Woodruff, M. L., & Bownds, M. D. (1979) *J. Gen. Physiol. 73*, 629–653.
Woodruff, M. L., Bownds, D., Green, S. H., Morrisey, J. L., & Shedlovsky, A. (1977) *J. Gen. Physiol. 69*, 667–679.
Yau, K.-W., & Nakatani, K. (1984) *Nature (London) 311*, 661–663.
Yau, K.-W., & Nakatani, K. (1985) *Nature (London) 317*, 252–255.
Yoshikami, S., Robinson, W. E., & Hagins, W. A. (1974) *Science (Washington, D.C.) 185*, 1176–1179.
Yoshikami, S., George, J. S., & Hagins, W. A. (1980) *Nature (London) 286*, 395–398.
Zimmerman, W. F., Lion, F., Daemen, F. J. M., & Bonting, S. L. (1975) *Exp. Eye Res. 21*, 325–332.
Zuckerman, R., Schmidt, G. J., & Dacko, S. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6414–6418.
Zuckerman, R., Buzdygon, B., Philip, N., Liebman, P., & Sitaramayya, A. (1985) *Biophys. J. 47*, 37a.

Monoclonal Antibodies to the Porcine Intestinal Receptor for 1,25-Dihydroxyvitamin D_3: Interaction with Distinct Receptor Domains†

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ABSTRACT: Monoclonal antibodies to different domains of the porcine intestinal 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] receptor have been produced. A nuclear extract enriched in the 1,25-$(OH)_2D_3$ receptor was prepared from small intenstinal mucosa of young pigs. The receptor was purified an additional 6600-fold by chromatography on DNA–cellulose, ammonium sulfate precipitation, gel filtration high-performance liquid chromatography, and DEAE-Sepharose chromatography, with an overall yield of 23% and an average purity of 24%. A BALB/c mouse immunized with this material developed serum polyclonal antibodies to the 1,25-$(OH)_2D_3$ receptor, as demonstrated by a change in sedimentation of the porcine receptor on sucrose gradients. Spleen cells from this animal were fused with mouse myeloma cells (P3-NSI/1-Ag4-1, SP2/0-Ag14), and 24 hybridomas secreting antibodies to the 1,25-$(OH)_2D_3$ receptor were identified by both a radiometric immunosorbent assay and an immunoprecipitation assay. Twenty-one hybridoma lines were cloned by limiting dilution and further characterized as subclass IgG_1 antibodies with the exception of one which is an IgA. All but two of the antibodies cross-react with the 1,25-$(OH)_2D_3$ receptor from both mammalian (human, monkey, and rat) and avian (chicken) intestine; two antibodies recognize only porcine intestinal receptor. All antibodies are unreactive to the vitamin D serum transport protein. Eight of the antibodies bind denatured receptor on an immunoblot. A solid-phase competition assay was used to identify four groups of antibodies that bind to distinct epitopes on the 1,25-$(OH)_2D_3$ receptor. One antibody from each of the four groups was used to examine the effect of antibody binding on the DNA-binding activity of the receptor–hormone complex. One antibody completely inhibited the binding of the 1,25-$(OH)_2D_3$ receptor complex to DNA–cellulose, suggesting that the epitope for this antibody may be located in the polynucleotide binding domain of the protein. Antibodies from two additional groups only slightly perturbed DNA binding, while one had no effect, suggesting that these antibodies bind to receptor epitopes distant from the region of the polypeptide directly involved in polynucleotide binding. These antibodies that are directed to several different binding sites on the 1,25-$(OH)_2D_3$ receptor provide important new tools to probe the biochemistry and topology of the 1,25-$(OH)_2D_3$ receptor and to investigate its role in mediating target tissue response to hormone.

The 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] receptor plays a central role in target tissue responsiveness to hormone (Franceschi et al., 1981; Link & DeLuca, 1985). In mammals, 1,25-$(OH)_2D_3$ stimulates a complex biphasic response of intestinal calcium transport in the small intestine (Halloran & DeLuca, 1981); in concert with parathyroid hormone, it functions in the mobilization of calcium from bone and is believed to act on the distal renal tubule to facilitate reabsorption of calcium (DeLuca, 1983; DeLuca & Schnoes, 1983). Tissue responsiveness is mediated by 1,25-$(OH)_2D_3$ binding to a intracellular receptor protein (Brumbaugh & Haussler, 1973; Kream et al., 1976). The hormone–receptor complex is believed to stimulate the transcription of proteins involved in calcium and phosphorus transport and homeostasis. The ultimate function of 1,25-$(OH)_2D_3$ is the maintenance of blood calcium and phosphate levels to support normal bone growth and mineralization. The receptor may also mediate less well-elucidated functions of the hormone in other tissues and cells in which the protein has been described (Stumpf et al., 1979, 1980, 1981; Narbaitz et al., 1983; Haussler et al., 1984; Eisman, 1984; Suda et al., 1984).

The 1,25-$(OH)_2D_3$ receptor has been studied extensively in crude extracts from a variety of tissues and species by using radiolabeled hormone–receptor binding techniques (Link & DeLuca, 1985). The mammalian receptor sediments at 3.1–3.2 S on high-salt sucrose gradients (Kream et al., 1977)

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and has an equilibrium dissociation constant of approximately 10^{-10} M (4 °C) (Wecksler et al., 1979; Feldman et al., 1979). Molecular weight estimates based on gel filtration data for the mammalian 1,25-$(OH)_2D_3$ receptor vary widely (Wecksler et al., 1979; Simpson & DeLuca, 1980). Our laboratory recently reported a molecular weight of 55 000 for the porcine intestinal 1,25-$(OH)_2D_3$ receptor (Dame et al., 1985). This estimate is based on gel filtration high-performance liquid chromatography (HPLC) and identification of the receptor on sodium dodecyl sulfate–polyacrylamide gels.

Studies using frozen section autoradiography and high specific activity tritiated ligand have shed light on the tissue and cellular localization of the hormone (Zile et al., 1978; Stumpf et al., 1979), but conclusions regarding the location of unoccupied or potentially non-hormone binding forms of the receptor cannot be drawn from these indirect studies. Attempts to isolate the quantity of receptor at the necessary purity for physical or biochemical studies have met with limited success due to the low abundance of this receptor in tissue extracts (Pike & Haussler, 1979; Simpson & DeLuca, 1982; Simpson et al., 1983; Pike et al., 1983; Dame et al., 1985). The elegant hybridoma technology introduced by Köhler and Milstein (1975, 1976) provides a means by which antibodies can be produced to very low abundance, impure proteins. Monoclonal antibodies are also advantageous compared to the more traditional polyclonal antibodies because each antibody is directed to a specific region or epitope on the protein of interest. Because monoclonal antibodies can be generated to specific antigenic sites and potentially different functional domains on the protein, they should serve as useful tools to examine protein structure and function.

The hybridoma technique has been applied to the study of many different steroid hormone receptors including estrogen (Greene et al., 1980a,b, 1984; Moncharmont et al., 1982), progesterone (Radanyi et al., 1983; Logeat et al., 1983; Edwards et al., 1984), and glucocorticoid (Grandics et al., 1982; Westphal et al., 1982; Okret et al., 1984; Gametchu & Harrison, 1984) receptors. Pike and co-workers used a purified avian intestinal 1,25-$(OH)_2D_3$ receptor preparation to produce antibodies that cross-react with mammalian receptors and are directed against a single epitope on the 1,25-$(OH)_2D_3$ receptor (Pike et al., 1982; Pike, 1984). We report here the generation of a series of hybridoma cell lines that produce monoclonal antibodies to different domains on the mammalian intestinal 1,25-$(OH)_2D_3$ receptor.

Materials and Methods

Vitamin D Compounds. Radioactive 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (160 Ci/mmol) was prepared as previously described (Napoli et al., 1980). Nonradioactive 1,25-$(OH)_2D_3$ was a gift from the Hoffmann-La Roche Co. (Nutley, NJ). For receptor purification, 1,25-$(OH)_2$-[26,27-^{3}H]D_3 was adjusted to 2 Ci/mmol by the addition of nonradioactive 1,25-$(OH)_2D_3$.

Immunochemicals. Unlabeled and peroxidase-labeled affinity-purified goat anti-mouse IgG (H+L) antibodies were obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Affinity-purified goat anti-mouse IgG (H+L) antibodies coupled to Sepharose 4B was obtained from Cappel Scientific Division, Cooper Biomedical (Malvern, PA). Mouse immunoglobulin subtyping reagents were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Affi-Gel protein A for the purification of mouse monoclonal IgG was purchased from Bio-Rad Laboratories (Richmond, CA). Purified mouse myeloma proteins IgG_1 (MOPC 21, k), IgG_{2a} (RBC5, k), IgG_{2b} (MOPC141, k), IgG_3 (J606, k), IgA (MOPC315, λ2), and IgM (TEPC 183, k) and mouse clarified ascites containing IgG_1 (MOPC 21) were obtained from Litton Bionetics, Inc. (Kensington, MD). Immulon 1 Removawell strips and Removawell strip holders were purchased from Dynatech Laboratories, Inc. (Alexandria, VA).

Cell Culture Reagents. Fetal calf serum (FCS) was obtained from HyClone Laboratories (Logan, UT). Dulbecco's modified Eagle's medium (DMEM) was obtained from GIBCO (Grand Island, NY). Hypoxanthine, aminopterin, and thymidine were purchased from Sigma Chemical Co. (St. Louis, MO). All additional cell culture supplements were obtained from GIBCO. The myeloma cell line P3-NSI/1-Ag4-1 (NS1) was provided by Dr. Colleen Hayes and Dr. David Nelson, Department of Biochemistry, University of Wiconsin–Madison. The myeloma cell line SP2/0-Ag 14 (SP2/0) was provided by Dr. Robert Auerbach, Department of Zoology, University of Wisconsin–Madison.

Buffers. The composition of buffers follows: TE, 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4 (25 °C), and 1.5 mM ethylenediaminetetraacetic acid (EDTA); TED, TE plus 5 mM dithiothreitol; $TEDNa_{150}$, TED plus 150 mM NaCl; $TEDK_{75}$, TED plus 75 mM KCl; $TEDK_{300}$, TED plus 300 mM KCl; TED-Triton, TED plus 0.5% (v/v) Triton X-100; HPLC running buffer, 20 mM Na_2HPO_4 and 200 mM KCl (pH 6.8); HPLC sample buffer, HPLC running buffer (pH 7.4) plus 5 mM dithiothreitol; PBD, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4 (pH 7.4) with 5 mM dithiothreitol; PBS, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 (pH 8.0), 137 mM NaCl, and 2.7 mM KCl; PBS-Triton, PBS plus 0.5% (v/v) Triton X-100; PBS-Tween, PBS plus 0.05% (v/v) Tween 20.

