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CHARACTERIZATION OF HUMAN 1,25-DIHYDROXYVITAMIN D_3 RECEPTOR ANTI-PEPTIDE ANTIBODIES

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Summary—Rabbit and chicken antibodies were raised against two peptides synthesized according to the structure of human 1,25-dihydroxyvitamin D3 receptor (hVDR): rabbit αhVDR-103 against the N-terminal amino acids 5–18 and αhVDR-104 against the amino acids 172–186 in the hinge region and chicken αhVDR-cab11 against the amino acids 172–186, respectively. The specificity of the antibodies was tested by peptide saturation, SDS–PAGE immunoblotting, gel shift assay and sucrose gradient centrifugation. Immunoblotting of a soluble extract (cytosol) from osteosarcoma cell line MG-63 showed a single band with an M_r of about 48,000 and human intestine cytosol a broad band (50–63,000) for both antibodies. The antibodies recognized activated (3.2S) hVDR by shifting the centrifugation sedimentation profile to 5–6S. The antibodies showed nuclear immunostaining of unoccupied VDR in human osteosarcoma cells MG-63, U2-Os and SaOs-2. The immunoreaction could be saturated with the corresponding synthetic peptide. In immunoblot αhVDR-103 reacted with human and rat VDR, whereas αhVDR-104 recognized human VDR only. Similarly in immunohistochemistry, αhVDR-103 showed staining with hVDR and rVDR, whereas αhVDR-104 reacted only with hVDR. All antibodies recognized the native hVDR as verified with sucrose gradient centrifugation or immunoprecipitation but only αhVDR-103 and αhVDR-cab11 in gel shift assay of hVDR associated with the vitamin D-responsive element of human osteocalcin gene promoter.

INTRODUCTION

Vitamin D action holds that vitamin D has a wide range of hormone-like actions and the regulation of plasma calcium is only part of its complex actions. Because vitamin D is generated in the body by the ultraviolet radiation of sunlight, it is more appropriately classified as an exohormone. The actions of vitamin D are mediated by a specific receptor (VDR) [1, 2], which has been recently cloned from avian [3] and human cells [4]. VDR is a member of the thyroid–steroid receptor superfamily [5], being more closely related to thyroid hormone receptor than to steroid receptors. It has a specific responsive element at the 5′-region of vitamin D-responsive genes, which is slightly different from that of other members of the superfamily [6], but which is also recognized by retinoic acid receptor [7]. Human VDR consists of 427 amino acids with a calculated M_r of 48,295 giving an electrophoretic migration of about 52–60 K [4, 8, 9]. Avian VDR is slightly larger in size, migrating electrophoretically at 60 K.

The cellular location of VDR has been studied by autoradiography using labelled ligands [10–12] and by immunohistochemistry with VDR antibodies [13, 14]. In addition to the classical target tissues for vitamin D (bone, intestine and kidney), VDR has been located in several other tissues. VDR appears to be up- and down-regulated by several hormones, dietary calcium, and ageing [15–21]. Specific antibodies against steroid receptors have proved to be valid tools for cellular and subcellular location of the corresponding receptors after various hormonal treatments [22–25] and for receptor quantification [26]. Monoclonal antibodies have been developed against affinity-purified avian and porcine VDRs [27, 28], which appear to crossreact with human VDR, but the epitopes are difficult to verify. We therefore made antibodies against selected synthetic

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Peptide-103

```
                5                                                    18
human VDR  -Ala-Ala-Ser-Thr-Ser-Leu-Pro-Asp-Pro - - -  Gly-Asp-Phe-Asp-Arg-
           :  :  :  :  :  :  :  :  :                    :  :  :  :  :
avian VDR  -Ala-Ala-Ser-Thr-Ser-Leu-Pro-Asp-Pro -Ala-  Gly-Asp-Phe-Asp-Arg-
               13                                                    27
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Peptide-104

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               172                                                  186
human VDR  -Ser-Arg-His-Thr-Pro-Ser-Phe-Ser-Gly-Asp-Ser-Ser-Ser-Ser-Cys-
           :                       :  :              :
avian VDR  -Ser-Val-Val-Ser-Gln-Asp-Phe-Ser-Ser-Glu-Asp-Ser-Asn-Asp-Val-
               181                                                  195
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Fig. 1. The amino acid sequences of human VDR peptides used for immunization and homologous avian sequences [29].

human VDR-peptides and determined their properties.

