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Variations in Vitamin D Receptor Transcription Factor Complexes Associated With the Osteocalcin Gene Vitamin D Responsive Element in Osteoblasts and Osteosarcoma Cells

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Abstract Vitamin D responsive transcription of the bone-specific osteocalcin gene differs markedly in osteosarcoma cells and normal diploid osteoblasts. In osteoblasts the osteocalcin gene is transcribed, and upregulated by Vitamin D, only in post-proliferative cells, but in osteosarcoma cells expression is constitutive. This distinction in transcriptional regulation of the osteocalcin gene correlates with striking differences in the relative representation of two principal Vitamin D-dependent protein/DNA complexes designated V1 and V2 at the Vitamin D responsive element in the osteocalcin promoter. Formation of both complexes is Vitamin D dependent and they contain the Vitamin D receptor as well as an RXR related protein. Pore size exclusion and sedimentation velocity analyses suggest that the V1 and V2 complexes represent oligomeric protein assemblies (respectively, tetramers and trimers), and reflect primarily DNA-directed association of the monomeric protein components at the osteocalcin Vitamin D responsive element. UV crosslinking and methylation interference analyses of the V1 and V2 complexes at the osteocalcin Vitamin D responsive element indicate differences in protein/DNA recognition. For example, the V1 complex interacts with both steroid half-elements, whereas the V2 complex appears to recognize the proximal half-element. Our findings suggest variations in protein/protein and protein/DNA interactions of the VDR and RXR related complexes V1 and V2 at the osteocalcin *Vitamin D* responsive element that reflect unique properties of the osteosarcoma and normal diploid osteoblast phenotype.

Key words: osteosarcoma cells, osteocalcin gene, osteoblasts, vitamin D response element (VDRE), transcription factor complexes

INTRODUCTION

Osteocalcin gene expression is restricted to the post-proliferative period of bone cell phenotype development in normal diploid osteoblasts [Owen et al., 1990a, 1991]. This bone-specific gene is aberrantly expressed in proliferating osteosarcoma cells, where interrelationships between growth and differentiation are abrogated; however, expression is significantly increased following the downregulation of proliferation. In both cell types, transcription of the osteocalcin gene is responsive to a broad spectrum of physiologic mediators, including steroid hormones and growth factors, which act at multiple cis-acting elements [Yoon et al., 1988; Morrison et al., 1989; Demay et al., 1989; Kerner et al., 1989; Markose et al., 1990; Schule et al., 1990; Terpening et al., 1991; Shalhoub et al., 1992; Wiese et al., 1992; Heinrichs et al., 1993; Frenkel et al., 1993; Li and Stashenko, 1993; Breen et al., in press; Frenkel et al., in press; reviewed in Stein et al., 1990; Rodan and Noda, 1991; Darwish and DeLuca, 1993]. Developmental responsiveness of osteocalcin gene transcription to vitamin D has been well documented both in normal diploid osteoblasts and osteosarcoma cells that exhibit expression of mature osteoblast phenotypic properties [Bortell et al., 1993; Owen et al., 1993].

Fundamental to vitamin D-responsive transcriptional control of the osteocalcin gene is an

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understanding of interactions of the vitamin D receptor complex with other promoter binding factors at the vitamin D responsive element (VDRE). It has been demonstrated that the vitamin D receptor, together with RXR and potentially other transcription factors, interacts with the VDRE in a manner that may be functionally related to the level of transcription [Yu et al., 1991; Ross et al., 1992a; MacDonald et al., 1993; Bortell et al., 1992, 1993; Ozono et al., 1990; Schrader et al., 1993; Leid et al., 1991]. Mutation and deletion analyses by several laboratories have established that the steroid half elements, as well as the spacer and the flanking sequences contribute to vitamin D responsive transcription of the osteocalcin gene [Terpening et al., 1991; Demay et al., 1992; Schrader et al., 1993; Nishikawa et al., 1993]. Particularly significant is the observation that there are striking differences in the vitamin D receptor transcription factor complexes at the osteocalcin VDRE in normal diploid osteoblasts and in osteosarcoma cells [Bortell et al., 1993].

To gain further insight into regulatory mechanisms that operate in steroid hormone control of osteocalcin gene transcription, we have initiated characterization of protein-DNA interactions at the osteocalcin gene VDRE in normal diploid osteoblasts and osteosarcoma cells. Our results indicate that a high molecular weight vitamin D receptor complex predominates in ROS 17/2.8 cells while a lower molecular weight vitamin D receptor complex predominates in normal diploid osteoblasts. Both VDRE complexes contain the vitamin D receptor and RXR. The higher molecular weight complex contacts the VDRE at both steroid half elements. In contrast, the lower molecular weight complex contacts only the proximal steroid half element. These findings are consistent with variations of steroid hormone transcriptional control in the normal diploid osteoblast compared to phenotypic characteristics of osteosarcomas.

MATERIALS AND METHODS

Cell Cultures

Rat osteosarcoma ROS 17/2.8 cells (gift of Dr. G. and Dr. S. Rodan, Merck, Sharp and Dohme, West Point, PA) were maintained as described by Majeska et al. [1985]. Normal diploid osteoblasts (ROB) were isolated from fetal rat calvaria and maintained as described by Aronow et al. [1990] and Owen et al. [1990a]. Cells were treated with 10^{-8} M 1,25-dihydroxy vitamin D3 (gift of M. Uskokovic, Hoffman-LaRoche, Nutley, NJ) for 24 h prior to harvest. Osteocalcin protein secreted into the culture medium was quantitated by radio-immune assay [Gundberg et al., 1984].

