that there are protein/DNA interactions that occur at least partially outside the OC box. Further, we can exclude the possibility of mutually exclusive binding of trans-acting factors to the CCAAT-specific or steroid-related cis elements since rOCbox45 reveals concomitant CCAAT-specific and steroid related protein/DNA interactions.

Nucleotide Specific Protein/DNA Interactions at Proximal Promoter Cis-acting Elements

We examined the major protein/DNA interactions at the 45-bp OC box region at single nucleotide resolution by methylation interference analysis. We analyzed D_2, the dominant CCAAT specific band and found that the two adenine residues in the sense strand showed interference (Fig. 4A). Surprisingly, the two guanine residues in the antisense strand that correspond to the two cytosine residues of the CCAAT motif did not exhibit interference. However, the interference at the adenine and guanine residues downstream of the CCAAT motif show that the flanking regions are also important for recognition by this trans-acting protein.

Since the bands A_1 and A_2 were competed differently by rOCbox Mu1 (Fig. 3B), we compared their respective methylation interference patterns. Methylation interference of A_1 and A_2 revealed interference at adenine and guanine residues of both steroid responsive half elements (Fig. 4B). The same nucleotides show interference for both protein/DNA interactions, but the extent of interference differs; the interference of a guanine residue at -97 is more pronounced for A_1 than for A_2, whereas two

guanine residues at -102 and -100 show more interference for A_2 than for A_1. These two guanine residues are part of the GTG sequence that was created when the CCAAT motif was mutated in rOCbox Mu1. This result may explain why A_2 is competed by rOCbox Mu1, while A_1, which has slightly different protein/DNA interactions, is unaffected by rOCbox Mu1.
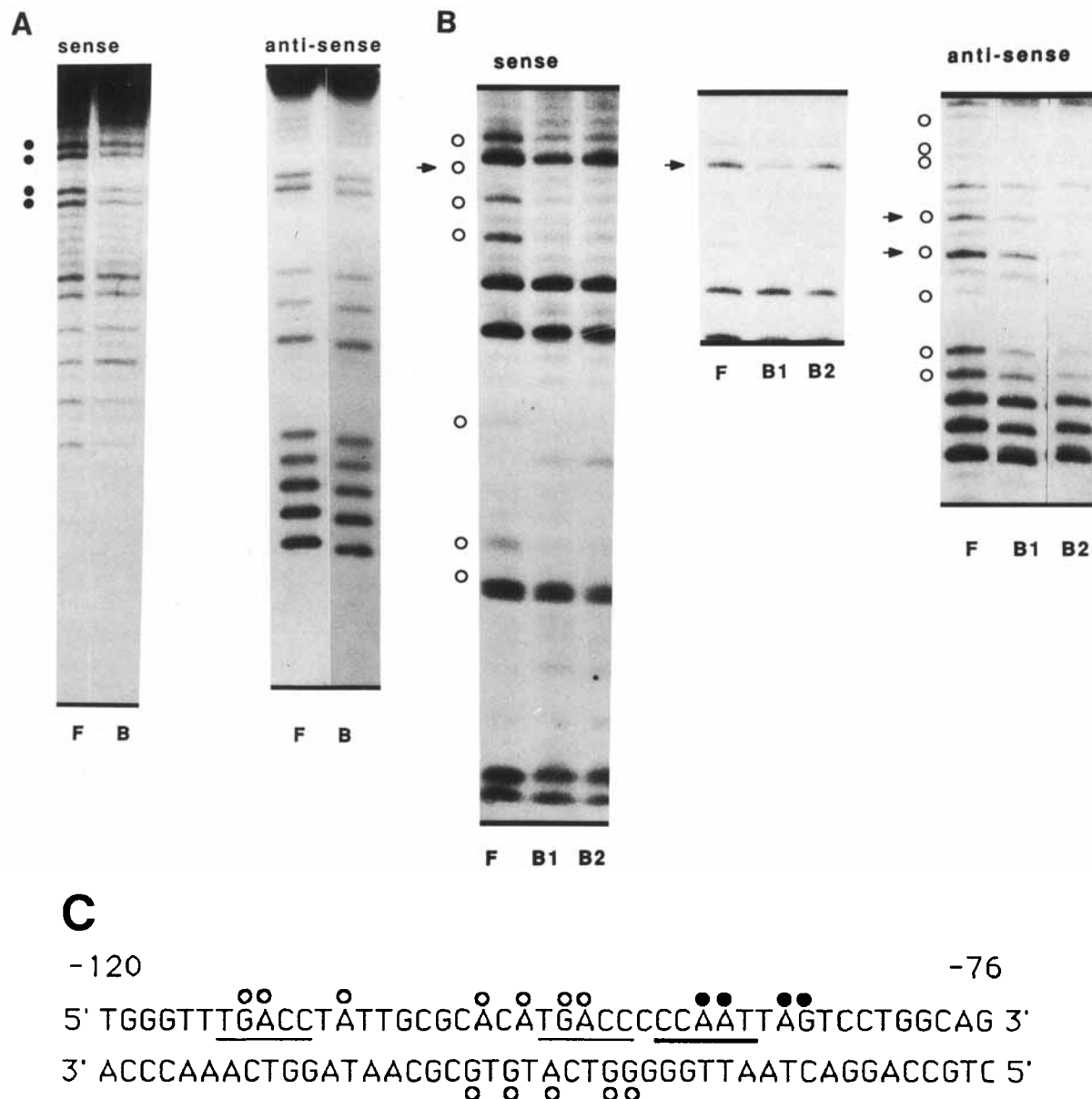
Differential Temperature Lability and Phosphatase Sensitivity of Protein/DNA Interactions in the OC Box Domain