EXPERIMENTAL

Synthetic peptides

The synthetic peptides were p103 $hVDR_{5-18}$ (NH_2-AASTSLPDPGDFDRC-COOH) with C-terminal cysteine added for coupling, and p104 $hVDR_{172-186}$ (NH_2-SRHTPSFSGDSSSSC-COOH). The peptides were conjugated to bovine serum albumin (BSA) or ovalbumin. The selected human and the corresponding chicken VDR sequences are shown in Fig. 1. Homologous peptide sequences for p103 are very similar in human and avian VDR, whereas those for p104 are dissimilar [29].

Immunization

Male California rabbits were injected with 100 μg of the peptide–BSA conjugate in 0.5 ml of Freund's complete adjuvant under nail skin with approval of the local Ethical Committee. Antigen injections were repeated five times in Freund's incomplete adjuvant. Sera were precipitated by dropwise addition of saturated $(NH_4)_2SO_4$ to a final concentration of 40% (v/v). Antibodies were tested by direct immunoassay against the corresponding ovalbumin conjugated peptides or uncojugated peptides (2 μg/ml) immobilized on solid phase (Immunoplate, Nunc, Roskilde, Denmark) (Fig. 2).

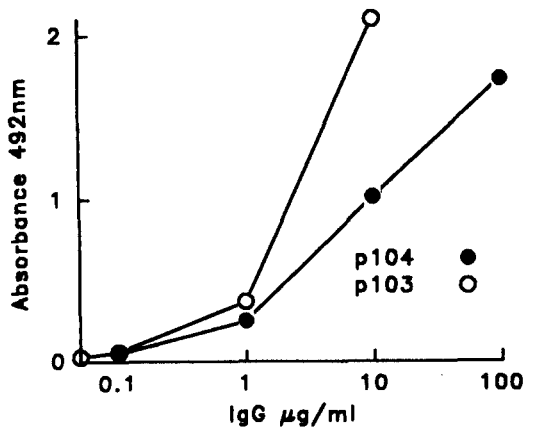

Fig. 2. Titration curves for αhVDR-103 (○—○) and αhVDR-104 (●—●) against corresponding ovalbumin conjugated peptides (2 μg/ml) immobilized on microtitre plates.

Leghorn hens were injected i.m. with 100 μg of p104–Keyhole limpet hemocyanin conjugate (KLH, Sigma, St Louis, MO, U.S.A.) in 0.7 ml of Freund's complete adjuvant. Antigen injection was repeated once in Freund's incomplete adjuvant. Egg yolk IgG was collected according to Jensenius *et al.* [30]. Purified antibody was named as anti-hVDR-cab11.

Cell culture

Human osteosarcoma cell lines MG-63, U2-Os, and SaOs-2 (American Type Culture Collection, Rockville, MD, U.S.A.) were grown in Dulbecco's modified essential medium supplemented with 10% heat-inactivated fetal bovine serum (2% FBS treated with dextran coated charcoal, DCC, for vitamin D treatment), 2mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and nonessential amino acids in a humidified 95% air/5% CO_2 incubator near to confluence on microscope slides in Petri dishes. The cells were fixed in 4% paraformaldehyde in a phosphate-buffered saline (PBS) for 15 min and washed in PBS.

Immunoblotting

Samples of the human small intestine taken from the operated parts (with approval of the

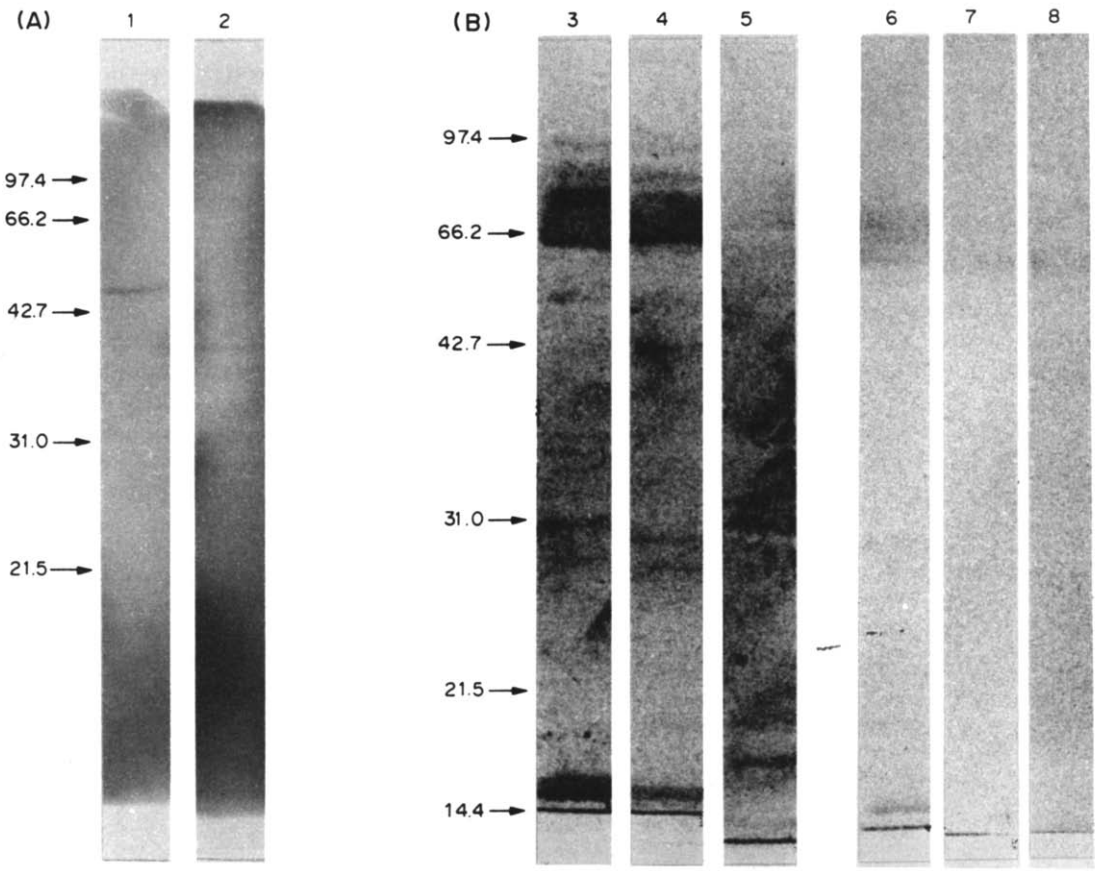