1,25-$(OH)_2D_3$ Receptor Isolation. All steps in the isolation procedure were performed at 0–4 °C with the exception of gel filtration HPLC which was performed at room temperature. The small intestines were removed from eight immature pigs (40–90 lb) and placed in $TEDNa_{150}$ buffer. Mucosa was scraped from the serosa and washed 2 times with 2 volumes of $TEDNa_{150}$ buffer and 1 time with 2 volumes TED buffer by suspension and settling. A 33% tissue homogenate was prepared in TED buffer with two 15-s bursts by using a Polytron homogenizer (Brinkman Instruments, Westbury, NY) and a crude nuclear pellet obtained by centrifugation at 3000*g* for 30 min. This pellet was washed 3 times by resuspension in TED buffer and centrifugation at 3000*g* for 10 min. The 1,25-$(OH)_2D_3$ receptor was extracted from the washed nuclear pellet by homogenization in 2 volumes of $TEDK_{300}$ buffer containing 10 mM $MgCl_2$ by using three 20-s bursts from a Polytron homogenizer. Solubilized receptor was obtained by centrifugation at 27000*g* for 2 h in a GSA rotor (Sorvall–Du Pont, Wilmington, DE). The supernatant (nuclear extract) was frozen in 500-mL (1.5 g of protein) aliquots in liquid nitrogen and stored at −70 °C.

The nuclear extract (1500 mL) was diluted to 75 mM KCl with TED buffer and incubated overnight with 2×10^{-9} M 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (2 Ci/mmol). Hormone was added in absolute ethanol, which never exceeded a final concentration of 4%. The resulting tritiated hormone–receptor-containing solution was combined with ultraviolet-irradiated DNA–cellulose prepared according to the method of Litman (1968), and the receptor was purified as described previously (Dame et al., 1985). DNA–cellulose chromatography was followed by 40% ammonium sulfate precipitation, preparative gel filtration HPLC, and finally chromatography on diethylaminoethyl-(DEAE-) Sepharose. The partially purified receptor preparation obtained after DEAE-Sepharose chromatography was either lyophilized or used for immunization

without further concentration.

Preparation of Receptor Whole Cell Extracts. Receptor extracts were prepared from pig, monkey, human, rat, and chicken intestines. Duodenal mucosa was washed 3 times in 10 volumes of $TEDNa_{150}$ buffer by resuspension and centrifugation at 800*g*. Washed mucosa was homogenized in 2 volumes of $TEDK_{300}$ buffer by using 10 strokes of a motor-driven Teflon–glass homogenizer. Whole cell extracts were obtained by centrifuging the homogenates at 170000*g* for 45 min at 4 °C in a Beckman L5-50 centrifuge using a type 70.1 rotor (Beckman Instruments). The supernatants were frozen in liquid nitrogen and stored at −70 °C.

Quantitation of Hormone–Receptor Complexes. The hydroxylapatite (HAP) assay as described previously (Williams & Gorski, 1974; Wecksler & Norman, 1979; Dame et al., 1985) was used to quantitate the hormone–receptor complexes during sequential purification steps.

Immunization. A 2-month-old female BALB/c mouse received a series of six intraperitoneal injections of partially purified 1,25-$(OH)_2D_3$ receptor over 4 months. For the primary immunization, 15 μg of receptor was administered in an emulsion of Freund's complete adjuvant (GIBCO). Three and 5 weeks following the primary immunization, 10 μg of receptor was administered per injection in PBS (0.2–0.4 mL). At 8, 12, and 15 weeks, 10 μg of receptor was administered per injection (0.5 mL) in an emulsion of Freund's incomplete adjuvant. Four weeks after the last boost and 3 days prior to fusion, the mouse received an intravenous injection of 7 μg receptor in PBS (0.2 mL). The mouse was bled by retroorbital puncture prior to the primary immunization, 3 days following the boosts in PBS and 7 days following the injections in Freund's incomplete adjuvant. Serum was stored at −70 °C until assay for antireceptor antibody activity.

Sedimentation Analysis. Crude pig nuclear extract diluted with 1 volume of $TEDK_{300}$ was incubated at 4 °C overnight with 2×10^{-9} M 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (160 Ci/mmol) in the presence or absence of 2×10^{-7} M 1,25-$(OH)_2D_3$. Unbound hormone was removed by treatment with dextran-coated charcoal as described (Clarke et al., 1985). Hormone–receptor complexes were incubated with either preimmune or immune serum or with purified monoclonal antibodies for 1.5–3 h at 4 °C. This incubation mixture was layered on top of sucrose gradients prepared in $TEDK_{300}$ buffer. ^{14}C-Labeled proteins were included with samples as internal sedimentation standards. Gradients were centrifuged at 257000*g* for 18 h at 4 °C in a Beckman L5-50 ultracentrifuge using a SW60 rotor. The gradients were fractionated (0.1 mL) from the bottom by displacement with mineral oil and fractions assayed for radioactivity (^{3}H and ^{14}C) in 4 mL of Scint A by using a double-label counting program in a PRIAS scintillation counter.

Cell Culture. Myeloma and hybridoma cells were maintained in a humidified atmosphere of 92.5% air and 7.5% CO_2 at 37 °C. All cells were grown in DMEM supplemented with 10 or 15% FCS, glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (10 mM), 2-mercaptoethanol (50 μM), and *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.3 (10 mM) which comprised complete DMEM (cDMEM). For at least 1 week prior to fusion, mouse myeloma cells were maintained in logarithmic growth phase between 1×10^5 and 5×10^5 cells/mL in cDMEM containing 10% FCS. On the day of cell fusion, the spleen was removed aseptically from the immunized mouse and placed in a sterile Petri dish containing DMEM. A single cell suspension was obtained by gently pipetting up and down several times with a Pasteur pipet. Immune splenic lymphocytes (2.26×10^8 cells) and myeloma cells were collected separately by centrifugation at 400*g* for 10 min and washed 3 times by centrifugation and resuspension in DMEM. Viability of the lymphocytes was assessed at 84% prior to the third wash in DMEM. The viability of the mouse myeloma cell lines was greater than 95%. Fusion of spleen cells and myeloma cells was performed according to the technique of Köhler and Milstein (1975, 1976), as modified by Oi and Herzenberg (1980). Briefly, 1.6×10^8 spleen cells and 1.6×10^7 NS1 myeloma cells or 0.6×10^8 spleen cells and 0.6×10^7 SP2/0 myeloma cells were centrifuged together and fused by the addition of 1 mL of 50% poly(ethylene glycol) 1500 (Baker, Phillipsburg, NJ) in DMEM over 1 min followed by slow addition of 45 mL of DMEM and incubation for 5 min. Cells were centrifuged and resuspended in 5 mL of medium (HT) consisting of hypoxanthine (60 μM), thymidine (20 μM), penicillin (100 units/mL), and streptomycin (100 μg/mL). The cells were incubated at 37 °C in 7.5% CO_2 for 2.5 h, centrifuged, and gently resuspended in HT containing aminopterin (0.5 μM) and peritoneal macrophages (6×10^4 cells/mL). The cell suspension was dispensed into 96-well microtiter plates (Costar, Cambridge, MA) at approximately 10^5 cells/well in a total volume of 200 μL/well. Culture supernatants (125 μL) were removed for screening 13 days after fusion and replaced with 150 μL of fresh HT medium. Supernatants that tested positive for antireceptor antibody by two assays (as will be discussed) were expanded to nickel wells in cDMEM containing 20% FCS and antibiotics. These hybridomas were cloned by limiting dilution, expanded for antibody production in cDMEM containing 15% FCS, frozen at various points in the cloning and expansion procedure in cDMEM containing 40% FCS and 10% dimethyl sulfoxide and stored in liquid nitrogen or at −70 °C.

In Vivo Production of Hybridoma Antibodies. Ascites tumors were produced in 2–3-month-old BALB/c mice as follows. The mice were given an intraperitoneal injection of 0.5 mL of 2,6,10,14-tetramethylpentadecane (Aldrich Chemical Co.) 10 and 3 days prior to the injection of individual hybridoma cell lines. Hybridomas were harvested in midlogarithmic growth, centrifuged at 400*g* for 10 min, and washed once with DMEM by resuspension and centrifugation. Hybridoma cells were resuspended at a density of $(2–6) \times 10^6$ cells/0.5 mL and injected intraperitoneally. Ascites fluid was collected when the abdomen was distended and centrifuged to remove red cells and lipid. The fluid was stored at −70 °C until use.

Receptor–Hormone Complexes. Tritiated hormone–receptor complexes used in screening supernatants from the fusion and cloning plates were obtained by partial purification of pig nuclear extract. The extract was diluted approximately 3-fold with TED, incubated overnight at 4 °C with 1×10^{-9} M 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (160 Ci/mmol), and purified by DNA–cellulose chromatography. For use in additional assays, pig nuclear extract was diluted 2-fold with $TEDK_{300}$ buffer and incubated overnight at 4 °C with 2×10^{-9} M 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (160 Ci/mmol). Parallel extracts were incubated with a 100-fold excess of 1,25-$(OH)_2D_3$ to assess nonspecific binding.

Radiometric Immunosorbent Assay (RISA). The RISA was performed as described previously (Pierce et al., 1986). Immulon 1 Removawell strips were coated with 50 μL of goat anti-mouse IgG (H+L) (40 μg/mL) in PBS overnight at 4 °C. After removal of the goat anti-mouse IgG reagent, the wells were filled with 1.5% BSA in PBS (150 μL) and incu-

bated for 30 min at room temperature. The BSA was removed, and the wells were washed 4 times with cold PBS by using a Dynatech platewasher. Hybridoma supernatant (50 μL) from the fusion plates, cloning plates, or cell expansions was incubated in the wells for 2 h at room temperature. This was followed by removal of the supernatant and washing 4 times with cold PBS. The cells were placed on ice, and 50 μL of either partially purified 1,25-$(OH)_2$-[26,27-^{3}H]D_3–receptor complexes (fusion and cloning plates) or crude nuclear extract labeled with tritiated 1,25-$(OH)_2D_3$ with and without a 100-fold excess of nontritiated hormone was added and incubated in the wells for 1 h at 4 °C. Unbound tritiated hormone–receptor complexes and free ligand were removed and the wells washed 4 times with cold PBS-Triton. Individual wells were combined with 4 mL of Scint A in a scintillation vial and counted by using a Prias scintillation counter with approximately 40% efficiency for ^{3}H.