To further characterize the dominant protein/DNA interactions A_1, A_2, B, and D_2 using [32P]-end-labeled rOCbox45, we determined if these protein/DNA interactions were selectively affected by temperature. The steroid-related bands A_1, A_2, and B are heat sensitive, whereas D_2 is more heat stable (Fig. 5A).

The protein/DNA interactions were also distinguishable by their sensitivity to phosphatase treatment. The rOCbox32 probe (which reveals the A_1 and A_2 bands) was used with phosphatase-treated cell nuclear extracts in protein/DNA studies. These phosphatase experiments (Fig. 5B) revealed that the representation of A_1 decreased progressively with increasing amounts of sweet potato phosphatase, suggesting that dephosphorylation diminishes DNA binding affinity, thereby decreasing and/or preventing formation of protein/DNA complexes. The protein/DNA interactions at this rOCbox32 appear, at least in part, to be phosphorylation-dependent. No changes were observed in CCAAT-specific interactions (band D_2) following phosphatase treatment (data not shown). Since the D_2 band and its 2 minor flanking bands (described in Fig. 3B) cannot be distinguished by specific competition nor by heat or phosphatase treatments, the possibility is raised that each of these bands represents a (multisubunit) protein/DNA complex, each containing at least one common subunit, a CCAAT-specific binding protein.

DISCUSSION

We identified two cis acting elements in the OC box region of the rat osteocalcin promoter. One cis element is located in the OC box and contains the CCAAT motif within its binding site. The trans-acting factor that binds this element is necessary for expression from the rat osteocalcin promoter as demonstrated by in vivo competition co-transfection assays. The thymidine residue of the CCAAT motif is the determinant for observed differences in protein/DNA

C

-120   -76

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      oo      o        o  o  oo              ••    ••
5' TGGGTTTGACCTATTGCGCACATGACCCCCAATTAGTCCTGGCAG 3'

3' ACCCAAACTGGATAACGCGTGTACTGGGGGGTTAATCAGGACCGTC 5'
              o   o   o      oo
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Fig. 4. Methylation interference analysis of sequence-specific protein/DNA interactions at the CCAAT element and OC box flanking sequences in the OC gene promoter. Methylated, asymmetrically [^{32}P]-end-labeled rOCbox45 DNA and ROS 17/2.8 cell nuclear extracts were used in a binding reaction which was scaled up 2-fold. 10 binding reactions were fractionated electrophoretically in a 4% 20:1 polyacrylamide gel. Selected protein/DNA complexes were electroeluted from excised segments of the gels. Methylation interference analysis was performed as described in Materials and Methods. A: The sequence of rOCbox45 with guanine and adenine residue contact sites for band D$_2$. B: For bands A$_1$ and A$_2$. Protected guanine and adenine residues are indicated by solid circles for band D$_2$ and open circles for bands A$_1$ and A$_2$. In B, the short middle panel is a lighter exposure of the upper region of the sense autoradiogram to verify the G residue contacts. Arrows indicate differences in the extent of DMS interference between bands A$_1$ and A$_2$ at nucleotides −97 in sense strands and −100 and −102 in the antisense strand. F, free probe; B, probe bound to protein in band D$_2$; B1, probe bound to protein in band A$_1$; B2, probe bound to protein in band A$_2$. C: Summary of the methylation interference pattern is shown with open circles indicating interactions of the A$_1$ and A$_2$ complexes and solid circles indicate protein/DNA contact sites resulting from complex D$_2$.

interactions between the highly homologous human and rat OC box elements. The second cis-acting element is not CCAAT specific and overlaps a region immediately upstream of the OC box that contains two steroid-like half-elements.