Fig. 3. Immunoblots of αhVDR-103 (lanes 1–5) and αhVDR-104 (lanes 6–8) with human osteosarcoma MG-63 cytosol (A) and with human (lanes 3 and 6), rat (lanes 4 and 7) and chicken small intestine cytosol (lanes 5 and 8) showing a band with M_r of about 48,000 in MG-63 cells and 50–63,000 in intestinal samples. A weaker band with M_r of about 16–17,000 is seen in some samples. Enhanced chemiluminescence (A) or alkaline phosphatase (B) detection system is used (For methodological details see Experimental). Molecular weight standards are given in the text for Experimental.

Ethical Committee of the University Hospital, Tampere, Finland) and of rat and chicken small intestine were homogenized. MG-63 cells from American Type Culture Collection (Rockville) were sonicated for 2 × 20 s in a Tris-buffer (0.15 M NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, SDS, 50 mM Tris–HCl pH 7.5). The homogenates were centrifuged at 100,000*g* for 1 h at 0°C. The supernatant was boiled in the sample buffer (50 mM Tris, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) for 4 min. The proteins were resolved in 12% polyacrylamide slab gels containing 0.1% SDS [31] and were transferred to nitrocellulose sheets with electrophoretic transfer apparatus (Mini Trans-blot, Bio-Rad, Richmond, CA, U.S.A.). The sheets were saturated with 3% gelatin in TBS (50 mM Tris, 0.9% NaCl, pH 8.0). The primary antibody (2 μg/ml in TBS containing 1% gelatin and 1% normal swine serum) was incubated at 4°C overnight. After washings, alkaline phosphatase conjugated swine anti-rabbit IgG (in TBS containing 1% gelatine and 1% normal swine serum) was incubated for 1 h at 37°C. The substrate was NBT-BCIP (Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, Sigma, Germany). Alternatively, nitrocellulose sheets were incubated for 1 h at room temperature after the primary antibody with peroxidase-conjugated goat anti-rabbit IgG (Cappel, West Chester, PA. U.S.A.) diluted 1/10,000. Immunoreactive protein bands were detected using the enhanced chemiluminescence detection method by Amersham (Bucks., England). Control strips were incubated with a similar amount of the primary antibody presaturated with a 40-fold molar excess of the corresponding peptide antigen. The molecular weight standards were phosphorylase b M_r 97,400, BSA M_r 66,200, ovalbumin M_r 42,699, carbonic anhydrase M_r 31,000, soybean trypsin inhibitor M_r 21,500, and lysozyme M_r 14,400 (BioRad).

