Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 6555–6559, August 1991
Biochemistry

Overproduction of rat 1,25-dihydroxyvitamin D_3 receptor in insect cells using the baculovirus expression system

(vitamin D/steroid receptor cDNA/expression system)

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Contributed by Hector F. DeLuca, April 29, 1991

ABSTRACT **The rat 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] receptor has been expressed at elevated levels in *Spodoptera frugiperda* cells using the baculovirus expression vector system. The recombinant 1,25-$(OH)_2D_3$ receptor is full-length, binds 1,25-$(OH)_2D_3$, and is recognized by a monoclonal antibody specific for 1,25-$(OH)_2D_3$ receptor. Densitometric scanning of Coomassie brilliant blue-stained SDS/polyacrylamide gels indicated a recombinant receptor protein level comprising 5% of the total soluble protein from the insect cells. The hydroxylapatite binding assay revealed average levels of 2 nmol of unoccupied 1,25-$(OH)_2D_3$ receptor per mg of protein in insect cells at 72 hr after infection with recombinant baculovirus. A measure of total 1,25-$(OH)_2D_3$ receptor using a ligand-independent, immunoradiometric assay disclosed average levels of 2.3 nmol of receptor per mg of protein produced by these same cells. A monoclonal antibody directed against the 1,25-$(OH)_2D_3$ receptor, and reported to cross-react with this receptor derived from several species, recognized the recombinant rat 1,25-$(OH)_2D_3$ receptor upon Western analysis. A monoclonal antibody directed specifically against the porcine receptor failed to recognize the recombinant rat 1,25-$(OH)_2D_3$ receptor protein. The cytosolic preparation of insect cells infected with recombinant baculovirus exhibited an equilibrium dissociation constant of 1×10^{-11} M as determined by a 1,25-$(OH)_2D_3$ saturation analysis plotted by the method of Scatchard. This expression system provides an adequate source from which abundant quantities of 1,25-$(OH)_2D_3$ receptor can be purified for subsequent x-ray crystallographic analyses.**

The biological activities of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the hormonal form of vitamin D, are mediated by means of its intracellular receptor protein (1). The three well-characterized functions of 1,25-$(OH)_2D_3$ in mammals are (*i*) stimulation of intestinal calcium and phosphate transport from the lumen of the small intestine to the plasma (2), (*ii*) mobilization of calcium from bone to plasma (3), and (*iii*) reabsorption of calcium in the distal renal tubule (4). The two latter functions are carried out in conjunction with parathyroid hormone (5, 6). These effects of vitamin D ultimately lead to the elevation of plasma calcium and phosphorus levels that are necessary for bone mineralization and proper neuromuscular function (1). A probable mechanism by which 1,25-$(OH)_2D_3$ elicits the intestinal calcium and phosphorus transport response consists of the 1,25-$(OH)_2D_3$ hormone entering the target cell and binding its nuclear receptor. The interaction of hormone with its receptor may introduce changes in 1,25-$(OH)_2D_3$ receptor conformation, thereby allowing it to interact with chromatin. This interaction can alter the expression of genes whose protein products influence functions such as calcium transport and mobilization (7).

The disease vitamin D-dependent rickets type II exemplifies the receptor-dependent function of 1,25-$(OH)_2D_3$ (8). Stricken patients suffer from hypocalcemia despite elevated levels of 1,25-$(OH)_2D_3$ in the plasma. With this form of rickets, there is a target organ resistance to the hormonal derivative of vitamin D. A defect in the 1,25-$(OH)_2D_3$ receptor has been reported to exist in at least one subgroup of rickets type II patients (9).