Immunoprecipitation. Tritiated hormone–receptor complexes (50–75 μL of post-DNA–cellulose purified receptor or 50 μL of crude nuclear extract) were incubated with an equivalent volume of hybridoma supernatant at 4 °C for 2–4 h. A 10% (v/v) suspension of either goat anti-mouse IgG Sepharose (20 μL) or formalin-fixed Staph A cells (20 μL) in PBS was added and the mixture incubated at 4 °C for 1 h, vortexing every 20 min. The tubes were centrifuged at either 1500*g* (Sepharose) or 400*g* (Staph A) for 5 min at 4 °C. The supernatant was discarded and the pellet washed 2 times by resuspension in PBS–Triton and centrifugation. The wash volume was equivalent to the final incubation volume. The Sepharose beads were transferred to scintillation vials with two 500-μL washes of ethanol. Staph A was transferred by using two 250-μL washes of PBS-Triton. Scint A (4 mL) was added to all vials and radioactivity counted.

Heavy- and Light-Chain Typing of Monoclonal Antibodies. The immunoglobulin heavy- and light-chain subtypes were determined by enzyme immunoassay using a kit from Boehringer Mannheim Biochemicals. The specificity of rabbit antiserum against individual mouse immunoglobulin heavy-chain subtypes was verified with purified mouse myeloma proteins (IgA, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and IgM).

Immunoblotting. Partially purified porcine intestinal receptor was used to test the ability of the antibodies produced by 24 hybridoma lines to recognize the denatured receptor on an immunoblot. Immunoblotting was performed as described previously with peroxidase-labeled second antibody (Dame et al., 1985).

Species Cross-Reactivity. Intestinal cytosols were labeled as described in studies using pig nuclear extract, and cross-reactivity with monoclonal antibody from hybridoma supernatants was examined by the RISA. Monoclonal antibodies were also tested for reactivity with the human serum vitamin D binding transport protein by RISA and an enzyme-linked immunosorbent assay (ELISA) (Pierce et al., 1985).

Antibody Epitopes on the 1,25-$(OH)_2D_3$ Receptor. A solid phase competition assay was developed to determine how many monoclonal antibodies bind to different epitopes on the receptor molecule. In this assay, the receptor–hormone complex was bound to antireceptor antibody immobilized on a solid phase. The ability of biosynthetically labeled antireceptor antibody to bind to the preformed receptor–antibody complex was used to determine whether the two antireceptor antibodies recognize different epitopes on the protein. Monoclonal antibodies were biosynthetically labeled by incubating individual clonal hybridoma lines (5×10^6 cells/line) in 10 mL of methionine-free cDMEM prepared with 15% dialyzed FCS and supplemented with L-[^{35}S]methionine (0.5 mCi, 1100 Ci/mmol, translation grade, du Pont/New England Nuclear) for 21 h. The culture supernatant containing the L-[^{35}S]methionine-labeled antibody was dialyzed extensively against PBS containing 0.01% NaN_3 and 50 μM 2-mercaptoethanol to remove free L-[^{35}S]-methionine. The 1,25-$(OH)_2D_3$ receptor used in these studies was partially purified from pig nuclear extract (no added hormone) by DNA–cellulose chromatography and subsequently incubated with 1×10^{-8} M 1,25-$(OH)_2D_3$. Parallel samples were labeled with 1×10^{-8} M 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (160 Ci/mmol). The solid-phase immunosorbent assay was performed as follows. Immulon 1 Removawells were coated with 50 μL of goat anti-mouse IgG (H+L) (50 μg/mL) in PBS overnight at 4 °C. The goat anti-mouse reagent was removed, and the wells were washed 3 times with cold PBS. Clonal hybridoma supernatants (50 μL/well) were added to the wells and incubated for 90 min at room temperature. The supernatants were removed and the wells washed 3 times with PBS. This was followed by the addition of 50 μL of mouse IgG_1 (20 μg/mL), incubation for 15 min at room temperature, the addition of 175 μL of 1.5% BSA in PBS, and a 15-min additional incubation at room temperature in the presence of both mouse IgG_1 and BSA. The blocking agents were removed and the wells washed 3 times with cold PBS and placed on ice. Partially purified receptor (50 μL) bound to nonradiolabeled hormone was added and incubated for 60 min followed by its removal and 3 washes with cold PBS. Parallel plates were incubated with BSA (protein concentration equivalent to receptor solution) containing 1×10^{-8} M 1,25-$(OH)_2D_3$ in $TEDK_{300}$ and carried through the remainder of the assay in similar fashion to the receptor positive plates. Finally, L-[^{35}S]methionine-labeled antibody in PBS (approximately 250 000 cpm/50 μL) was added to the wells and incubated for 45 min on ice. This solution was removed, and the plates were washed 4 times with cold PBS-Tween. The wells were broken apart and combined with Scint A (4 mL) and counted. An additional set of plates was run as described above except that the receptor solution was labeled with 1.0×10^{-8} M tritiated 1,25-$(OH)_2D_3$ (160 Ci/mmol), and the L-[^{35}S]-methionine-labeled antibody incubation was replaced with similarly diluted supernatant from the parent myeloma line (NS1). In a second set of competition experiments, monoclonal antibodies (40 μg/mL) purified from hybridoma supernatant using Affi-Gel protein A were used to coat wells directly followed by BSA block and the remainder of the assay as described earlier.

Effect of Antibody on Receptor–DNA–Cellulose Binding. Pig nuclear extract diluted with 3 volumes of TED was incubated at 4 °C overnight with 2×10^{-9} M 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (160 Ci/mmol). A parallel sample was incubated with 100-fold excess 1,25-$(OH)_2D_3$ to assess nonspecific binding. Hormone–receptor complexes (50 μL) were incubated with ascites fluid diluted 25 times in $TEDK_{75}$ (25 μL) for 3 h at 4 °C. Ascites from the MOPC21-derived tumor was used as a control. Triplicate samples were then incubated with 50 μL of a 50% suspension of DNA–cellulose or with 40 μL of a 50% suspension of goat anti-mouse IgG–Sepharose for 1 h at 4 °C followed by centrifugation at 800*g* for 5 min. The supernatant was removed, and the pellets were washed 3 times with 250 μL of TED-Triton by resuspension and centrifugation and transferred with two 500-μL washes of absolute ethanol to a scintillation vial and combined with Scint A (4 mL). Additional samples were assayed for hormone binding activity using the HAP procedure.

Table I: Purification of the Porcine 1,25-$(OH)_2D_3$ Receptor Protein

	sp act. (pmol/mg)	purification (x-fold)	yield (%)	purity (%)
nuclear extract (10)[a]	0.635	1	100	0.0035
DNA–cellulose (10)	47.2	74	51	0.26
$(NH_4)_2SO_4$ ppt (10) and size-exclusion HPLC (25)	629	990	27	3.8
DEAE-Sepharose (6)	4170	6600	23	24

[a] Number of batches or columns.

General Procedures. Protein concentration was determined by the method of Bradford (1976) using crystalline bovine serum albumin as a standard. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed using the buffer system of O'Farrell (1975) with minor modifications (Dame et al., 1985). Gels wre prepared for fluorography using EN^{3}HANCE according to manufacturer's directions.

Results

1,25-$(OH)_2D_3$ Receptor Purification. The 1,25-$(OH)_2D_3$ receptor was partially purified from pig intestinal mucosa starting with the preparation of a crude nuclear extract. The recovery of receptor binding activity after each step was assessed by the HAP binding procedure and fold purification assessed by measurement of total protein and use of a molecular weight estimate of 55 000 for the receptor protein. Labeled nuclear extract was incubated with DNA–cellulose, washed in batch form with $TEDK_{75}$, and eluted in column form by using a high ionic strength buffer ($TEDK_{300}$). The chromatographic profile of this column is shown in Figure 1A. The 1,25-$(OH)_2D_3$ receptor typically isolated from this column was purified approximately 74-fold with a 51% yield. This step was followed by precipitation with 40% ammonium sulfate which resulted in nearly quantitative recovery of receptor hormone binding activity. The precipitate could be conveniently stored in pellet form at −70 °C for several weeks until purified further. The ammonium sulfate precipitate was diluted (3–5 mg/mL) and applied in two or three 2-mL injections on a preparative gel filtration HPLC column with guard column. This resulted in separation of the fractions containing significant bound radioactivity from the major protein peak which appeared in the void volume of the column (Figure 1B). The ammonium sulfate precipitation and gel filtration steps gave a combined 13-fold purification with an overall yield of 53%. The receptor-containing fractions from the gel filtration column were pooled (approximately 30 mL/run), diluted with 1 volume of PBD, and applied to the final separation procedure. An additional 7-fold purification was achieved by using DEAE-Sepharose chromatography with 85–90% recovery of receptor binding activity (Figure 1C). The receptor preparation was collected in a buffer and in many cases a volume appropriate for injection into animals. A summary of the overall purification and yield of the receptor isolation procedure is shown in Table I. This table represents data obtained from 10 DNA–cellulose columns, 25 gel filtration HPLC separations, and 6 DEAE-Sepharose columns. The procedure resulted in a preparation that was on average 24% pure with an overall yield of 23%. A silver-stained gel of fractions from the DEAE-Sepharose column is shown in Figure 2; lane 4 represents the peak fraction (no. 20) from the DEAE-Sepharose column.