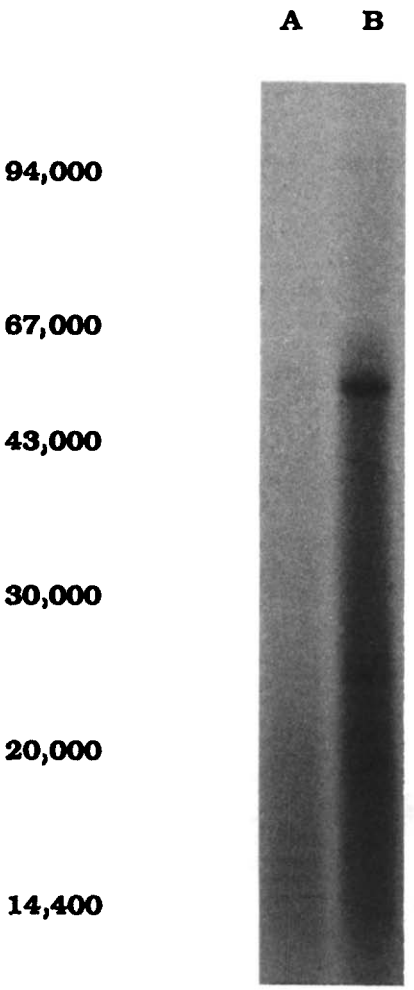

Fig. 4. Metabolic labelling of MG-63 osteosarcoma cells with L-[^{35}S]methionine and immunoprecipitation of VDR with antipeptide antibody, αhVDR-103. Subconfluent MG-63 cells were labelled with L-[^{35}S]methionine, lysed, and the extract equivalent to 1×10^7 cpm radioactivity immunoprecipitated as described in Experimental. Lane A, immunoprecipitation with preimmune serum; lane B, immunoprecipitation with αhVDR-103. Protein standards are: phophorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,400).

Metabolic labelling and immunoprecipitation

For metabolic labelling, the MG-63 cells were seeded at 6×10^5 cells per 100-mm culture plates. The subconfluent cultures were rinsed and incubated for 15 min in methionine-free Minimum Essential Medium (MEM). Then the cells were incubated for 6 h in methionine-free MEM supplemented with 2% charcoal-treated FCS and L-(35S) methionine (100 μCi/plate) in a humidified atmosphere of 95% air/5% CO_2. After labelling MG-63 cells were washed three times with PBS, and lysed with 1 ml of ice cold lysis buffer (50 mM Tris–HCl, pH 7.5), 1% NP-40, 0.1% SDS, 1 mM EDTA, 5 mM dithiotreitol (DTT), 0.3 mM $ZnCl_2$, 50 μg/ml PMSF, 2 μg/ml leupeptin, 2.0 μg/ml aprotinin, 50 μg/ml TLCK). The lysates were transferred into Eppendorf tubes and sonicated for 15 s. The cell extracts were centrifuged for 30 min at 100,000*g* and the supernatants were subjected to immunoprecipitation (stored at −80°C).

Aliquots of 100 μl (about 10^7 cpm) of metabolically-labelled MG-63 cells were subjected to immunoprecipitation by addition of αhVDR-103 or preimmune serum, and incubated on ice for 1 h. Protein G Sepharose (Pharmacia) was used to collect the immunocomplexes. 20 μl of 1:1 slurry of Protein G Sepharose was added to the extract and shaken for 2 h at 4°C. After sedimenting, the beds of Protein G Sepharose were washed, boiled for 5 min in SDS-buffer and electrophoresed on 12% polyacrylamide gels as described by Laemmli [31]. The polyacrylamide gels were fixed in 40% methanol–10% acetic acid, treated with Amplify fluorographic reagent (Amersham) and fluorographed overnight at −80°C on Kodak Omat XAR film.

Gel shift assay

Nuclear proteins were extracted from $1,25(OH)_2D_3$-treated human osteosarcoma MG-63 cells using the method of Hurst *et al.* [32]. DNA binding was assayed by electrophoretic mobility shift on 5% polyacrylamide gels according to Palvimo *et al.* [33]. Nuclear proteins were incubated in the presence of 2 μg poly(dI-dC)(dI-dC) with a synethetic double-stranded 23-mer corresponding to the vitamin D responsive element (VDRE) of human osteocalcin promoter (upper strand: 5′-ACCGGGT-GAACGGGGGCATTGCG-3′). VDRE was labelled using T4 polynucleotide kinase with [γ-^{32}P]ATP. Indicated amounts of antibodies were included in the preincubation (90 min at 4°C) before addition of labelled VDRE.

Sucrose gradient centrifugation and double antibody precipitation

Sedimentation analysis of human osteosarcoma MG-63 cell soluble extract (cytosol) and rachitic chicken intestinal VDR (Amersham) labelled for 14 h at 37°C or 1 h at 22°C with 0.25 nM 1α,25-dihydroxy[26,27-methyl-^{3}H]D_3(176 Ci/mmol, Amersham) was performed using sucrose gradient ultracentrifugation. Nonspecific binding was determined in the presence of a 200-fold molar excess of unlabelled ligand.