Molecular cloning and nucleotide sequence determination of the avian (10), rat (11), and human (12) cDNAs for the 1,25-$(OH)_2D_3$ receptor have indicated a structural similarity to other members of the steroid family of receptors. The deduced amino acid sequence displays a cysteine-rich region at the amino terminus, characteristic of a DNA binding motif. The hydrophobic amino acids near the carboxyl terminus form what is likely the hydrophobic pocket responsible for hormone binding. The domains within the human 1,25-$(OH)_2D_3$ receptor protein were defined more precisely by deletion analysis and expression studies (13). The discovery of cis-acting vitamin D response elements (DREs) lying within the upstream regions of the human (14) and rat (15, 16) osteocalcin genes and the mouse osteopontin gene (17) is also consistent with 1,25-$(OH)_2D_3$ being a member of the steroid family of receptors. Further evidence of this has been provided by reconstitution of the human 1,25-$(OH)_2D_3$ osteocalcin gene responsive transcription system in yeast (18).

The available data, albeit extensive, do not provide insight as to the three-dimensional structure of the 1,25-$(OH)_2D_3$ receptor and its domains. Due to inadequate sources of the 1,25-$(OH)_2D_3$ receptor protein for large-scale purification, the molecular interaction between the receptor and 1,25-$(OH)_2D_3$, or synthetic analogs of 1,25-$(OH)_2D_3$, remains a mystery. The human 1,25-$(OH)_2D_3$ receptor has been expressed in *Saccharomyces cerevisiae*; however, the authors indicate potential problems in utilizing the recombinant receptor from yeast cells for x-ray crystallographic analysis (19).

The baculovirus expression system has become a popular and effective means of expressing foreign genes. Well over 300 recombinant proteins have successfully been overproduced in insect cells using this system (20), including the particularly relevant human glucocorticoid (21) and human estrogen (21) receptors.

MATERIALS AND METHODS

Construction of Recombinant Plasmid Transfer Vector PCR-YM1. A hybrid plasmid, hereby designated pRDR26, is a pUC18 derivative harboring the 2043-base-pair (bp) segment of rat 1,25-$(OH)_2D_3$ receptor cDNA (Fig. 1*A*). Construction of this plasmid, together with the nucleotide sequence of the cDNA, have been described (23). Use of the

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Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; AcNPV, *Autographa californica* nuclear polyhedrosis virus; IRMA, immunoradiometric assay; DRE, vitamin D response element.