However, it remains to be determined which steroid receptor binding sequence resides 5' to the CCAAT motif.

Although the steroid-like half-elements in the region of the OC box (two TGACC motifs with
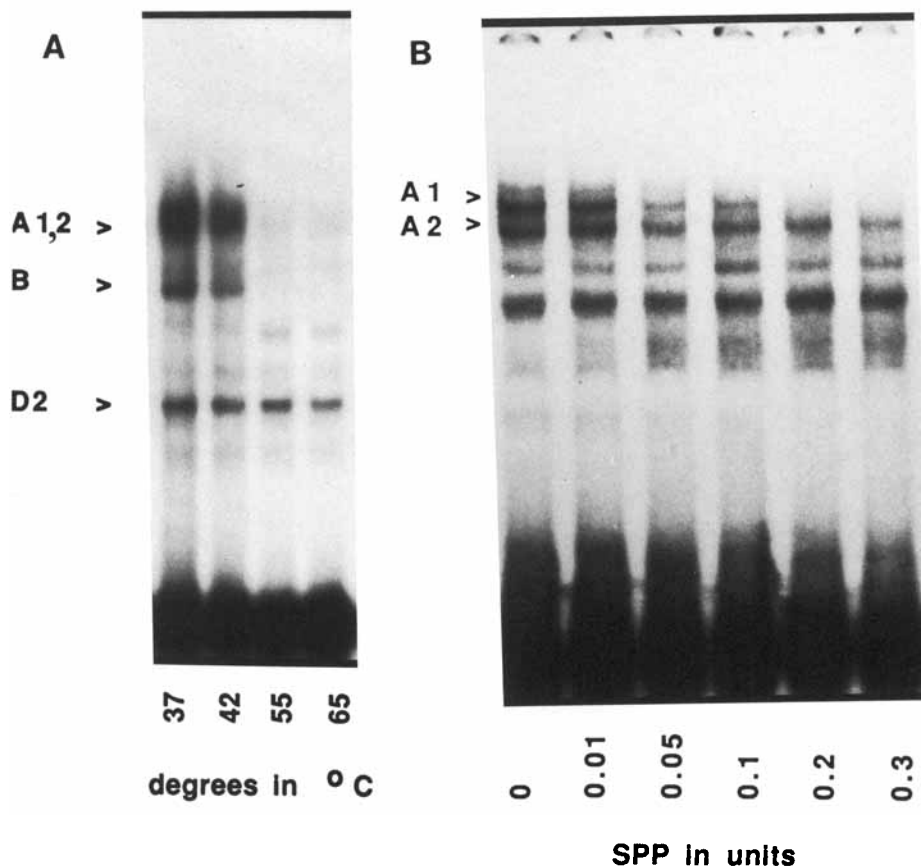
Fig. 5. Effect of heat treatment and incubation with sweet potato phosphatase (SPP) on protein/DNA interactions within the OC box region (-120 to -76) of the rat OC gene promoter. **A:** The effect of temperature on the stability of the different protein/DNA interactions determined using [^{32}P]-end-labeled rOCbox45 DNA and 5 µg of ROS 17/2.8 cell nuclear extracts; (a) the binding reaction as followed by heating the reaction mixture to 37°C, 55°C or 65°C for 1 min, (b) the binding reactions were carried out at 37°C, 42°C, 55°C, or 65°C for 20 min; (c) the nuclear extracts were heat treated for 5 min, centrifugated for 5 min, and the supernatant was used in a

binding reaction mixture. Since no differences were observed for the three heat-treatment protocols, only the resulting protein/DNA interactions obtained by method c are shown. Bands A_1, A_2, B, and D_2 are indicated with arrows. **B:** Increasing amounts of sweet potato phosphatase (SPP) (0–0.3 units) were preincubated with ROS 17/2.8 cell nuclear extracts for 15 min at 37°C. The samples were maintained on ice for 5 min and then the binding reaction mixture including [^{32}P]-end-labeled rOCbox32 DNA, competitor DNA and DTT was incubated for 10–15 min at room temperature. A_1 and A_2 are indicated with arrows.