Demonstration of Serum Antireceptor Antibodies. Serum anti-1,25-$(OH)_2D_3$ receptor antibodies were demonstrated by using gradient ultracentrifugation analysis. Incubation of tritiated hormone–receptor complexes with immunized mouse

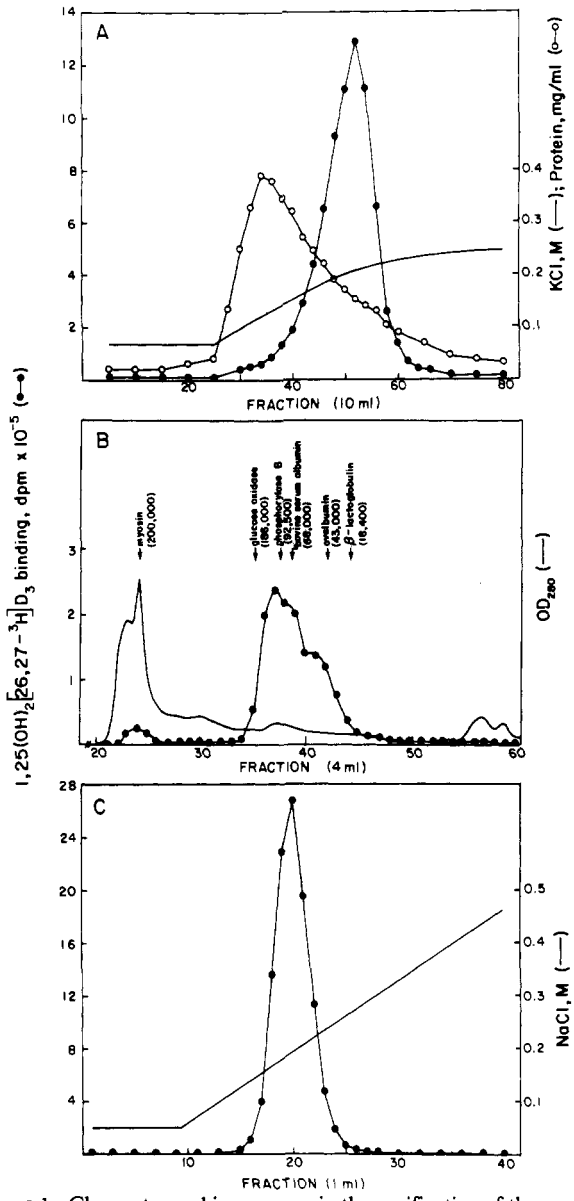

FIGURE 1: Chromatographic sequence in the purification of the porcine intestinal 1,25-$(OH)_2D_3$ receptor. (A) DNA–cellulose chromatography. Nuclear extract (1500 mL) was diluted with TED buffer (~1.6 mg/mL) and labeled overnight with 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (2 Ci/mmol) as described under Materials and Methods. DNA–cellulose was added to the labeled receptor fraction (10%, v/v) and incubated for 1 h at 0–4 °C. The mixture was centrifuged at 200*g* for 5 min and decanted and the pellet washed 2 times with 9–10 volumes $TEDK_{75}$ buffer. The washed pellet was resuspended to a 33% slurry in $TEDK_{75}$ buffer, poured into a 50 mm × 33 cm column, and washed extensively, followed by elution with $TEDK_{300}$ buffer at a flow rate of 100 mL/min. Aliquots of fractions were assayed for 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (●), protein (○), and KCl concentration (—). Fractions 42–60 were pooled, brought to 40% with solid ammonium sulfate, and centrifuged at 7000*g* in a GSA rotor. The pellet was stored at −70 °C. (B) HPLC gel filtration chromatography. The ammonium sulfate precipitate was resuspended in HPLC sample buffer to a protein concentration of approximately 3 mg/mL. Aliquots (2 mL) were chromatographed at room temperature on a preparative TSK gel filtration column, type G3000SW (64 mm × 2.5 cm) with guard (10 cm × 2.5 cm) in HPLC running buffer at a flow rate of 4 mL/min. Aliquots of fractions were assayed for 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (●), and protein concentration was followed by absorbance at 280 nm (—). The relative elution position of protein standards (100–200 μg/mL) under similar chromatographic conditions is shown by the arrows. (C) DEAE-Sepharose chromatography. Receptor-containing fractions (35–43) from six HPLC gel filtration column runs (two DNA–cellulose column equivalents) were pooled, diluted with 1 volume of PBD, and applied to a DEAE-Sepharose column (7 mL bed volume) at a flow rate of 20 mL/h. The receptor was eluted with a 30-mL 50–500 mM NaCl gradient in PBD. Aliquots of fractions were assayed for 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (●) and for NaCl (—) by conductivity measurement.

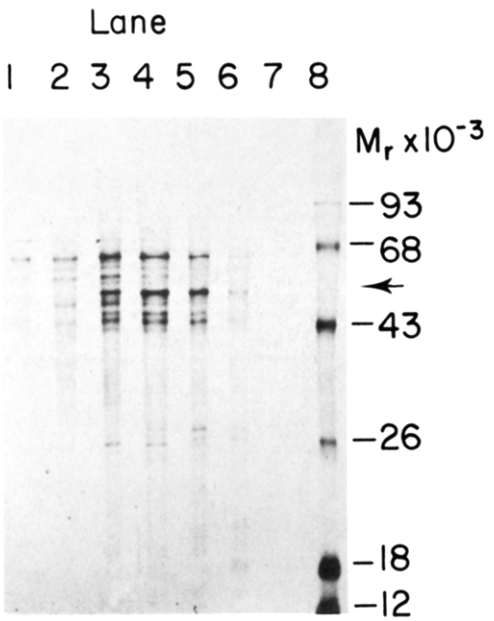

FIGURE 2: Electrophoretic analysis of fractions after DEAE-Sepharose chromatography. Aliquots (5 μL) of fractions 14, 16, 18, 20, 22, and 24 were combined with sample buffer and resolved in 10% sodium dodecyl sulfate–polyacrylamide gels with a 4.75% stacking gel (lanes 1–6). The separating gel was silver-stained. Lane 7 is a column elution buffer blank, and lane 8 shows silver-stained molecular weight markers: phosphorylase B (92 500), bovine serum albumin (68 000), ovalbumin (43 000), α-chymotrypsinogen (25 7000), β-lactoglobulin (18 400), and cytochrome *c* (12 300). The arrow indicates the position of the 1,25-$(OH)_2D_3$ receptor. The estimated purity of pooled fractions 18–23 from this DEAE-Sepharose column was 58%.

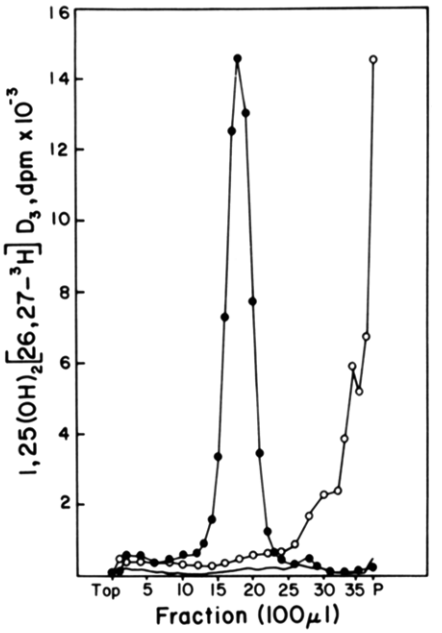

FIGURE 3: Detection of serum anti-1,25-$(OH)_2D_3$ receptor antibodies by sedimentation analysis. 1,25-$(OH))_2$-[26,27-^{3}H]D_3–receptor complexes (0.15 pmol) in nuclear extract that had been treated with dextran–charcoal were incubated with either 5 μL of preimmune serum (●) or immune serum (○) for 1.5 h at 0–4 °C and then layered on top of 3.6-mL 4–20% sucrose gradients in $TEDK_{300}$ buffer. An additional receptor-containing sample labeled with tritiated hormone containing a 100-fold excess of unlabeled hormone was incubated with immune serum to assess nonspecific hormone–receptor–antibody binding (—).

serum after week 12 (injection 5) resulted in a quantitative shift of the characteristic 3.2S 1,25-$(OH)_2D_3$ porcine receptor to the bottom of the tube (Figure 3). This shift was not observed with preimmune mouse serum. The radioactive fractions observed were specific for the 1,25-$(OH)_2D_3$ receptor since the addition of 100-fold excess nontritiated hormone to the original incubation displaced the radioactivity from the 3.2S region in the presence of preimmune serum and the larger sedimenting species when immune serum was used.

Antibody Production by Mouse–Mouse Hybridomas. Hybridomas secreting antibody against the 1,25-$(OH)_2D_3$ receptor were first detected by using the RISA assay. Wells with a signal 2 times above background radioactivity (99 wells) were rescreened by immunoprecipitation, and 1.2% (24/2016) of the wells originating from the fusion were positive for the presence of antireceptor antibody activity by these two criteria. Twenty of these hybridomas resulted from the fusion with the NS1 myeloma cell partner and four from SP2/0. Twenty-one of these 24 cell lines were cloned by limiting dilution. According to statistical probability theory, if less than 63% of the wells show growth, then colonies plated at that density may be considered clonally derived (Oi & Herzenberg, 1980). In this study, more stringent criteria were used. All hybridoma lines were cloned until at a given plating density there were no more than 25% of the wells showing growth.

Supernatants from these clonal populations were tested for specific 1,25-$(OH)_2D_3$ receptor antibody activity by using crude antigen from nuclear extract labeled with 1,25-$(OH)_2$-[26,27-^{3}H]D_3 with and without the addition of excess nontritiated hormone. All 21 supernatants tested resulted in the retention of specific 1,25-$(OH)_2D_3$ receptor binding activity (data not shown). A similar experiment performed using the immunoprecipitation assay confirmed the specificity of the monoclonal antibodies for the 1,25-$(OH)_2D_3$ receptor. A parallel immunoprecipitation using Formalin-fixed Staph A cells showed that only one monoclonal antibody is recognized well by protein A.

Specificity of the resultant antibodies for the 1,25-$(OH)_2D_3$ receptor was also demonstrated by sedimentation analysis. The sedimentation profile of the 1,25-$(OH)_2D_3$ receptor incubated with the purified clones IVA7E7, VD2F12, VIIID8C12, and XVIE6E6G10 or control IgG_1 is shown in Figure 4. In all cases, incubation of the hormone–receptor complex with monoclonal antibody resulted in a shift of the characteristic 3.2S receptor peak to a larger 7S to 8S sedimenting species.

Purified 1,25-$(OH)_2D_3$ receptor was resolved by electrophoresis under denaturing conditions and transferred to nitrocellulose filters. Monoclonal antibodies from clonal hybridoma supernatants were tested for the ability to recognize 25 ng of receptor protein on immunoblots by using goat anti-mouse peroxidase (second antibody) and 4-chloro-1-naphthol substrate for detection. For the eight monoclonal antibodies capable of immunoblotting, the highest molecular weight species appearing on the immunoblot in all cases corresponded to the protein identified previously as the purified porcine 1,25-$(OH)_2D_3$ receptor (data not shown). Three additional lower molecular weight bands showed faint staining on the blots probed with antireceptor antibodies, but no bands were present when a control (NS-1 supernatant) preparation was used. These bands most likely represent proteolytic cleavage products of the receptor.