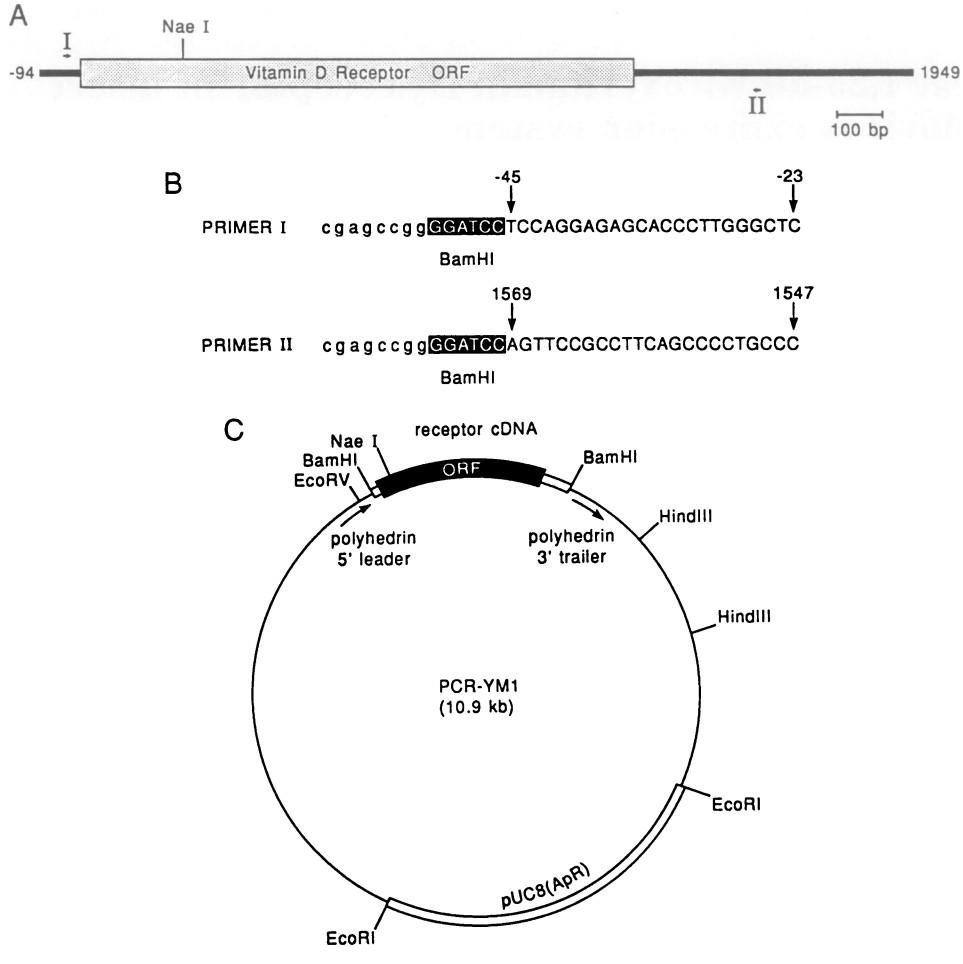

FIG. 1. Strategy for construction of recombinant plasmid transfer vector. (*A*) Representation of the nucleotide sequence for the rat 1,25-$(OH)_2D_3$ receptor cDNA (22). The thick, shaded segment indicates the location of the 1,25-$(OH)_2D_3$ receptor open reading frame (ORF). Arrows labeled I and II denote the location of the sequence from which the synthetic oligonucleotide primers were derived for polymerase chain reaction (PCR) amplification. The position of the unique *Nae* I restriction site is indicated. (*B*) Nucleotide sequence of primers I and II used in the PCR amplification of the 1,25-$(OH)_2D_3$ receptor ORF. The artificial *Bam*HI recognition sequences are highlighted. The random tailing sequences are shown in lowercase letters. (*C*) Recombinant plasmid PCR-YM1 consists of the PCR-amplified segment of receptor cDNA inserted into plasmid vector pAcYM1. The orientation of the insert at the *Bam*HI restriction site is shown relative to the direction of transcription and translation for the polyhedrin gene. kb, Kilobases; ApR, ampicillin resistance.

baculovirus expression system required that a reduction be made in the length of the nontranslated leader sequence from the rat receptor cDNA (94 bp). Because of a lack of convenient restriction enzyme recognition sites within this region of the nucleotide sequence, we chose to amplify only a segment of the cDNA that included the protein coding region using PCR technology. This approach circumvented the restriction site problem while alleviating the concern over the length of the leader sequence. For this purpose oligonucleotide primers were synthesized as follows: primer I, nucleotides −45 to −23, and primer II, nucleotides 1569–1547. Both primers were synthesized with the recognition sequence for *Bam*HI and eight nucleotides of random sequence at their 5′ termini (Fig. 1*B*). Syntheses were performed by the University of Wisconsin, Department of Biochemistry oligonucleotide service. Amplification of a 1614-bp segment of the rat 1,25-$(OH)_2D_3$ receptor cDNA from pRDR26 was performed using a Perkin–Elmer/Cetus, DNA thermal cycler. A single colony from *Escherichia coli*, strain DH5α (24), transformed with pRDR26, was transferred into 150 μl of H_2O to make a homogeneous cell suspension. Fifteen microliters of the cell suspension was added to the following PCR reaction components: 1 μM primer I, 1 μM primer II, 1× Taq polymerase reaction buffer, 2 units of Taq polymerase (Promega), and 200 nM deoxynucleotide triphosphates (dATP, dGTP, dCTP, and dTTP) in a total reaction volume of 100 μl. The following parameters were followed for amplification: 94°C, 1 min, 20 cycles [94°C, 1 min; 55°C, 1 min; 72°C, 1.5 min]; 72°C, 5 min; 4°C, storage. An aliquot of the amplified DNA was treated with *Bam*HI to generate the appropriate cohesive restriction termini and then ligated with 1 μg of *Bam*HI-digested plasmid transfer vector pAcYM1 (25) in the presence of T4 DNA ligase for 18 hr at 16°C. A portion of the mixture was used to transform competent cells of strain DH5α to ampicillin resistance (Ap^R) (26). Small-scale isolation and restriction analysis of plasmid DNAs from several Ap^R transformants revealed four having the proper PCR-amplified product contained within the pAcYM1 vector. By using several different combinations of restriction endonuclease digestions and subsequent analysis by agarose gel electrophoresis, a recombinant was identified with the insert DNA in the proper orientation with respect to the polyhedrin gene signals from pAcYM1. The recombinant plasmid transfer vector was designated PCR-YM1 (Fig. 1*C*).