Nuclear Protein Extraction

Soluble nuclear proteins from Vitamin D treated ROB and ROS 17/2.8 cells were prepared by extraction of isolated nuclei with high salt buffer (0.42 M KCl, 25% glycerol, 0.2 mM EDTA, 1 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, and 25 mM Hepes, pH 7.5) as described previously [van den Ent et al., 1993; van Wijnen et al., 1991a] in the presence of a broad spectrum protease inhibitor cocktail. After removal of nuclei, extracts were frozen in liquid N_2 and stored directly at −70°C. Desalting of nuclear protein preparations was accomplished by dilution with 3 vol of KN0 buffer (20% glycerol, 0.2 mM EDTA, 0.01% Nonidet P-40, and 25 mM Hepes/NaOH, pH 7.5) per volume of nuclear extract immediately before each experiment. Final adjustments in protein concentrations were made by the addition of calculated amounts of KN100 buffer (as KN0 buffer, but also containing 100 mM KCl).

Gel Shift Assays

Gel shift assays were performed as described previously [Bortell et al., 1993; van Wijnen et al., 1991b]. The rat osteocalcin Vitamin D responsive element (OC-VDRE; nt −467/−439) [Owen et al., 1990b] used as probe (specific activity of approximately 4,000 cpm/femtomole) was purified and labelled with T4 polynucleotide kinase and ^{32}P-γ-ATP [Ausubel et al., 1987]. Binding reactions were prepared by combining a 10 μl DNA solution (containing 10 femtomole probe DNA, 2 μg poly (dI-dC)*poly (dI-dC), and 2 mM DTT) with 10 μl of protein solution diluted in KN100 buffer. Protein/DNA complexes were resolved in 4% polyacrylamide (40:1 acrylamide:methylene-bisacrylamide) gels in 1 × TGE-buffer [Ausubel et al., 1987]. After electrophoresis at 200 V for 2 h, gels were dried, and the complexes visualized by autoradiography.

Competition assays were performed by inclusion of a 100-fold molar excess (1 picomole per reaction; approximately 20 ng DNA) of specific oligonucleotides [Owen et al., 1990a,b] spanning the rat OC-VDRE and human OC-VDRE, a mutant rat OC-VDRE, or DNA fragments spanning AP-1 and CCAAT-box sequences as indicated in

the figure legends. Immuno-reactivity of gel shift complexes was assessed with monoclonal antibodies directed against the porcine intestinal Vitamin D receptor. These antibodies either cross-react with both the rat and porcine Vitamin D receptor (antibody IVG8C11), or recognize only the porcine Vitamin D receptor (antibody XVIE6E6G10) [Dame et al., 1986]. These antibody preparations were kindly provided by Dr. H. DeLuca (University of Wisconsin, Madison, WI) and were used in gel shift assays as described previously [Bortell et al., 1993].

Methylation Interference and UV Crosslinking Analyses

Methylation interference analysis [Pauli et al., 1990; Ausubel et al., 1987] of Vitamin D receptor protein/DNA complexes was performed by preparative gel shift assays with rat OC-VDRE probes that were labeled on either the top or the bottom strand, and partially methylated by dimethylsulphate treatment. Gel shift complexes were transferred to positively charged NA45 membranes (Schleicher and Schuell, Keene, NH) followed by autoradiography and elution of relevant bands. Samples were treated with piperidine, and the chemical degradation products were analyzed on a 12% sequencing gel.

UV crosslinking analysis [Ausubel et al., 1987] was performed with the following modifications. OC-VDRE protein/DNA complexes were irradiated with UV (310 nm) in the gel after electrophoretic separation. As control, we performed UV cross-linking with binding reactions containing a mutant OC-VDRE probe. After localization of the OC-VDRE complexes by autoradiography, the corresponding gel segments of the wildtype and mutant probes were excised and the cross-linked complexes were eluted. Eluates were complemented with Mg^{2+} and Ca^{2+}, followed by treatment with DNase I and micrococcal nuclease. The samples were concentrated by centrifugal filtration (Centricon-10; Amicon, Beverly, MA), and analyzed by SDS-PAGE. The molecular weight of the cross-linked proteins was determined by imaging analysis (GDS 2000 Gel Documentation system, Ultra-Violet Products, Inc., San Gabriel, CA) of auto-radiograms, by comparing the relative migration of cross-linked product with those of Coomassie-stained marker proteins.

The UV crosslinking probe was prepared by annealing a short primer-sequence (5′dCGCTTTCGG; UVP-1) with a single-stranded DNA template spanning the wildtype or mutant OC-VDRE sequences, followed by extension with Klenow polymerase (New England Biolabs, Beverly, MA) in the presence of BrdUTP and ^{32}P-α-dCTP. The template oligonucleotides were as follows (primer sequences are underlined, mutations are in italics and underlined): wildtype OC-VDRE (XOV-1) = 5′dCTGCACTGGGTGAATGAGGACATTA*CCGAAAGCG;* mutant OC-VDRE (XOM-2) = 5′dCTGCACTG*TA*TGAATGA*CT*ACATTA*CCGAAAGCG.*

Pore-Size Exclusion and Sedimentation Velocity Analysis

Pore-size exclusion electrophoresis was performed by subjecting gel shift reactions to electrophoresis in gradient polyacrylamide gels (4 to 20%) for 24 h. As markers we used SDS-denatured protein standards that were run in parallel. Gels were subjected to both Coomassie staining and autoradiography. The apparent molecular weights of protein/DNA complexes were estimated based on the relative migration of the marker proteins.