Unbound ligand was bound with 1% DCC. Samples were incubated with or without the αVDR antibody (20 μg/100 μl cytosol for 2.5 h at 0°C). Samples were centrifuged in a linear sucrose gradient (5–20%) prepared in 10 nM Tris, 12 mM monothioglycerol, 1.5 mM EDTA, 10% glycerol and 0.2 M KCl, pH 7.4. The tubes were centrifuged at 200,000*g* for 16 h. Fractions were collected by piercing the bottom of the tube. The internal standards were horse radish peroxidase (3.6S) and glucose oxidase (7.9S) (Sigma). Radioactivity was counted with an LKB-Wallac liquid scintillation counter. Double antibody precipitation was carried out with goat anti-rabbit IgG (5 μg/ml) for rabbit antibodies and the samples labelled as indicated above were centrifuged on a 1 M sucrose cushion (200 μl) in Eppendorf centrifuge in a cold room. The supernatant and the bottom of the tube were counted for radioactivity.

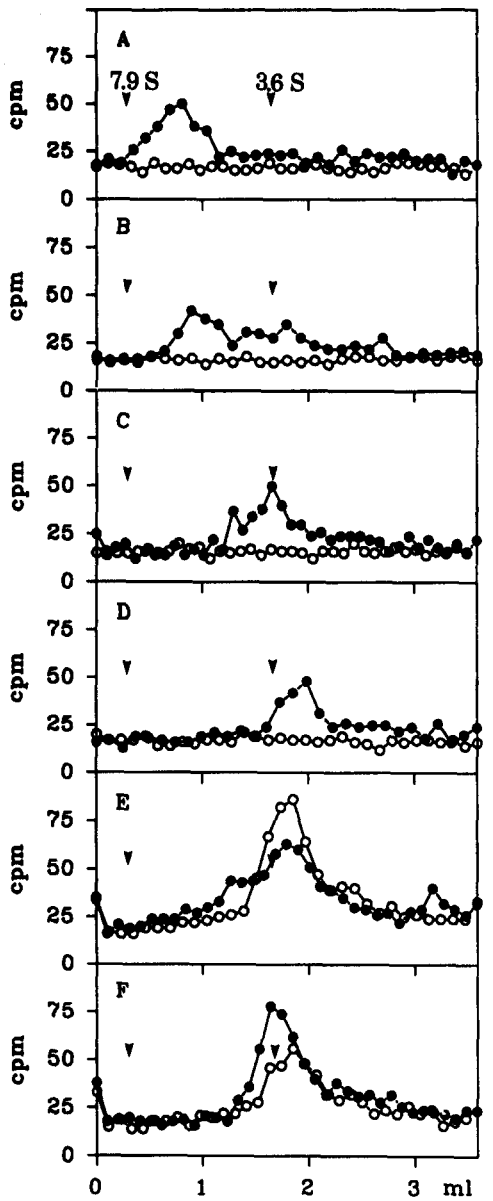

Fig. 5. Sucrose gradient ultracentrifugation analysis of antibody and VDR complexes. (A–D). Human MG-63 osteosarcoma cells are incubated 14 h with 0.25 nM tritiated $1,25(OH)_2D_3$ with (○—○) or without 200-fold molar excess of cold ligand (●—●), cytosol is made by repeated freeze–thawing and antibodies are incubated as given in Experimental. All antibodies cause a shift of VDR. (E–F). Chicken (cVDR) is incubated with the antibodies and no shift of VDR is seen. (A) hVDR + αhVDR-103 (●—●). Nonspecific binding (○—○). (B) hVDR + αhVDR-104 (●—●). Nonspecific binding (○—○). (C) hVDR + αhVDR-cab11 (●—●). Nonspecific binding (○—○). (D) hVDR + buffer (●—●). Nonspecific binding (○—○) (E) cVDR + αhVDR-103 (●—●). cVDR + αhVDR-104 (○—○). (F) cVDR + αhVDR-cab11 (●—●). cVDR + buffer (○—○). Standards were horse radish peroxidase (3.6S = 1.7 ml) and glucose oxidase (7.9S = 0.3 ml).