Generation of Recombinant Baculovirus Vitamin D Receptor *Autographa californica* Nuclear Polyhedrosis Virus (DR-AcNPV). Fifteen micrograms of plasmid DNA from PCR-YM1, purified by ethidium bromide/CsCl equilibrium density gradient centrifugation, was cotransfected with 1 μg of wild-type AcNPV DNA (27) into sf21 cells (28) using the Lipofectin (GIBCO BRL/Life Technologies, Gaithersburg, MD) reagent according to manufacturer's specifications. sf21 (*Spodoptera frugiperda*) insect cells and wild-type AcNPV were gifts from Paul Friesen (Department of Biochemistry, University of Wisconsin, Madison, WI). After a 96-hr incubation of the cotransfection mixture at 27°C, the viral supernatant was harvested (29). Dilutions of this viral preparation were used to infect freshly plated sf21 cells according to the agarose overlay procedure (29). Plaques derived from a potential occlusion-deficient, recombinant virus were picked and purified through three rounds of purification. Confirmation of retrieval of recombinant virus DR-AcNPV, containing the 1,25-$(OH)_2D_3$ receptor coding region, was carried out by hybridization screening (29).

Preparation of Protein Extracts from Infected Insect Cells. sf21 cells were plated at a density of 3×10^6 per 100-mm plate

in TC100 (GIBCO/BRL) insect cell medium and allowed to attach for 30–60 min. The medium was removed and 1 ml of recombinant or wild-type AcNPV (multiplicity of infection = 1–10) was added to the surface of the cell monolayer. This was maintained at 27°C with gentle rocking for 60 min. This was followed by the addition of 5 ml of TC100 medium supplemented with 10% fetal calf serum and continued incubation at 27°C. Cells were harvested 72 hr after infection with virus unless otherwise noted. Extracts of soluble protein were prepared by first disrupting the infected cells by repeated pipetting and washing of the plate surface. The suspended cells were transferred to a plastic conical tube and then collected by centrifugation at 500 × *g* for 10 min. The medium was discarded and the cells were suspended in $TEDK_{20}$ [50 mM Tris·HCl, pH 7.4/1.5 mM EDTA/5 mM dithiothreitol/20 mM KCl] containing 1 mM phenylmethylsulfonyl fluoride, 5 mM diisopropyl fluorophosphate, and 1 μg of pepstatin per ml. The cell suspension was incubated for 30 min on ice before homogenization in a stainless steel Dounce homogenizer (three strokes). Enough TED buffer, containing 600 mM KCl, was added to bring the final KCl concentration to 300 mM. The cells were homogenized with three additional strokes and the homogenate was centrifuged for 60 min at 45,000 rpm in a Beckman 70.1 Ti rotor. The cleared supernatant was divided into small aliquots and quickly frozen in liquid nitrogen. Storage of the extracts was at −70°C.

Total cellular protein extracts were prepared by infecting and harvesting the cells as described above. The cells, collected by centrifugation, were lysed in 4% SDS electrophoresis sample buffer (30).