Sedimentation velocity analysis was performed by layering concentrated nuclear extracts (400 μl; 4–8 μg/μl protein) onto 11 ml linear 10 to 30% glycerol gradients containing 100 mM KCl, 0.2 mM EDTA, and 25 mM Hepes/NaOH (pH 7.5). Samples were subjected to centrifugation in a Beckman L8M ultra-centrifuge using the SW41 rotor at 41,000 rpm for 36 h at 4°C. Gradient fractions (400 μl) were manually collected from the top and analyzed by gel shift assays [as described above, but in the presence of 50 ng poly (dI-dC)*poly (dI-dC) non-specific competitor DNA]. Native molecular weight markers with known sedimentation coefficients (Combithek, Boehringer, Indianapolis, IN) were run in parallel gradients and analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

Different Protein/DNA Complexes at the Vitamin D Responsive Element of the Osteocalcin Promoter in Osteoblasts and Osteosarcoma Cells

Two principal protein/DNA complexes are formed when Vitamin D treated nuclear proteins from osteosarcoma cells (ROS 17/2.8) and normal diploid rat osteoblasts (ROB) are assayed by gel shift assays using the osteocalcin

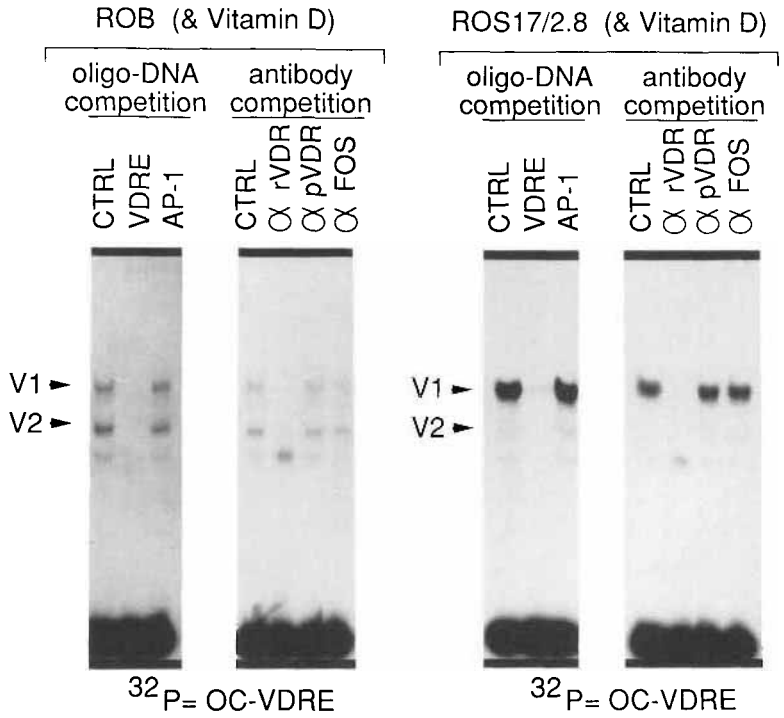

Fig. 1. Different protein/DNA interactions at the osteocalcin Vitamin D responsive element in osteoblasts and osteosarcoma cells. The gel shift assays depicted here were performed with nuclear protein (approximately 5 μg) from Vitamin D treated osteosarcoma ROS 17/2.8 cells and normal diploid osteoblasts (ROB) (designated controls, CTRL) using the OC-VDRE as probe. With both cell types, two protein/DNA complexes (designated V1 and V2) are detected that differ in relative representation in each cell type. The first and third panel from left show specificity of the interactions by oligonucleotide competition with inclusion of a 100-fold molar excess of the unlabelled OC-VDRE (1 pmole, 20 ng), but not with an AP-1 related DNA fragment. The second and fourth panel show antibody competition experiments verifying the presence of the Vitamin D receptor in both complexes V1 and V2, as was documented by Bortell et al. [1993]. The following antibody preparations were used: ctrl, no antibody; α-rVDR, IVG8C11-antibody recognizing the rat and porcine VDR; α-pVDR, XVIE6E6G10-antibody recognizing only porcine VDR; and α-FOS, a commercially available FOS-antibody.

Vitamin D responsive element (OC-VDRE) as a probe (Fig. 1). However, there is a striking difference in the relative representation of the lower and higher mobility complexes designated, respectively, V1 and V2. In normal diploid osteoblasts, the V1 and V2 complexes are equally represented, whereas in ROS 17/2.8 osteosarcoma cells the V1 complex predominates. In both cell types the V1 and V2 complexes are strongly enhanced upon Vitamin D treatment and are barely detectable in untreated control cells (Bortell et al., 1993 and data not shown).

Sequence-specificity of the V1 and V2 protein/DNA interactions is demonstrated by oligonucleotide competition with the OC-VDRE DNA fragment, but not with an AP-1 sequence (Fig. 1). In osteoblasts and osteosarcoma cells both V1 and V2 contain the Vitamin D receptor. The V1 and V2 complexes are immunoreactive with antibody preparation IVG8C11 which recognizes both rat and porcine VDR, but not with the XVIE6E6G10 antibody which recognizes only porcine VDR. Addition of the rat Vitamin D receptor antibody abolishes complexes V1 and V2 due to inhibition of Vitamin D receptor binding to the OC-VDRE, as has been established previously [Bortell et al., 1993]. The specificity of the antibody characterization of complexes V1 and V2 is further confirmed by inability of a c-FOS antibody to influence gel shift patterns.

Apart from the Vitamin D receptor, both V1 and V2 complexes in osteosarcoma cells and normal diploid osteoblasts include Retinoid X receptor (RXR) related proteins. This has been demonstrated previously by Bortell et al. [1992, 1993] who characterized the same two complexes in parallel with both VDR and RXR-specific antibodies [Dame et al., 1986; Leid et al., 1991] using gel shift assays. Similar to observations with the Vitamin D receptor antibody, the V1 and V2 complexes reacted quantitatively with the RXR antibody. Therefore, the V1 and V2

complexes reflect VDR and RXR containing heteromeric transcription factors which exhibit variations in representation in normal diploid osteoblasts and osteosarcoma cells.