Characterization of the monoclonal antibodies using class- and subclass-specific anti-mouse immunoglobulin antisera in an ELISA showed that all but one are IgG_1 and one is an IgA (IVB12G12).

Species Cross-Reactivity. The antibodies were tested by the RISA for their ability to recognize the 1,25-$(OH)_2D_3$ receptor in extracts of monkey, rat, and chicken intestinal mucosa. All but two of the hybridomas cross-react with in-

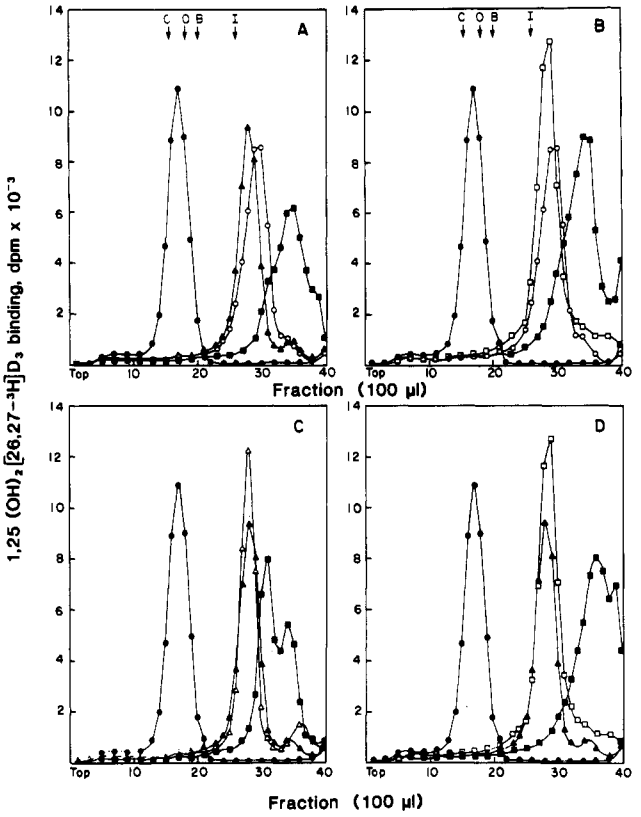

FIGURE 4: Sedimentation profile of 1,25-$(OH)_2$-[26,27-^{3}H]D_3–receptor complexes from pig nuclear extract incubated with pure monoclonal IgG_1 antibodies. (A) Aliquots of the tritiated hormone–receptor complex (0.2 pmol), with unbound hormone removed by dextran–charcoal, were incubated with 25 µg of control IgG_1 (MOPC 21) (●) or with 12.5 µg each of antibodies IVA7E7 and control IgG_1 (○), antibodies XVIE6E6G10 and control IgG_1 (▲), or antibodies IVA7E7 and XVIE6E6G10 (■) in a final volume of 300 µL. After 2.5 h at 0–4 °C, 25 µL of ^{14}C-methylated protein standard was added to each tube, and the samples (220 µL) were layered on top of 3.6 mL of 10–30% (w/v) sucrose gradients in $TEDK_{300}$ buffer and centrifuged at 257000*g* for 18 h at 2 °C. Arrows show the migration of internal sedimentation standards: carbonic anhydrase (C, 3.0 S), ovalbumin (O, 3.7 S), bovine serum albumin (B, 4.4 S), and γ-globulin (I, 7.0 S). (B) Same as (A) except monoclonal antibodies were IVA7E7 and control IgG_1 (○), VD2F12 and control IgG_1 (□), and IVA7E7 and VD2F12 (■). (C) Same as (A) except monoclonal antibodies were VIIID8C12 and control IgG_1 (△), XVIE6E6G10 and control IgG_1 (▲), and VIIID8C12 and XVIE6E6G10 (■). (D) Same as (A) except monoclonal antibodies were VD2F12 and control IgG_1 (□), XVIE6E6G10 and control IgG_1 (▲), and VD2F12 and XVIE6E6G10 (■).

testinal receptor from both mammalian and avian sources. Two of the hybridoma lines produce antibodies that bind to only porcine intestinal receptor. Seventeen of the original 24 lines were retested by using cell extracts prepared from pig, rat, monkey, human, and chicken intestine with similar results (Table II). The interaction appears to be of similar affinity regardless of species tested because a similar percentage of receptor specific binding activity was retained when equivalent quantities of receptor were used in either the RISA or immunoprecipitation assay. None of the monoclonal antireceptor antibodies cross-react with the serum vitamin D binding transport protein. This is true when the 1,25-$(OH)_2$-[26,27-^{3}H]D_3 receptor complex is replaced by 25-OH_2-[26,27-^{3}H]D_3–vitamin D binding protein complex in the RISA or when pure vitamin D binding protein is used in an ELISA assay.

Mapping the Receptor Epitopes. In order to determine which hybridoma lines produce antibodies that bind to different determinants on the receptor protein, a solid-phase immunosorbent competition assay was used. In this solid-phase assay only receptor bound to antibody will be retained in the well and, therefore, available for binding to the L-[^{35}S]-methionine-labeled antibody. If the L-[^{35}S]methionine-labeled antibody binds to a different determinant than the unlabeled antibody, the L-[^{35}S]methionine-labeled antibody will be retained. If, however, the L-[^{35}S]methionine-labeled antibody binds to the same or an overlapping determinant on the receptor molecule, no radioactivity will be found. This analysis assumes no epitope is repeated more than once on the receptor molecule and that binding order is not important.

In the first set of experiments, 17 separate clonal hybridoma lines were assayed against eight clonal hybridoma lines labeled in culture with L-[^{35}S]methionine. An enriched receptor preparation (generated by passage of unlabeled pig nuclear extract over a DNA–cellulose column) was used to enhance the sensitivity of the competition assay. The results of these competition studies indicate that monoclonal antibodies recognizing at least four distinct epitopes on the receptor molecule were generated. These results have been confirmed in a second experiment in which the goat anti-mouse antibody step was eliminated and eight pure monoclonal antibodies or control IgG_1 was used directly in the assay. Each purified antibody was probed against itself as a control.

The data in Figure 5A shows the variable amount of 1,25-$(OH)_2$-[26,27-^{3}H]D_3 receptor complexes retained by individual antibodies when either the goat anti-mouse method (left panel) or purified monoclonal antibodies (right panel) was used. Representative examples of L-[^{35}S]methionine-labeled monoclonal antibodies used to probe receptor bound to antibody from different hybridoma supernatants or purified monoclonal antibodies are shown in Figure 5(B–E). The L-[^{35}S]methionine-labeled antibody used in Figure 5B is one of the two antibody lines showing no cross-reactivity with intestinal receptor from other mammalian or avian species and therefore serves as an additional control for the validity of the competition assay. Antibody XVIE6E6G10 clearly binds to an epitope distinct from antibodies IVA7E7, IVG8C11, VD2F12, and VIIID8C12, which are all antibodies that display cross-reactivity. The absence of retained radioactivity by the other non-cross-reactive antibody (XVIE10B6A5) suggests that it binds to the same or an overlapping determinant on the porcine receptor molecule as antibody XVIE6E6G10. The data shown in Figure 5C,D suggest that antibodies VIIID8C12 and VD2F12 bind to receptor epitopes separate from the other antibodies shown. For antibodies that are poorer receptor binders such as VIIID8C12 and VD2F12, elimination of the goat anti-mouse IgG step and direct use of the purified monoclonal antibody decrease assay variability. The results in Figure 5E in which IVA7E7 is radiolabeled, combined with the similar responsiveness of unlabeled antibodies IVA7E7 and IVG8C11 in panels B–D, indicate that these two antibodies bind to a similar or overlapping receptor epitope. The purity of the monoclonal antibodies used in the second set of competition studies was confirmed by electrophoresis under denaturing conditions followed by staining with Coomassie blue (Figure 6A). These same antibodies labeled with L-[^{35}S]-methionine in culture were electrophoresed on sodium dodecyl sulfate–polyacrylamide gels and examined by fluorography. This experiment demonstrates that the major labeled polypeptides correspond to light and heavy chains of the mouse immunoglobulins (Figure 6B).

The four antibody groups identified by these competition studies were further examined by sucrose density gradient analysis. When antibodies IVA7E7, IVD2F12, VIIID8C12,

Table II: Cross-Reactivity of Monoclonal Antibodies

hybridoma	intestinal 1,25-$(OH)_2D_3$ receptor[a] source					vitamin D binding protein
	pig	rat	monkey	human	chicken	human
IVA7E7[c]	+++	+++	+++	+++	+++	-
IVB12G12[b,c]	++	++	++	++	++	-
IVG8C11[c]	+++	+++	+++	+++	+++	-
VA3C12	+	+	+	+	+	-
VB3F12	+	+	+	+	+	-
VD2F12[c]	++	++	++	++	++	-
VIIA1E9A7	+	+	+	+	+	-
VIIIB8B2A9	+	+	+	+	+	-
VIIID8C12[c]	+	+	+	+	+	-
VIIIF7E12	+	+	+	+	+	-
IXD5F10	+	+	+	+	+	-
XA9E8	+	+	+	+	+	-
XH12G11[c]	+	+	+	+	+	-
XIID9G5D6	+	+	+	+	+	-
XVA9A7E6	+	+	+	+	+	-
XVIE6E6G10[c]	+++	-	-	-	-	-
XVIE10B6A5[c]	+++	-	-	-	-	-
control (cDMEM)	-	-	-	-	-	-

[a] Specific 1,25-$(OH)_2D_3$ receptor binding (fmol) and total protein (mg) added per well (50 μL) were the following: pig, 67 and 0.25; rat, 46 and 0.20; monkey, 47 and 0.27; human, 2.1 and 0.04; chicken, 34 and 0.28, respectively. The symbol (+) indicates the relative amount of hormone–receptor complex retained by individual antibodies. In general, (+++) = ≥20% of added specific binding was retained in the well, (++) = ≥10% and <20%, and (+) = <10% and ≥0.5%. [b] IgA; all others $IgG_{1,k}$. [c] Antibodies that bind pig receptor on an immunoblot.

and XVIE6E6G10 were added in excess to tritiated hormone–receptor complexes in nuclear extract and analyzed by sedimentation analysis, a 7S to 8S sedimenting species resulted (Figure 4). When antibodies IVA7E7 and XVIE6E6G10 were coincubated with tritiated hormone–receptor complexes, a larger sedimenting species was formed, thus confirming the difference in binding epitopes on the receptor molecule for these two antibody species. A difference in antibody epitopes was also confirmed by sedimentation analysis for IVA7E7 and VD2F12 (Figure 4B), VIIID8C12 and XVIE6E6G10 (Figure 4C), and VD2F12 and XVIE6E6G10 (Figure 4D). Examination of antibody binding sites on the receptor by sedimentation analysis, however, did not reveal all the differences that were evident when the solid-phase competition assay was used. No difference in sedimentation position was noted when antibodies IVA7E7 and VIIID8C12 were incubated with the labeled receptor individually or together. This was also the case when VIIID8C12 was used in combination with VD2F12 (data not shown).