Immunohistochemistry

The sarcoma cells were fixed in 3.7% paraformaldehyde in PBS at 4°C for 10 min, followed by methanol at −20°C for 4 min and acetone at −20°C for 2 min and permeabilized with 0.5% Triton X-100 in PBS for 40 min. Also samples of human, rat and chicken small intestine were cut in a cryostat and fixed as described above. After washing in PBS, nonspecific binding was minimized by treatment with 10% goat serum. Primary antibody (10 μg/ml) was incubated overnight at 4°C. After washing, biotinylated goat anti-rabbit IgG (2 μg/ml) was incubated for 30 min at RT. Sections were incubated with avidin–biotin–peroxidase complex (Vectastain Elite, Vector Labs, Burlingame, CA, U.S.A.). The substrate was diaminobenzidine. Controls included stainings with preimmune serum (1:200), with αhVDRs saturated with the corresponding peptide (10-fold excess for 2 h), without primary serum, and with serial dilutions of αhVDRs up to extinction.

RESULTS

Both rabbit antisera showed high titres, since αhVDR-103 antiserum could be diluted up to 1:50,000 and αhVDR-104 antiserum up to 1:100,000. The ammonium sulphate-precipitated IgG fraction could be used in concentrations ranging from 0.1 to 10 μg/ml (Fig. 2). The crossreactivity of the αhVDR-103 (1:500) against peptide-104 was <5%.

The specificity of the αhVDRs using immunoblotting is shown in Fig. 3. In human

MG-63 osteosarcoma cells, both IgGs recognize a single band of M_r 48,000 (lane 1), which can be presaturated with 40-fold molar excess of the corresponding peptide (lane 2). When the blots were made with human, rat and chicken small intestine cytosol, αhVDR-103 (Fig. 3, lanes 3–5) recognized a broad band with an M_r of about 50–63,000 and a weaker band with an M_r of about 16–17,000 in human and rat samples. Anti-hVDR-104 reacted only with human VDR with similar pattern of bands as αhVDR-103 (Fig. 3, lanes 6–8). Immunoprecipitation of metabolically labelled MG-63 cell VDR with αhVDR-103 showed a clear band at M_r 55,000 and a weaker band at M_r 25,000 (Fig. 4). Furthermore, ligand binding activity of 0.3 M KCl-activated (3.2S) human osteosarcoma MG-63 cell cytosol VDR was completely shifted by αhVDR-103 and αhVDR-104 to 5-6S, as assayed by sucrose gradient centrifugation (Fig. 5). Figure 6 lane 2 shows that hVDR bound to the VDRE of human osteocalcin gene promotor is shifted with αhVDR-103 in a gel shift assay suggesting that the epitope is not masked in the complex. On the other hand, VDRE–VDR complex disappears partially with 20 μg of αhVDR-cab11 (Fig. 6, lane 3) or completely with 50 μg of αhVDR-cab11 (Fig. 6, lane 4) suggesting that the anti-hinge peptide antibody interferes with DNA binding of VDR.

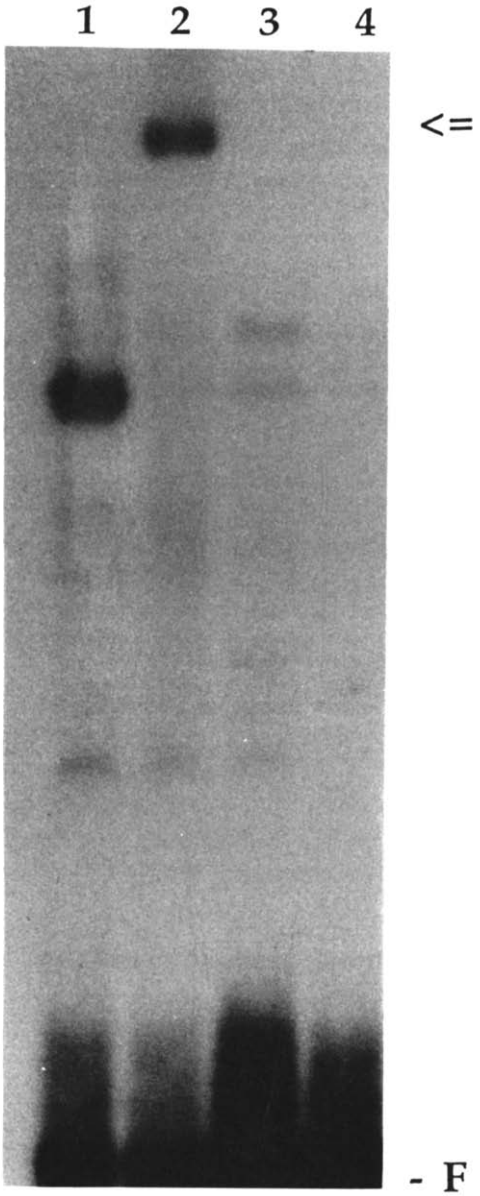

Fig. 6. Identification of vitamin D receptor as a component in VDRE–nuclear protein complexes using αhVDR-103 and αhVDR-cab11 antibodies and gel shift assay. Following components, in addition to the nuclear extract, were included in the preincubation before addition of the radiolabelled probe: no additions (lane 1), 50 μg αhVDR-103 (lane 2), 20 μg (lane 3), and 50 μg (lane 4) αhVDR-cab11. The arrow indicates the position of supershift VDRE–VDR complex caused by the anti-hVDR-103 antibody addition. F = free probe.