The extent to which the different representation of the V1 and V2 complexes in normal diploid osteoblast and osteosarcoma cells reflects inhibitory factors and/or stoichiometric differences in the presence of intrinsic components was assessed (Fig. 2). Gel shift assays were carried out with increasing concentrations of a 1:1 mixture of nuclear proteins from both ROB and ROS 17/2.8 cells. For comparison, we performed binding reactions with identical concentrations of nuclear proteins from either ROB or ROS 17/2.8 cells alone. The data indicate that pre-incubation of normal diploid osteoblast with osteosarcoma derived nuclear proteins does not result in diminished levels of either the V1 or the V2 complex. This result suggests the absence of inhibitory activities in either preparation that could impinge on the formation of the V1 and V2 complexes. We also observe that the extent of complex formation for V1 and V2 is similar to that expected from the additive contributions of the components. The absence of inhibitory or synergistic effects on formation of the V1 and V2 complexes in mixing experiments with ROS 17/2.8 and ROB nuclear proteins suggests that the V1 and V2 protein/DNA complexes with the osteocalcin Vitamin D responsive element have unique intrinsic properties that reflect phenotypic differences in normal diploid osteoblasts and osteosarcoma cells.

Phosphorylation Requirements of Vitamin D Receptor Complexes at the Osteocalcin Vitamin D Responsive Element

We evaluated the contribution of phosphorylation to formation of the V1 and V2 protein/DNA complexes at the osteocalcin Vitamin D responsive element (Fig. 3). Nuclear protein from ROS 17/2.8 cells was incubated with increasing amounts of protein phosphatase (λ-phosphatase). The results clearly demonstrate that formation of both V1 and V2 complexes is decreased or abolished by phosphatase treatment depending on the phosphatase concentration. This finding indicates that complexes V1 and V2 are phosphorylation dependent. Previously, we have shown that incubation of nuclear proteins with a limited amount of a different phosphatase (sweet potato [SP] phosphatase) results in apparent conversion of the V1 complex into the

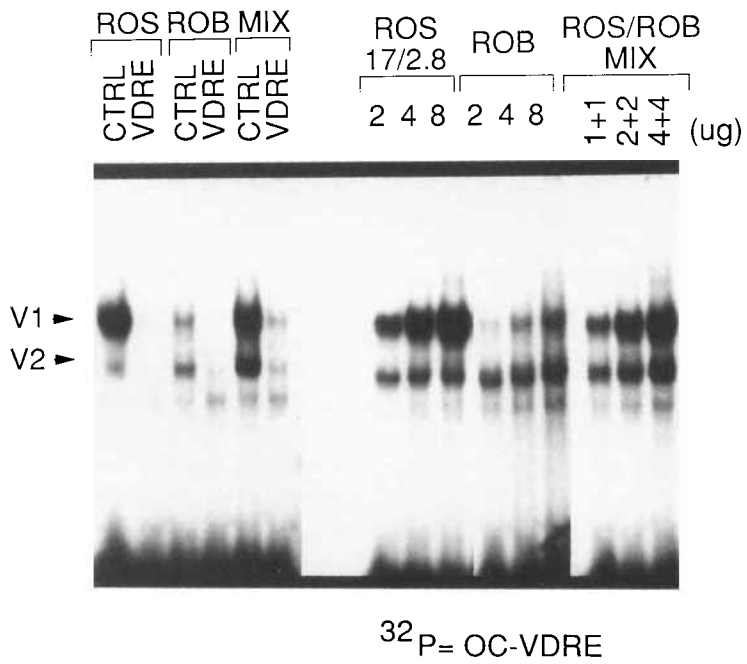

Fig. 2. Additive contributions of nuclear proteins from osteosarcoma and osteoblast to formation of OC-VDRE complexes V1 and V2. Formation of complexes V1 and V2 was assessed with increasing amounts of nuclear protein (right portion, μg amounts above the lanes) from either Vitamin D treated ROS 17/2.8 or ROB cells alone, or increasing amounts of a 1:1 mixture of proteins from both cell types. The OC-VDRE was used as a probe and oligonucleotide competition (left portion) was performed as described in Figure 1.

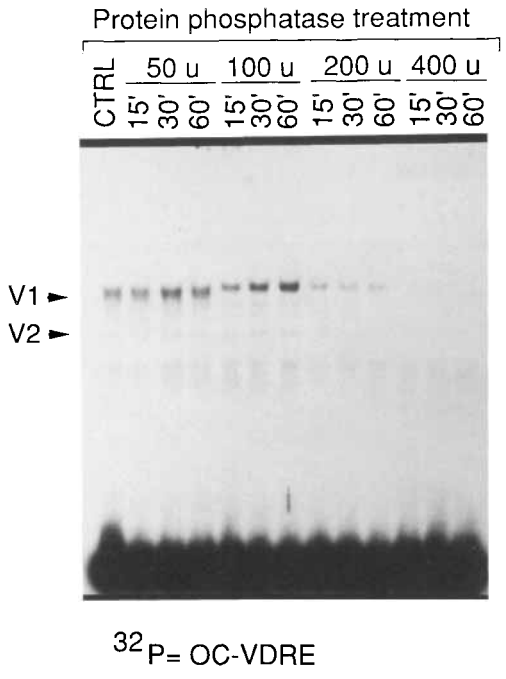

Fig. 3. OC-VDRE complexes V1 and V2 are sensitive to dephosphorylation. Nuclear protein from Vitamin D treated ROS 17/2.8 cells was pre-incubated with increasing units (u) of λ-phosphatase for the indicated periods (vertically depicted in minutes), followed by gel shift assays with the OC-VDRE probe. The control lane (CTRL) represents pre-incubation of protein in λ-phosphatase buffer (New England Biolabs, Beverly, MA) without enzyme. The decrease in signal of the V1 and V2 complexes was evaluated as a function of phosphatase concentration relative to the control.