Antibody–Receptor Interaction and DNA–Cellulose Binding. To explore the effects of these four antibody groups on receptor function, antibody from ascites fluid was preassociated with receptor in nuclear extract and the effect of the antibody on the receptor–DNA–cellulose binding interaction examined. Parallel samples were examined for immunoprecipitable receptor binding activity. The immunoprecipitable binding activity represented 78–88% of the total receptor binding activity as assessed by the HAP procedure. The results (Figure 7) show that one antibody (VIIID8C12) completely inhibits the ability of the antibody–receptor complex to bind to DNA–cellulose. Two other antibody groups only slightly perturb this interaction (IVA7E7, VD2F12), and one antibody group has no effect (XVIE6E6G10). These results suggest that antibody VIIID8C12 may bind to or near the putative DNA binding site on the receptor molecule. In addition, these data confirm the results from the solid-phase competition studies showing that antibody VIIID8C12 is directed against a region on the 1,25-$(OH)_2D_3$ receptor distinct from the regions recognized by the other three antibody groups.

Discussion

Rapid advances are being made in our understanding of the structure and function of a variety of steroid hormone receptors due to the application of new technologies such as monoclonal antibody production. This technique is uniquely suited for application to the receptor field because these proteins are found in such low abundance, and usually only limited purification is possible when conventional techniques are used. We report here a four-step purification of a mammalian 1,25-$(OH)_2D_3$ receptor to 24% purity with an overall yield of 23%. This receptor has been identified on denaturing polyacrylamide gels as a polypeptide with a molecular weight of 55 000 (Dame et al., 1985). These data demonstrate the use of this partially purified porcine intestinal 1,25-$(OH)_2D_3$ receptor to elicit an immune response in a BALB/c mouse. By use of the hybridoma technique (Köhler & Milstein, 1975, 1976), the splenic lymphocytes from this animal were fused with mouse myeloma cells to produce 24 hybridoma lines that secrete antibodies directed against the 1,25-$(OH)_2D_3$ receptor. A RISA (Pierce et al., 1986), in which tritiated hormone–receptor complexes are retained by hybridoma antibody bound to goat anti-mouse IgG on a solid phase, was used as a sensitive and fast assay to identify hybridomas producing antireceptor antibodies. This technique has also been used to detect monoclonal antibodies to the human vitamin D binding serum transport protein and is generally applicable to the detection of antibodies to any protein that binds an identifiable ligand (Pierce et al., 1986). An immunoprecipitation assay was used to confirm the positive wells first identified by the RISA. The two-step screening process required that the antibody, whether free in solution or bound to a solid phase, be capable of binding the receptor. The immunoprecipitation assay confirmed that 24% of the original positive lines identified by the RISA contained antireceptor antibody activity. The data from the RISA represent one value for bound tritium obtained per well in the original fusion plate. A certain percentage of both false positives and negatives might be expected due to assay error. It is possible that several of the hybridomas identified by the RISA did not represent false positives but were antibodies that worked better when immobilized than free in solution.

The specificity of the resultant monoclonal antibodies was demonstrated by RISA using a crude nuclear receptor preparation labeled with a saturating concentration of 1,25-$(OH)_2$-[26,27-^{3}H]D_3 in the presence and absence of excess unlabeled hormone. The presence of antireceptor antibodies

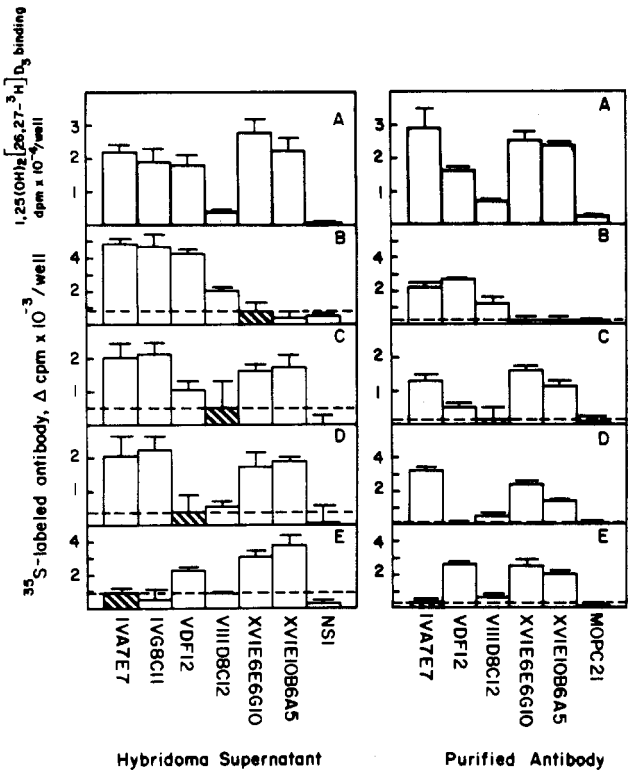

FIGURE 5: Identification of different antibody binding sites on the porcine intestinal 1,25-$(OH)_2D_3$ receptor. Representative data from the L-[^{35}S]methionine-labeled antibody competition studies (described under Materials and Methods). (A) 1,25-$(OH)_2$-[26,27-^{3}H]D_3–receptor complexes retained by monoclonal antireceptor antibodies from six hybridoma supernatants and a control supernatant (NS1) (left panel) or tritiated hormone–receptor complexes retained by five purified monoclonal antireceptor antibodies and a control IgG_1 (right panel). (B–E) Representative data from the solid-phase competition studies using four different L-[^{35}S]methionine-labeled monoclonal antibodies to probe 1,25-$(OH)_2D_3$ receptor bound to antibodies from hybridoma supernatants (left panel) or purified monoclonal antibodies (right panel). L-[^{35}S]Methionine-labeled antibodies include XVIE6E6G10 (B), VIIID8C12 (C), VD2F12 (D), and IVA7E7 (E). Data are expressed as Δcpm = (cpm retained in the presence of receptor) − (cpm retained in the presence of BSA). The dashed line (---) represents background for each L-[^{35}S]methionine-labeled antibody probed against itself (hatched bars). Data are mean ± SD.

in the serum of the immunized mouse and the specificity of the monoclonal antibodies produced from the fusion was also confirmed by demonstration of a shift of the characteristic 3.2S sedimenting porcine receptor species on high-salt sucrose density gradients in the presence of immune serum or purified monoclonal antibody (Figures 3 and 4). This shift did not take place when receptor was incubated in the presence of either preimmune serum or a control IgG_1 (MOPC21). The shift of the receptor peak to the bottom of the tube in the presence of immune serum indicates the polyvalent nature of this interaction. Use of individual pure monoclonal antireceptor antibodies bound to labeled receptor in all cases resulted in a peak that migrated in the 7S to 8S region of the gradient confirming the monovalent nature of the these interactions. Further characterization of these antibodies using class- and subclass-specific anti-mouse immunoglobulin antisera confirms that all but one of the antibodies are of the IgG_1 subclass and, therefore, are monovalent in receptor interaction. All of the hybridomas expressed a κ light chain. Finally, specificity of the eight antibodies that immunoblot is implied by their recognition of the gel band previously identified as the porcine intestinal 1,25-$(OH)_2D_3$ receptor (Dame et al., 1985). These eight antibodies should be useful in probing the structure of the intact receptor, receptor cleavage products, and potentially different functional domains of this receptor.

All of the antibodies examined to date recognize both the nonhormone-bound and hormone-bound receptor (data not shown). This is not an unexpected finding because both the RISA and immunoprecipitation screening assay used tritiated hormone bound to receptor to identify antireceptor antibody activity. Antibodies causing a conformational change in the receptor and loss of ligand or antibodies directed against the hormone binding site would not have been detected by these screening assays.

It is interesting that all but two of the monoclonal antibodies bind intestinal 1,25-$(OH)_2D_3$ receptor from all mammalian sources tested as well as receptor isolated from chicken intestine. These antibodies must recognize regions of the receptor protein that have been conserved through evolution. There is a clearly demonstrable biochemical difference between the mammalian and avian 1,25-$(OH)_2D_3$ receptor as evidenced by sedimentation coefficient (3.1–3.2 S vs. 3.7 S, respectively) and gel filtration analysis. More subtle differences must also exist between mammalian 1,25-$(OH)_2D_3$ receptors because two monoclonal antibodies were generated that recognize only the porcine 1,25-$(OH)_2D_3$ receptor. Although the reported sedimentation coefficients for mammalian receptors are quite similar, there is evidently enough amino acid sequence differences giving rise to differences in antigenicity between these receptors.

The original antibody screening assays employed in this investigation utilized partially purified receptor that had been extracted from a crude nuclear pellet. We cannot exclude that there might be a cytoplasmic form of the receptor that is not recognized by these antibodies. We feel that this is unlikely because biochemical fractionation studies and frozen section autoradiographic studies of the 1,25-$(OH)_2D_3$ receptor and recent biochemical and immunochemical studies on other steroid hormone receptors (King & Green, 1984; Welshons et al., 1984; Perrot-Applanat et al., 1985) support the conclusion that these receptors, whether bound to hormone or free, are nuclear proteins. Because interpretation of future data will be dependent on which receptor species are recognized by the antibodies reported here, it is imperative to determine whether the antibodies recognize all receptor forms. Quantitative immunoprecipitation studies and immunoblotting experiments are currently under way in our laboratory to address this question.