Immunohistochemical studies demonstrated that the immunoreactive VDR was located predominantly in the cell nuclei of the MG-63 osteosarcoma cells [Fig. 7(a)] or of the small intestine epithelial cells [Fig. 7(c–k)]. Anti-hVDR-103 stained both human and rat VDR, especially in the Lieberkühn glands [Fig. 7(c and f)], whereas αhVDR-104 was human-specific only [Fig. 7(d)]; rat [Fig. 7(g)] and chicken intestine [Fig. 7(j)] showed no staining with the latter antibody. In most cells, faint cytoplasmic staining was observed. U2-Os and SaOs-2 cells showed staining similar to that of MG-63 cells. Presaturation controls showed no staining.

DISCUSSION

The antibodies described here are monospecific polyclonal rabbit antibodies, which bind to a single antigen band in the immunoblotting of osteosarcoma cell cytosol. The estimated M_r (48,000 or somewhat higher in intestinal samples) of the band is in agreement with previous studies [2, 9, 28, 33]. The monospecific nature of the antibodies is further proved as follows: (1) Antigen binding is saturated with an excess of the corresponding peptide in immunohistochemistry. (2) The ligand-binding form of VDR is shifted by treatment with antibodies in the sedimentation analysis. (3) The recognization of the VDR–VDRE complex by αhVDR-103 in gel shift assay. In the presence of the antibody against the N-terminal peptide of

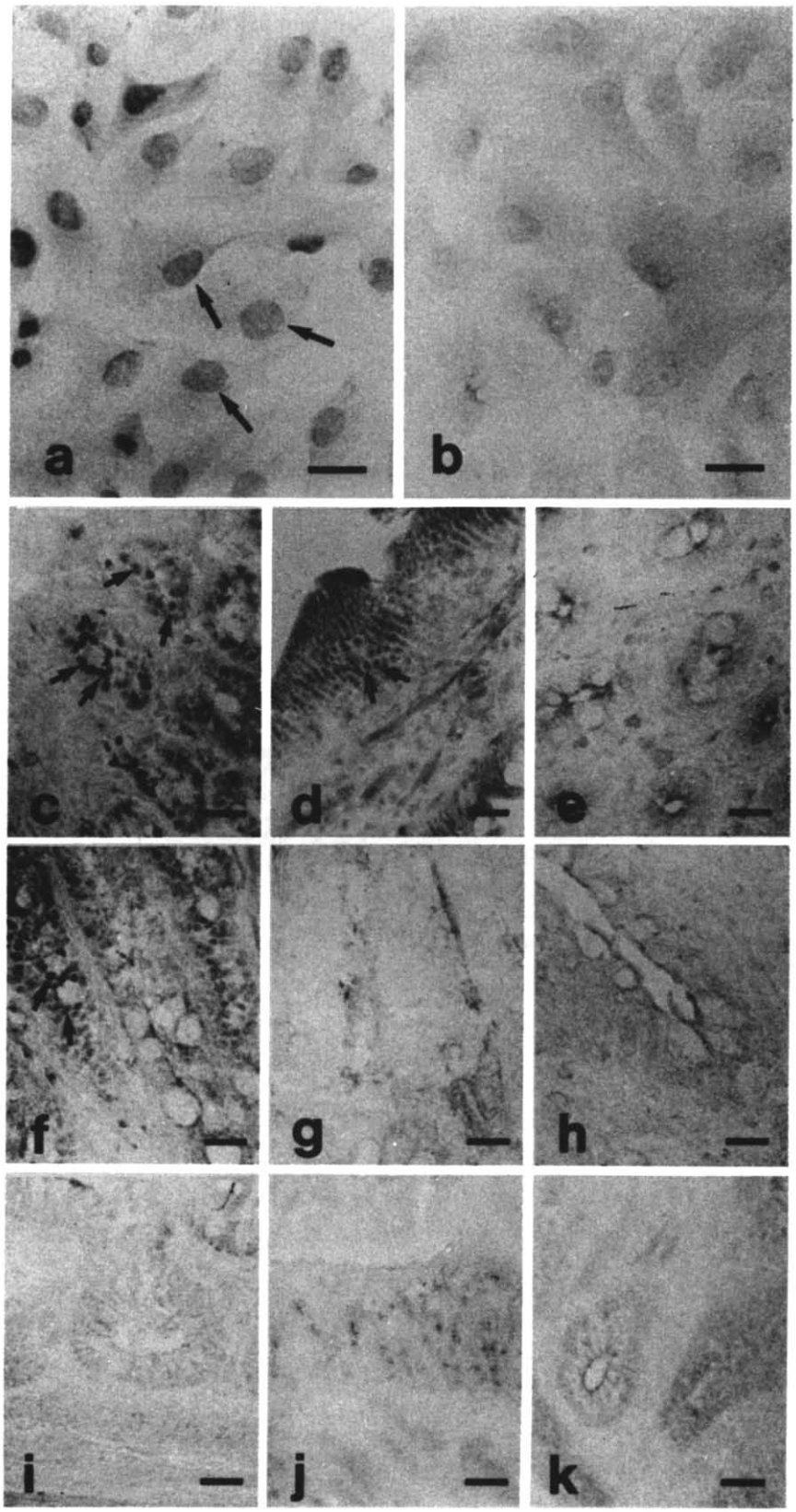

Fig. 7. Immunohistochemistry of VDR in different cells tissues. Human osteosarcoma cell line MG-63 is stained with αhVDR-103 (a) and with αhVDR-103 presaturated with ovalbumin conjugated peptide-103 (b). Human (c), rat (f) and avian small intestine (i) is stained with αhVDR-103 and respective tissues with αhVDR-104 (d, g, j) and with preimmune rabbit IgG (e, h, k). Arrows point to VDR-positive nuclei in the Lieberkühn crypts. Bar = 25 μm in a and b; bar = 50 μm in c–k.

VDR (αhVDR-103), a supershift was found causing retardation of migration of the VDR–VDRE complex. This may be caused by the increased size of the complex. In the presence of the antibody against the hinge region of VDR (αhVDR-cab11), the antibody probably prevented VDR–VDRE complex formation by steric hindrance near the Zn-finger region of VDR (4). The epitopes selected are from different parts of the receptor, designed to contain a low degree of homology with other human steroid receptors. The titre and affinity of the antibodies seem to be sufficient to allow development of a VDR-IEMA, which is under way.