V2 complex [Bortell et al., 1993]. The λ-phosphatase used in this study is selective for phosphoserine and phospho-threonine residues, and displays activity towards phospho-tyrosines only at high concentrations. In contrast, SP phosphatase is a broad spectrum phosphomonoesterase. Catalytic differences in the two enzymatic activities may account for the qualitative differences in phosphatase sensitivity observed for complexes V1 and V2. In addition, the different results obtained with SP phosphatase [Bortell et al., 1993] and λ-phosphatase (Fig. 3) raise the possibility that two kinetically distinct (groups of) phosphorylated amino-acids may exist that mediate competency for the interactions of the Vitamin D receptor complexes with the osteocalcin Vitamin D responsive element.

Sedimentation Velocity Analysis of Vitamin D Receptor Complexes in Osteosarcoma Cells and Normal Diploid Osteoblasts

To address the molecular structure of the Vitamin D receptor complexes, we analyzed nuclear proteins from ROB (Fig. 4A) and ROS 17/2.8 (Fig. 4B) cells by centrifugal sedimentation in glycerol gradients. The resultant gradient fractions for both cell types were assayed by gel shift assays, and detection of complexes V1 and V2 in positive fractions was confirmed by competition analysis (data not shown). Analysis of nuclear proteins from normal diploid osteoblasts indicates that the V2 complex is mediated by proteins that show a broad peak of activity with a relative sedimentation coefficient of approximately 3.6S(±0.4 S). The low sedimentation coefficient suggests that the V2 complex is composed of 42–60 kD factors. Because the molecular weights of the VDR and RXR fall within the same size range [approximately 55 kD for both receptors; Burmester et al., 1988; Leid et al., 1991], this finding suggests that the Vitamin D receptor and Retinoic X receptor co-migrate as monomeric units through the glycerol gradient. Based on this result, formation of the V2 complex appears to be related to DNA-directed assembly of the monomeric components on the osteocalcin Vitamin D response element.

Nuclear proteins from osteosarcoma cells are resolved into two widely separated peaks in glycerol gradients. Similar to observations with ROB nuclear protein, there is a major peak with a relative sedimentation coefficient of 3.6 S (±0.4 S), but there is also a significant minor peak migrating at 9.8 S (±0.6 S). Furthermore, both peaks mediate primarily the low mobility V1 complex. These results suggest that the V1 complex, similar to the results discussed above for the V2 complex, involves assembly of slowly sedimenting monomeric subunits on the OC-VDRE. However, detection of the 9.8 S molecular species indicates that the V1 complex includes a high molecular weight protein/protein complex (190–220 kD). This protein/protein complex may represent a tetrameric arrangement of the VDR and RXR, as well as perhaps other factors.

Interestingly, with ROS 17/2.8 cells the 3.6 S fraction (containing monomeric receptors) mediates formation of the V1 complex, whereas in ROB cells the 3.6 S fraction mediates primarily formation of V2. These observed differences reflect variations in the competency of the VDR and related proteins to form distinct complexes on the DNA, which may result directly from differences in the molecular properties (e.g., phosphorylation-status) of the monomeric protein components. In addition, the nuclear pro-