11-bp spacer) exhibit some homology to a VDRE, none of the protein/DNA interactions were mediated by the vitamin D receptor based on the absence of competition by either VDRE oligonucleotides or vitamin D receptor antibodies (data not shown). Additionally, gene constructs containing the rat OC box but lacking the VDRE are not responsive to 1,25(OH)$_2$D$_3$ [Morrison et al., 1989; Yoon et al., 1988; Demay et al., 1990, Terpening et al., 1991]. Recently, we identified glucocorticoid receptor (GR) binding sites in the proximal rat OC gene promoter; a DNase I footprint overlapping the OC box and methylation interference of guanine residues were observed in the element. However, the protected region and the guanine residues that showed interfer-

ence did not correspond to either of the steroid-like half-elements [Heinrichs et al., in press].

A retinoic acid responsive element is proposed based on the sequence of the two half-elements and their spacing. A TGACC motif separated by 3–15 nucleotides is typical for a retinoic acid responsive element. Interestingly, in the human oxytocin promoter 4 TGACC motifs are necessary for optimal RA induction. The TGACC motif at -113 was separated by 15 nt from a TGACC motif at -83 that is overlapped by a CCAAT box [Richard and Zingg, 1991]. The pentamer motif is also related to estrogen and thyroid hormone responsiveness, since TGACC is found in the right half of most EREs and THREs [Beato, 1989]. However, the spacing for

EREs and THREs are 3 and 4, respectively. In addition to observed sequence homologies with retinoic acid responsive elements in the OC gene promoter, retinoic acid is known to modulate the levels of OC [Evans et al., 1988; Nishimoto et al., 1987]. Retinoic acid may also potentiate the actions of $1,25(OH)_2D_3$ by inducing the vitamin D receptor, and in addition it has been shown that retinoic acid and $1,25(OH)_2D_3$ independently affect osteocalcin expression, suggesting that multiple regulatory mechanisms are operative. Additionally, the VDRE in the human OC gene confers responsiveness to retinoic acid and the retinoic acid receptor was shown to bind to the VDRE [Schüle, 1990].

While the proximal promoters of the rat and the human OC genes are homologous, the human OC promoter does not contain two identical TGACC motifs separated by 11 nt in the region of the OC box; only the downstream steroid responsive half-element located within the OC box is present. It remains to be determined whether the promoter domain contiguous to and upstream of the CCAAT binding site that contains the putative, steroid-like elements is involved in differential rat OC gene expression.

This study suggests that the CCAAT binding site and its corresponding trans-acting factor are involved in basal and species-specific transcription activity. The basis for the homology of the rat and the human OC box is not understood, since a single bp substitution results in very different protein/DNA interactions. However, the stringent requirement for the thymidine residue in the CCAAT motif within the rat OC box for rat versus human CCAAT factor binding suggests specific characteristics for both the element and cognate binding proteins.

Another distinctive property of this binding factor is the lack of methylation protection at the CC of the CCAAT motif. Our data show protection at the AA as well as the AG 2 nucleotides further downstream. This is not typical for the class of CCAAT box binding proteins which exhibit protection at the GG (corresponding to the CC) and the AA [Chodosh et al., 1988]. Indeed, our DMS protection data suggest that the AATTAG motif is the recognition site of the rat OC box binding protein. A recent study proposes a motif of (C/G)TAATTG for binding by three different murine homeodomain proteins [Catron et al., 1993], which is exactly homologous with the 3' orientation of the rat OC box motif. A role for the OC box binding factor as a

potential homeobox protein is consistent with studies in our laboratory which suggest that the rat OC box and its cognate trans-acting factor also play a role in tissue-specific as well as developmentally regulated transcriptional control [Heinrichs et al., in press].

The presence of multiple regulatory elements with overlapping transcription factor binding domains in the proximal promoter of the rat osteocalcin gene reflects the complexity of basal transcriptional control. Such complexity in the organization of regulatory sequences together with the potential for integration of activities at independent elements supported by chromatin structure and nucleosome organization and nuclear matrix studies [Bortell et al., 1992; Bidwell et al., 1993] provides the basis for responsiveness to a diverse series of physiological regulatory signals.

ACKNOWLEDGMENTS

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