An interesting property of the antibody αhVDR-103 is that it recognizes epitopes of both SDS-treated and paraformaldehyde-denaturated receptor as well as those of native VDR, which makes it useful for various kinds of immunological analyses of VDR. Monoclonal antibodies have been raised against affinity-purified chicken and porcine VDR [9, 28]. They show no species specificity and their antigenic determinants are not exactly known. We describe here monospecific polyclonal antibodies with known epitopes. The synthetic peptides can be effectively utilized as standards in quantitative immunohistochemistry and immunoassays. Because the exact location of the epitopes in the VDR molecule is known, the antibodies allow e.g. a study of proteolysis of VDR during ligand occupation. The amino acid sequence represented by peptide-103 is well conserved during evolution (Fig. 1), whereas that of peptide-104 is not; thus, αhVDR-104 is more species-specific than αhVDR-103.

The location of the unoccupied VDR is predominantly intranuclear in cultured human osteosarcoma cells. Thus, VDR appears to be similar to other steroid receptors [6, 23–25, 35], except for glucocorticoid receptor, which unoccupied may be located in the cytoplasm, but which is intranuclear after ligand occupation [36]. Enucleation experiments with VDR-containing cells have suggested that VDR may be in a nuclear-cytoplasmic equilibrium, which is dependent on the ligand [37]. In order to arrive at a clear solution to the location problem, an immunohistochemical technique [38] should be used as would prevent diffusion artefacts of soluble proteins. We always found a small amount of cytoplasmic staining, which may be due to the apparently continuous synthesis of VDR, since some mRNA for VDR is always detected in these cells [39]. On the other hand, some cytoplasmic staining for estrogen or progesterone receptor is often seen in cultured cells (our unpublished observations), which may be due to leakage of the receptors from the nuclei, because cultured cells are never in a perfectly physiological condition. However, very little VDR is seen in the cytoplasm of cultured osteosarcoma cells, suggesting that VDR might be strongly associated with nuclear components.

It is generally thought that concentrations of VDR are about one tenth of those of other steroid receptors [2]. We observed, however, an intense staining with our antibodies, so that the affinities of the present antibodies would seem to be high also to VDR denaturated by histological fixation. The immunohistochemistry of VDR in bone-derived cells has previously been described in rat [13, 40] and human osteoblasts [14, 39]. Our antipeptide antibodies for distantly located epitopes at the A/B- and D-domains will allow immunohistochemical studies at light- and electron-microscopic levels on the different (including partially degraded) VDR forms in the target tissues.

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