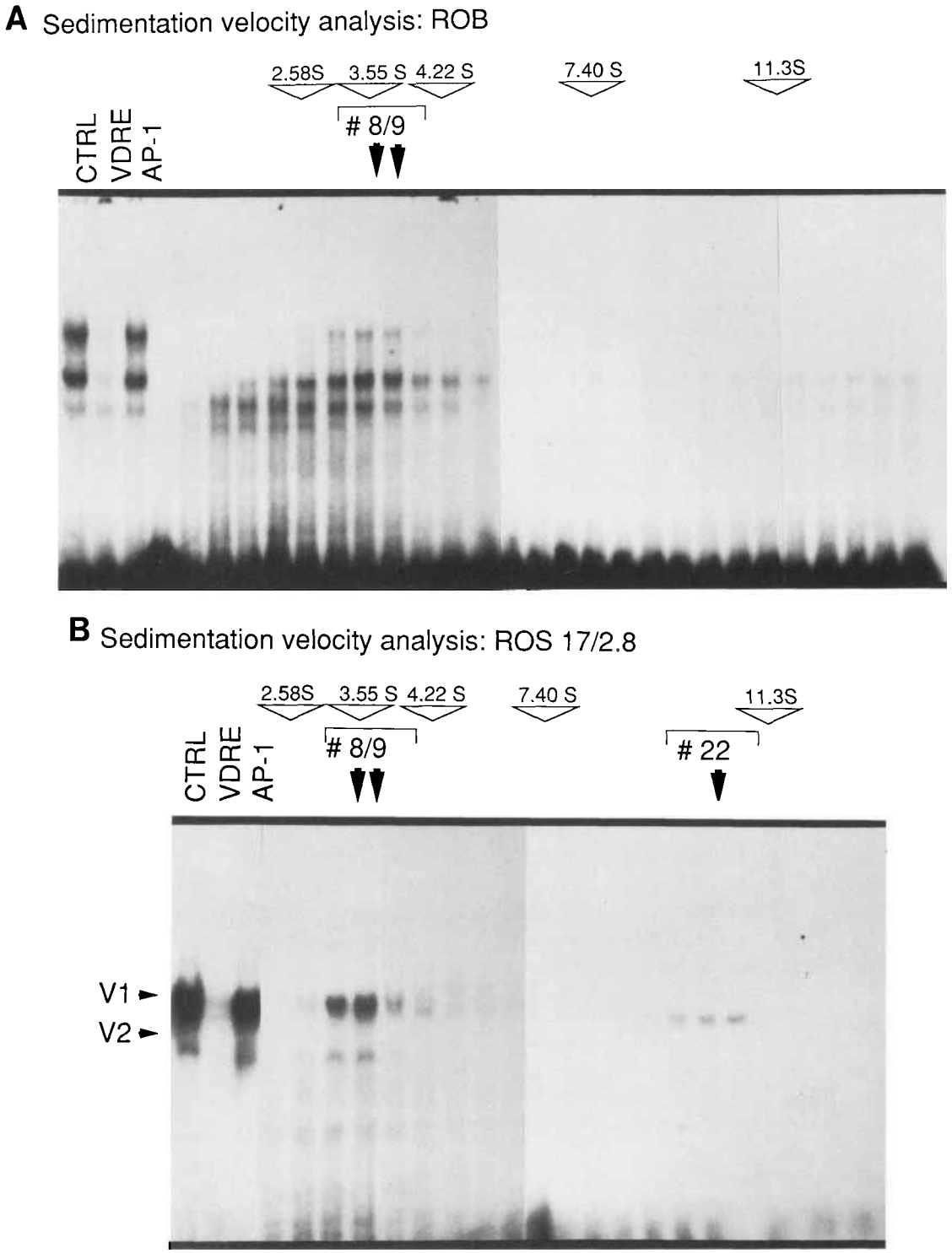

Fig. 4. Sedimentation velocity analysis of factors mediating OC-VDRE complexes V1 and V2. Nuclear factors from Vitamin D treated ROB (**A**) or ROS 17/2.8 (**B**) cells were subjected to *centrifugation* in 10–30% glycerol gradients. Gradient fractions were assayed in gel shift assays with the OC-VDRE probe (peak-fractions indicated by arrowheads). Oligonucleotide competition (left lanes in A and B, and data not shown) was performed as described in Figure 1. Sedimentation coefficients were determined with native molecular weight markers (myoglobin, 2.58 S, 25 kD; ovalbumin, 3.55 S, 45 kD; bovine serum albumin, 4.22 S, 66 kD; aldolase, 7.40 S, 158 kD; and catalase, 11.3 S, 240 kD). These protein standards were run in duplicate in parallel gradients and analyzed by SDS-PAGE. The approximate peak-position in the gradients of each of the standards is illustrated above the autoradiogram (open triangles).

teins from ROS 17/2.8 cells migrate as a double peak (3.6 S and 9.8 S fractions), and those from ROB as a single peak (3.6 S). This finding may reflect fundamental differences between osteosarcoma cells and normal diploid osteoblasts in the potential of the putative monomeric 3.6 S subunits (i.e., VDR or RXR) to mediate protein/protein associations in the absence of DNA.

Direct Characterization of Vitamin D Receptor Complexes at the Osteocalcin Gene Vitamin D Responsive Element

To establish an estimate of the molecular weight of the Vitamin D receptor complexes directly interacting at the osteocalcin Vitamin D responsive element, we performed electrophoretic pore size exclusion analysis on binding reactions containing the OC-VDRE and protein from ROS 17/2.8 cells under the native conditions of gel shift assays (Fig. 5). As markers we used SDS-treated molecular weight standards that were run on adjacent lanes. The results show that the Vitamin D receptor complexes V1 and V2 migrate as protein/DNA complexes with apparent relative molecular weights of, respectively, 200 kD and 140 kD. These molecular weight estimates include the relative molecular weight (20 kD) of the DNA probe. Because glycerol gradient analysis indicated that the V1 and V2 complexes are mediated by monomeric subunits of 42 kD to 60 kD, the pore-size exclusion results suggest that the V1 complex probably represents a tetramer, whereas the V2 complex is most likely a trimer.

Ultraviolet (UV) cross-linking analysis was carried out to determine the molecular weights of proteins that are in direct contact with the osteocalcin Vitamin D responsive sequences (Fig. 6). Our experimental approach was to electrophoretically fractionate the V1 and V2 complexes in

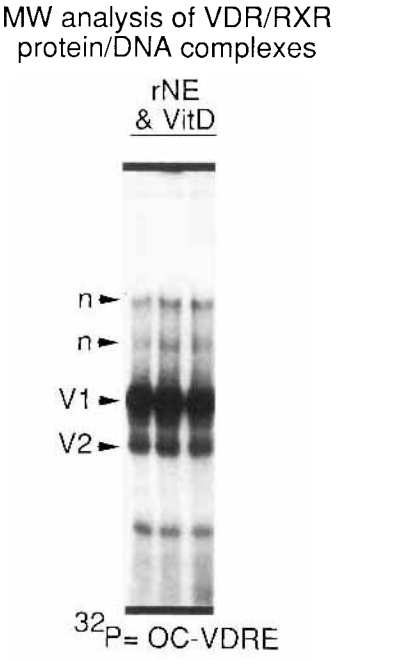

Fig. 5. Pore-size exclusion analysis of OC-VDRE protein/DNA complexes V1 and V2. Gel shift complexes with proteins from Vitamin D treated ROS 17/2.8 cells were electrophoresed in triplicate in 4–20% polyacrylamide gradient gels. Approximate molecular weights of complexes V1 and V2 were determined by comparison with Coomassie-stained markers that were run in adjacent lanes (not indicated). Additional complexes (labeled n) were not further characterized.