Using a competition assay, we have demonstrated the production of monoclonal antibodies to at least four distinct antigenic regions on the 1,25-$(OH)_2D_3$ receptor. The use of a competition assay to determine whether monoclonal antibodies bind to different protein epitopes was described by Kohno et al. (1982). In their assay, one purified antibody was employed to bind the protein, myoglobin, to the solid-phase support. A second purified antibody linked to the enzyme, alkaline phosphatase, could then bind to the solid phase only by binding to a myoglobin molecule already attached to the first antibody. A major advantage of this method is that it can be used with impure antigens. By extension, we have used this method to screen antibodies directly out of hybridoma supernatant. We were able to do this by inserting a goat anti-mouse IgG bridge to the solid phase. We replaced the enzyme-conjugated second antibody step with L-[^{35}S]methionine-labeled monoclonal antibodies. By use of this modification, fusions producing large numbers of hybridomas secreting antibodies of interest can be rapidly screened for antibodies to different epitopes on the protein of interest. Performing the assay in this fashion also eliminates the time-consuming process of purifying multiple

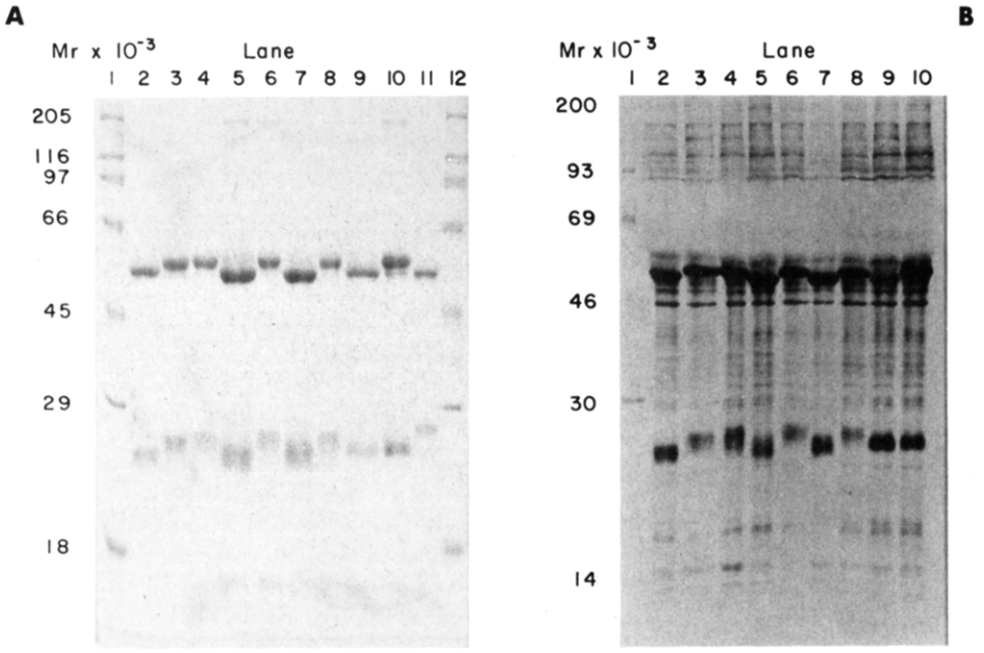

FIGURE 6: Electrophoretic analysis of purified monoclonal antibodies and L-[^{35}S]methionine-labeled hybridoma products on discontinuous sodium dodecyl sulfate–polyacrylamide gels. (A) Purified monoclonal antibodies (1.6 μg) were resolved on gels and stained with Coomassie blue as described under Materials and Methods. Lanes 2–11, monoclonal antibodies IVA7E7, VB3F12, VD2F12, VIIID8C12, VIIIF7E12, XH12G11, XVA9A7E6, XVIE6E6G10, XVIE10B6A5C6, and control IgG_1 (MOPC 21), respectively. Lanes 1 and 12, protein standards: myosin (205 000), β-galactosidase (116 000), phosphorylase B (97 400), bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (29 000), and β-lactoglobulin (18 400). (B) L-[^{35}S]Methionine-labeled protein from dialyzed supernatants (5 μL, 70 000 cpm) were resolved on gels and visualized on X-ray film (X-Omat AR-5) by fluorography. Lanes 2–10 are as defined above. Lane 1 represents a ^{14}C-methylated protein mixture: myosin (200 000), phosphorylase B (92 500), bovine serum albumin (69 000), ovalbumin (46 000), carbonic anhydrase (30 000), and lysozyme (14 300).

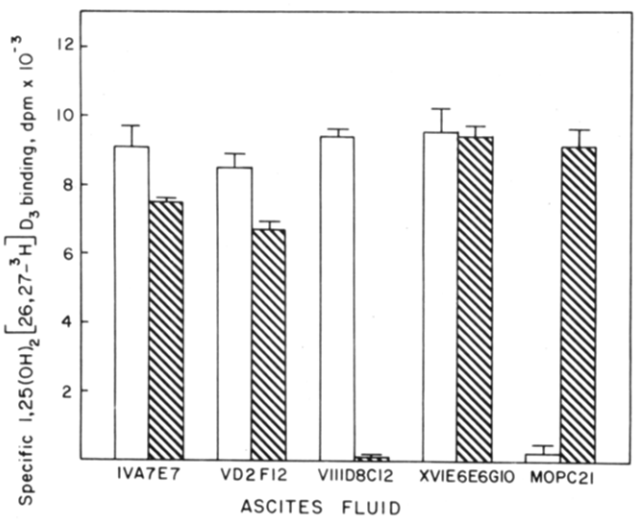

FIGURE 7: Effect of 1,25-$(OH)_2D_3$–receptor–monoclonal antibody association on DNA–cellulose binding. Diluted nuclear extract (1:3 v/v in TED) was incubated overnight with 2×10^{-9} M 1,25-$(OH)_2$-[26,27-^{3}H]D_3 with and without a 100-fold excess nonradiolabeled 1,25-$(OH)_2D_3$. Diluted ascites fluid (25 μL, 1:25 v/v in $TEDK_{75}$) containing antibodies representative of each of the four groups defined from the competition studies was incubated for 3 h at 0–4 °C with labeled receptor. Ascites from the MOPC 21 tumor secreting an IgG_1 was used as a control. Aliquots were assayed for specific 1,25-$(OH)_2$-[26,27-^{3}H]D_3–receptor DNA–cellulose binding activity (hatched bars) and for immunoprecipitation of specific receptor binding activity (open bars). Data are mean ● SD.

antibodies. A disadvantage of this modification is the potential for exchange of the unlabeled antibody for labeled antibody on the goat anti-mouse IgG molecule. We have confirmed that the assay yields similar results using either the goat anti-mouse bridge or pure monoclonal antireceptor antibody. Although the problem of exchange and, therefore, assay noise is eliminated or reduced when pure monoclonal antibody is used, the sensitivity and usefulness of the assay is increased with the inclusion of the goat anti-mouse step because it allows the use of solutions containing much lower concentrations of monoclonal antibody.

We have compared the use of this competition assay with the more traditionally used technique of sedimentation analysis in the identification of antibodies that bind to different epitopes on the receptor molecule. When the four antibody groups identified by the solid-phase competition studies were examined by sedimentation analysis, the differences between receptor epitopes for monoclonal antibodies IVA7E7, VD2F12, and XVIE6E6G10 as well as XVIE6E6G10 and VIIID8C12 were confirmed. However, certain pairs of monoclonal antibodies did not always yield the expected displacement of radioactivity from the 7S to 8S position in the presence of one antibody to a larger sedimenting species in the presence of two different antibodies. It is possible that the binding of one antibody may affect receptor conformation such that it reduces the binding affinity of the other antibody. In addition, if one of the antibodies has a lower binding affinity relative to the other antibody partner, the antibody–receptor–antibody complex might dissociate to a receptor–antibody complex during the 18-h sedimentation procedure. In this circumstance, the solid-phase competition assay appears to be a much more sensitive way to test for differences between antibody–receptor binding sites. In the competition experiment, the period of time in which the receptor is available for binding by the second antibody is relatively short compared to that in the sedimentation studies. Further, use of the solid-phase competition assay enables preliminary screening of monoclonal antibodies using hybridoma supernatant and is relatively easy to perform on large numbers of samples. Sucrose gradient studies as well as being less sensitive are time consuming and would have been impractical for screening for differences in antibody–receptor

interactions for all 24 of the original hybrid lines.

The 1,25-$(OH)_2D_3$ receptor, like other steroid receptors, has been reported to contain at least two distinct binding domains: one that binds steroid and the other polynucleotide (Franceschi et al., 1981). Recently, several laboratories have prepared proteolytic digests of the chicken intestinal 1,25-$(OH)_2D_3$ receptor and examined the characteristics of the fragments using standard biochemical techniques (Mellon, 1985) and additionally using immunochemical methods (Allegretto & Pike, 1985). Both laboratories report that trypsin cleavage results in a decrease in the Stokes radius of the receptor from 3.6–3.7 nm to either a 3.2–3.3- or 2.8–2.9-nm species. Both fragments retain hormone binding capacity but lose the ability to bind to DNA cellulose. Allegretto and Pike (1985) found that binding of their antireceptor monoclonal antibody to the 3.2–3.3-nm fragment was markedly reduced and binding was eliminated with the formation of the 2.8–2.9-nm species. Coupled with the fact that this antibody reduces the affinity of the intact receptor for DNA–cellulose (Pike, 1984), these authors propose that their monoclonal antibody binds to a region on the receptor protein between the hormone and polynucleotide binding domains (Allegretto & Pike, 1985). We have studied the action of one antibody from each of the four different antibody groups described previously, on receptor–DNA–cellulose binding. The results indicate that we may have generated antibodies that bind to different functional domains on the receptor protein. This is evidenced by the fact that one antibody (VIIID8C12) completely inhibits subsequent receptor interaction with DNA–cellulose, whereas another antibody (XVIE6E6G10) has no effect. It appears that antibody VIIID8C12 may interact with a region of the receptor's DNA binding domain. No monoclonal antibody that completely blocks DNA binding of a steroid hormone receptor has been reported previously. The antibodies representative of the remaining two groups (IVA7E7 and VD2F12) behave more like the monoclonal antibody described by Pike (1984); these antibodies perturb but do not inhibit the receptor–DNA interaction. The diverse nature of the binding sites to which these antibodies are directed on the receptor may be useful in isolating the functional hormone and polynucleotide binding sites of the receptor molecule. If the site involved in binding to calf thymus DNA is related to the receptor domain involved in recognition of a specific site(s) on chromosomal DNA, then the functional change conferred on the receptor by the antibody that blocks DNA–cellulose binding and the lack of the functional change or slight perturbation caused by the other antibody groups should be useful in studying receptor binding to purified 1,25-$(OH)_2D_3$ responsive gene sequences. The question of whether physiologically relevant nonhormone binding forms of the receptor exist, as well as the role of protein processing and turnover in relation to receptor function, should also be approachable with monoclonal antibodies. It should also be possible to study the subcellular distribution of the receptor in relationship to hormonal status and to determine whether the 1,25-$(OH)_2D_3$ receptor represents another nuclear steroid hormone binding receptor. The availability of monoclonal antibodies to the 1,25-$(OH)_2D_3$ receptor enables the study of this protein independent of hormone binding and should yield much new information on the role of this receptor in target tissue responsiveness to 1,25-$(OH)_2D_3$.