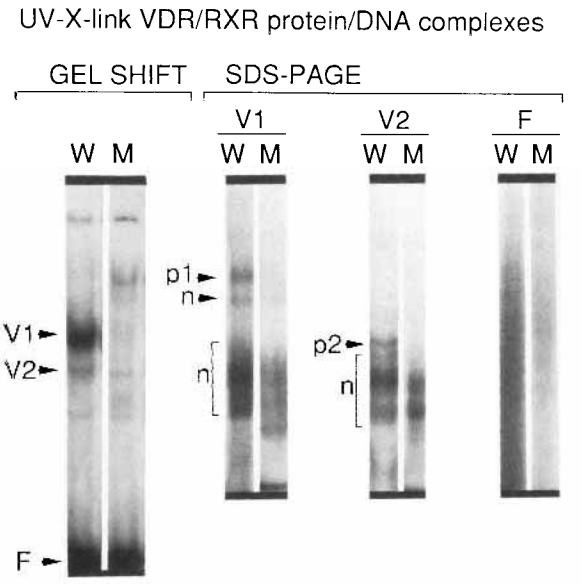

Fig. 6. UV crosslinking analysis of DNA binding factors present in OC-VDRE complexes V1 and V2. Nuclear proteins from Vitamin D treated ROS 17/2.8 cells were incubated in the presence of a BrdU-substituted OC-VDRE probe (wildtype, W) or a mutant OC-VDRE probe (M) in which both steroid half-elements were mutated. After UV-irradiation, the gel shift complexes with both probes were electrophoretically fractionated (first panel from left). Complexes V1 and V2, and free (F) probe observed for the wildtype OC-VDRE probe, as well as the corresponding gel segments of the mutant OC-VDRE probe, were each excised from the gel, eluted and subject to nuclease digestion. Samples representing V1, V2, and F (respectively, second, third, and fourth panel) for both wildtype (W) and mutant (M) probes were normalized with respect to the amount of radioactivity present, and analyzed in parallel by SDS-PAGE and autoradiography. Relative molecular weights of specific DNA binding factors (p1 and p2) cross-linked to the radiolabeled wildtype OC-VDRE probe, were determined by comparison with Coomassie-stained markers that were run in adjacent lanes (not indicated). Additional complexes (labeled with n and bracket) are not related to complexes V1 and V2 based on detection with both mutant and wildtype probes.

gel shift assays using a BrdU substituted OC-VDRE probe containing ^{32}P-dCTP labelled nucleotides located within the Vitamin D steroid hormone half-elements. The protein/DNA complexes within the gel were treated by UV irradiation (312 nm). After electro-elution and extensive nuclease digestion, the cross-linked protein components of the V1 and V2 complexes were analyzed by SDS-PAGE. To establish specificity of the cross-linking procedure, we performed binding reactions in parallel with a mutant probe that does not form complexes V1 and V2 in gel shift assays. Our findings indicate that the V1 complex contains one major specific DNA binding activity (p1) with a relative molecular weight of approximately 50 kD. The size of p1 and its cross-linking to labelled nucleotides suggests that the protein most likely represents the Vitamin D receptor (or RXR), which is in direct contact with the cognate steroid hormone half-elements. In contrast to the results for the V1 complex, the primary cross-linkable component of the V2 complex (p2) is a 30 kD protein, but we do not observe cross-linking of the 50 kD protein, presumably representing the Vitamin D receptor. Taken together, these findings suggest that the V1 and V2 complexes differ in protein/DNA associations at the osteocalcin Vitamin D responsive sequences.

To identify the VDRE nucleotides in direct contact with the V1 and V2 protein components, we performed methylation interference analysis of the V1 and V2 protein/DNA complexes. Figure 7 shows that the V1 complex interacts at a series of guanine residues (nt −460, −459, −458, −450, −449, and −447) residing in both steroid hormone half-elements (nt −460 to −455 and nt −451 to −446; schematically indicated in Fig. 7c). However, the contacts of the V2 complex with the OC-VDRE nt −450 and −449 are confined to the proximal half-element (nt −451 to −446). The methylation interference results clearly establish fundamental differences in the manner by which the Vitamin D receptor protein/protein complexes recognize the osteocalcin Vitamin D responsive sequences.

Implications of Tumor-Related Modifications in Protein/DNA Interactions at the Vitamin D Responsive Element for Steroid Hormone Responsive Transcriptional Control

In summary, our studies demonstrate two distinct Vitamin D receptor protein/DNA complexes at the osteocalcin Vitamin D responsive element: an osteosarcoma-related Vitamin D receptor complex (V1) and an osteoblast-related complex (V2). Taken together, our findings suggest differences in protein/protein- and protein/DNA interactions of these Vitamin D receptor related complexes V1 and V2 at the osteocalcin VDRE. These molecular differences reflect unique properties of the osteosarcoma and osteoblast phenotype.

Several observations have been made which provide insight into requirements for Vitamin D mediated transcriptional control at the osteocalcin Vitamin D responsive sequences, including phosphorylation of the Vitamin D receptor, ligand binding, protein/protein associations, protein/DNA interactions at the VDRE, transactivation, and subnuclear distribution [Markose et al., 1990; Brown and DeLuca, 1990, 1991; Hsieh et al., 1991; Leid et al., 1991; Jones et al., 1991; Yu et al., 1991; Ross et al., 1991, 1992a,b, 1993; Demay et al., 1992; MacDonald et al., 1991, 1993; Wiese et al., 1992; Bidwell et al., in press; Schrader et al., 1993; reviewed by Orti et al., 1992; Darwish and DeLuca, 1993]. In addition, association of the Vitamin D receptor with auxiliary factors including RXR, and effects of glucocorticoids and retinoic acid related compounds on Vitamin D responsive transcription of the osteocalcin gene suggests a high level of complexity in Vitamin D dependent transcriptional control [Bortell et al., 1993; Leid et al., 1991; MacDonald et al., 1993; Schrader et al., 1993; Liao et al., 1990].