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REFERENCES

Allegretto, E. A., & Pike, J. W. (1985) *J. Biol. Chem. 260*, 10139–10145.

Brumbaugh, P. F., & Haussler, M. R. (1973) *Biochem. Biophys. Res. Commun. 51*, 74–80.

Clark, S. A., Dame, M. C., Kim, Y. S., Stumpf, W. E., & DeLuca, H. F. (1985) *Anat. Rec. 212*, 250–254.

Dame, M. C., Pierce, E. A., & DeLuca, H. F. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7825–7829.

DeLuca, H. F. (1983) in *Annual Advances in Bone and Mineral Research* (Peck, W. A., Ed.) pp 7–73, Excerpta Medica, Amsterdam.

DeLuca, H. F., & Schnoes, H. K. (1983) *Annu. Rev. Biochem. 52*, 411–439.

Edwards, D. P., Weigel, N. L., Schrader, W. T., O'Malley, B. W., & McGuire, W. L. (1984) *Biochemistry 23*, 4427–4435.

Eisman, J. A. (1984) in *Vitamin D* (Kumar, R., Ed.) pp 365–382, Martinus Nijhoff, Boston.

Feldman, D., McCain, T. A., Hirst, M. A., Chen, T. L., & Colston, K. W. (1979) *J. Biol. Chem. 254*, 10378–10384.

Franceschi, R. T., Simpson, R. U., & DeLuca, H. F. (1981) *Arch. Biochem. Biophys. 210*, 1–13.

Gametchu, B., & Harrison, R. W. (1984) *Endocrinology (Baltimore) 114*, 274–279.

Grandics, P., Gasser, D. L., & Litwack, G. (1982) *Endocrinology (Baltimore) 111*, 1731–1733.

Greene, G. L., Fitch, F. W., & Jensen, E. V. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 157–161.

Greene, G. L., Nolan, C., Engler, J. P., & Jensen, E. V. (1980b) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5115–5119.

Greene, G. L., Sobel, N. B., King, W. T., & Jensen, E. V. (1984) *J. Steroid Biochem. 20*, 51–56.

Halloran, B. P., & DeLuca, H. F. (1981) *Arch. Biochem. Biophys. 208*, 477–486.

Haussler, M. R., Donaldson, C. A., Marion, S. L., Kelly, M. A., Dokoh, S., & Pike, J. W. (1984) in *Endocrine Control of Bone and Calcium Metabolism* (Cohn, D. V., Fujita, T., Potts, J. T., & Talmage, R. V., Eds.) pp 331–334, Elsevier Science Publishers B.V., Amsterdam.

King, W. G., & Greene, G. L. (1984) *Nature (London) 307*, 745–747.

Köhler, G., & Milstein, C. (1975) *Nature (London) 256*, 495–497.

Köhler, G., & Milstein, C. (1976) *Eur. J. Immunol. 6*, 511–519.

Kohno, Y., Berkower, I., Minna, J., & Berzofsky, J. A. (1982) *J. Immunol. 128*, 1742–1748.

Kream, B. E., Reynolds, R. D., Knutson, J. C., Eisman, J. A., & DeLuca, H. F. (1976) *Arch. Biochem. Biophys. 176*, 779–787.

Kream, B. E., Yamada, S., Schnoes, H. K., & DeLuca, H. F. (1977) *J. Biol. Chem. 252*, 4501–4505.

Link, R., & DeLuca, H. F. (1985) in *The Receptors* (Conn, P. M., Ed.) Vol. II, pp 1–13, Academic, New York.

Litman, R. M. (1968) *J. Biol. Chem. 243*, 6222–6233.

Logeat, F., Hai, M. T. V., Fournier, A., Legrain, P., Buttin, G., & Milgrom, E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6456–6459.

Mellon, W. S. (1985) *Endocrinology (Baltimore) 116*, 1408–1417.

Moncharmont, B., Su, J.-L., & Parikh, I. (1982) *Biochemistry 21*, 6916–6921.

Napoli, J. L., Mellon, W. S., Fivizzani, M. A., Schnoes, H. K., & DeLuca, H. F. (1980) *Biochemistry 19*, 2515–2521.

Narbaitz, R., Stumpf, W. E., Sar, M., Huang, S., & DeLuca, H. F. (1983) *Calcif. Tissue Int. 35*, 177-182.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007-4021.
Oi, V. T., & Herzenberg, L. A. (1980) in *Selected Methods in Cellular Immunology* (Mishell, B. B., & Shiigi, S. M., Eds.) pp 351-371, W. H. Freeman, San Francisco.
Okret, S., Wikström, A.-C., Wrange, O., Andersson, B., & Gustafsson, J.-A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1609-1613.
Perrot-Applanat, M., Logeat, F., Groyer-Picard, M. T., & Milgrom, E. (1985) *Endocrinology (Baltimore) 116*, 1473-1484.
Pierce, E. A., Dame, M. C., Bouillon, R., Van Balaen, H., & DeLuca, H. F. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8429-8433.
Pierce, E. A., Dame, M. C., & DeLuca, H. F. (1986) *Anal. Biochem. 153*, 67-74.
Pike, J. W. (1984) *J. Biol. Chem. 259*, 1167-1173.
Pike, J. W., & Haussler, M. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5485-5489.
Pike, J. W., Donaldson, C. A., Marion, S. L., & Haussler, M. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7719-7723.
Pike, J. W., Marion, S. L., Donaldson, C. A., & Haussler, M. R. (1983) *J. Biol. Chem. 258*, 1289-1296.
Radanyi, C., Joab, I., Renoir, J.-M., Richard-Foy, H., & Baulieu, E.-E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2854-2858.
Simpson, R. U., & DeLuca, H. F. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5822-5826.
Simpson, R. U., & DeLuca, H. F. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 16-20.
Simpson, R. U., Hamstra, A., Kendrick, N. C., & DeLuca, H. F. (1983) *Biochemistry 22*, 2586-2594.
Stumpf, W. E., Sar, M., Reid, F. A., Tanaka, Y., & DeLuca, H. F. (1979) *Science (Washington, D.C.) 206*, 1188-1190.
Stumpf, W. E., Sar, M., Narbaitz, R., Reid, F. A., DeLuca, H. F., & Tanaka, Y. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1149-1153.
Stumpf, W. E., Sar, M., & DeLuca, H. F. (1981) in *Hormonal Control of Calcium Metabolism* (Cohn, D. V., Talmage, R. V., & Matthews, J. L., Eds.) pp 222-229, Excerpta *Medica, Amsterdam.*
Suda, T., Abe, E., Miyaura, C., Tanaka, H., Shiina, Y., Kuribayashi, T., Honma, Y., Hozumi, M., Momoi, T., & Nishii, Y. (1984) in *Endocrine Control of Bone and Calcium Metabolism* (Cohn, D. V., Fujita, T., Potts, J. T., & Talmage, R. V., Eds.) pp 308-315, Elsevier Science Publishers B.V., Amsterdam.
Wecksler, W. R., & Norman, A. W. (1979) *Anal. Biochem. 92*, 314-323.
Wecksler, W. R., Ross, F. P., & Norman, A. W. (1979) *J. Biol. Chem. 254*, 9488-9491.
Welshons, W. V., Lieberman, M. E., & Gorski, J. (1984) *Nature (London) 307*, 747-749.
Westphal, H. M., Moldenhauer, G., & Beato, M. (1982) *EMBO. J. 1*, 1467-1471.
Williams, D., & Gorski, J. (1974) *Biochemistry 13*, 5537-5542.
Zile, M., Bunge, E. C., Barsness, L., Yamada, S., Schnoes, H. K., & DeLuca, H. F. (1978) *Arch. Biochem. Biophys. 186*, 15-24.

Chemical Reactivity of Monofunctional Platinum-DNA Adducts†

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ABSTRACT: Complexes formed in vitro between *cis*- or *trans*-$PtCl_2(NH_3)_2$ (DDP) and DNA were found to contain monofunctional adducts that reacted with exogenous guanosine. [^{14}C]Guo bound irreversibly to *cis*- and *trans*-DDP-DNA complexes to form bis-Gua adducts. The reaction was first order with respect to the concentration of both [^{14}C]Guo and platinum-DNA complex, but the rate of the reaction varied nonlinearly as a function of the level of platinum binding on DNA. The reaction between [^{14}C]Guo and these platinum-DNA complexes was used to probe the concentration and stability of the monofunctional adducts and to investigate their chemistry in situ. The concentration of monofunctional adducts was highest immediately after reaction of DDP with DNA for 2 h at 37 °C, at which time they represented greater than 15% of the *cis*-DDP-DNA lesions and on the order of 80% of the *trans*-DDP-DNA lesions. The *cis*-DDP-DNA complex reacted with [^{14}C]Guo by two kinetically distinct processes, indicating two types of reactive adducts. The most reactive adduct represented 5% of the platinum lesions. These monofunctional adducts disappeared during the incubation of the platinum-DNA complexes in the absence of drug, probably as a result of chelation to DNA. The half-lives of this chelation at 37 °C, 10 mM $NaClO_4$, were 15 and 30 h for the cis and trans complexes, respectively. Monofunctional adducts were formed on Gua bases in DNA. Hydrolysis was not a rate-limiting step for the reaction between [^{14}C]Guo and the platinum-DNA complex, which suggests that the platinum atom did not possess an inner shell chloride ligand. The stability of these monofunctional adducts and the products formed by their chelation with DNA depend on DNA secondary structure.

Fixation of *cis*-$PtCl_2(NH_3)_2$ on DNA is believed to be responsible for the antitumor activity and other biological effects of this drug (Roberts & Pera, 1983). The trans isomer also reacts with cellular DNA (Pascoe & Roberts, 1974), but it is not antitumoral (Connors et al., 1972; Cleare & Hoeschele, 1973). The majority of the *cis*-DDP[1] molecules rapidly chelate

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