Specific regulatory mechanisms remain to be established which account for normal developmental control of Vitamin D responsive transcription of the osteocalcin gene in normal diploid osteoblasts, compared with aberrant constitutive responsiveness to Vitamin D in osteosarcoma cells. However, the distinctions between Vitamin D receptor complexes in osteoblasts and osteosarcoma cells may reflect characteristic deviations of transformed and normal diploid cells in signalling mechanisms which mediate steroid hormone responsiveness of transcriptional activities at gene promoter elements. Definitive assignment of functional activities to VDRE associated proteins required for regulation of osteocalcin gene transcription necessitates establishing in vivo occupancy of Vitamin D responsive sequences and protein/protein interactions in intact cells.

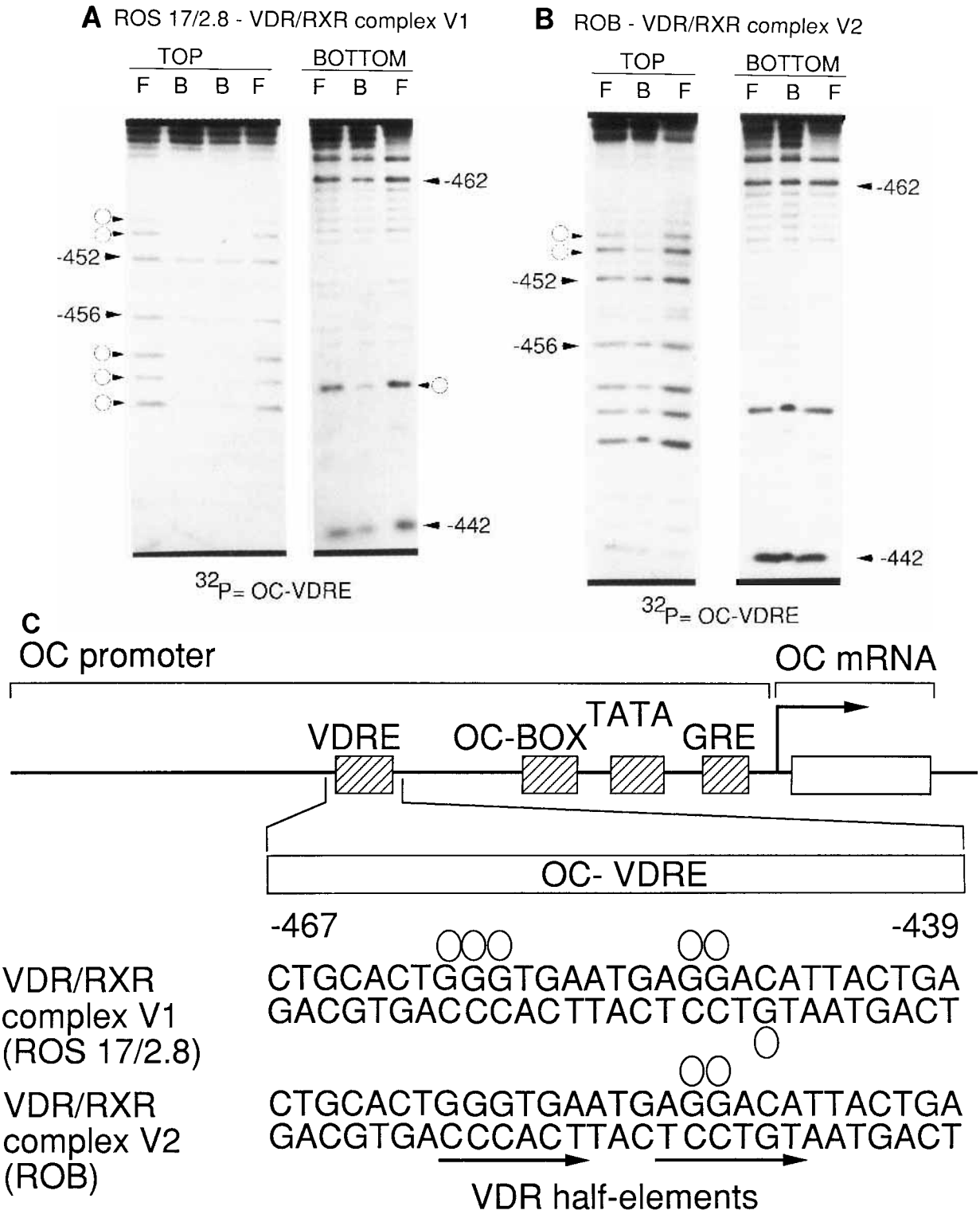

Fig. 7. DNA recognition site analysis of OC-VDRE complexes V1 and V2. Methylation interference analysis was performed with nuclear proteins from Vitamin D treated ROS 17/2.8 (**A**) and ROB cells (**B**). Probes spanning the OC-VDRE were labeled on either the top- or bottom-strand as indicated above the panels, and partially methylated. Gel shift complexes V1 and V2 were resolved by electrophoresis, followed by elution and analysis of piperidine-cleavage products on 8% sequencing gels. Methylation interference contacts are indicated by open circles and arrowheads. Guanine residues outside the recognition motifs for complexes V1 and V2 are indicated for reference. **C** shows a summary of the methylation interference analysis relative to the two steroid half-elements of the OC-VDRE (indicated by thin arrows) (bottom portion, symbols as in A and B) and the location of transcriptional elements in the osteocalcin promoter [top portion; reviewed in Stein et al., 1990].

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