Gel Electrophoresis and Western Blotting. Total protein extracts or total soluble protein extracts with sample buffer (31) added were boiled for 1 min and electrophoresed on 9% SDS/polyacrylamide gels. The gels were either stained with Coomassie brilliant blue or transferred to nitrocellulose for Western blotting. After immobilization of proteins for Western blotting, the filters were blocked with Tris-buffered saline/Tween 20 (TBST) containing 5% nonfat dry milk, followed by incubation with primary antibody for 90 min. The filters were washed extensively in TBST and then incubated with a secondary alkaline phosphatase-conjugated goat anti-mouse IgG antibody. The color was developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate using the ProtoBlot AP system according to the manufacturer's specifications (Promega). Monoclonal antireceptor antibody preparation has been described (32).

Measurement of 1,25-$(OH)_2D_3$ Receptor. The 1,25-$(OH)_2$-[26,27-^{3}H]D_3 binding activity in total soluble protein extracts from DR-AcNPV-infected sf21 cells was determined by a hydroxylapatite binding assay as described (33). Total 1,25-$(OH)_2D_3$ receptor was determined by the immunoradiometric assay (IRMA) already described (34). Protein content of the extracts was measured by the Bradford method (35). Data from the equilibrium binding study were plotted by the method of Scatchard (36). Regression analysis was performed to obtain best fit.

Vitamin D Compounds. 1,25-$(OH)_2D_3$ was a gift from Hoffmann–LaRoche. 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (160 Ci/mmol; 1 Ci = 37 GBq) was produced by DuPont/NEN as described (37).

RESULTS

Expression of the Rat 1,25-$(OH)_2D_3$ Receptor. A baculovirus expression vector system was used to overproduce the rat 1,25-$(OH)_2D_3$ receptor. The recombinant plasmid PCR-YM1, containing the amino acid coding segment for the receptor, was used to cotransfect with wild-type baculovirus AcNPV DNA into sf21 insect cells. A recombinant baculovirus containing the receptor segment (DR-AcNPV) was identified by the process of visual screening for plaque morphology and purified through three rounds of plaque purification.

The level of rat 1,25-$(OH)_2D_3$ receptor in infected insect cells was approximated by SDS/polyacrylamide gel analysis of cell extracts (Fig. 2). Fig. 2*A* represents the total protein extracts from infected, or uninfected, sf21 cells. A predominant band at M_r 55,000 evident in the extract from cells infected with DR-AcNPV (Fig. 2*A*, lane 3) is not present in the extract from sf21 cells or the extract from sf21 cells infected with wild-type AcNPV (Fig. 2*A*, lanes 2 and 1, respectively). The polyhedrin protein (M_r 30,000), whose expression is interrupted upon the creation of a recombinant AcNPV, is evident in the wild-type virus-infected cells (Fig. 2*A*, lane 1); however, the protein is absent in the sf21 cell extract and the extract from DR-AcNPV-infected cells (Fig. 2*A*, lanes 2 and 3, respectively).

SDS/polyacrylamide gel electrophoresis was also performed on cytosolic preparations representing the total soluble proteins (Fig. 2*B*). As is evident in the total protein extract from DR-AcNPV-infected cells (Fig. 2*A*, lane 3), there is also a predominant band at M_r 55,000 in the total soluble protein extract from identically infected cells (Fig. 2*B*, lane 3). The M_r 55,000 protein, representing the recombinant rat 1,25-$(OH)_2D_3$ receptor, comprises ≈5% of the total soluble protein from DR-AcNPV-infected sf21 cells, as determined by densitometric scanning of the Coomassie brilliant blue-stained SDS/PAGE gel. Extracts from wild-type AcNPV-infected cells and uninfected insect cells are also shown (Fig. 2*B*, lanes 1 and 2, respectively).

A more precise quantification of recombinant 1,25-$(OH)_2D_3$ receptor was performed using the hydroxylapatite ligand binding assay and the ligand-independent IRMA (Table 1). An average of 2000 pmol of the steroid binding forms of the receptor per mg of protein was measured using the hydroxylapatite assay. The source of 1,25-$(OH)_2D_3$ receptor in this laboratory has been intestinal pig nuclear extract, which served as the positive control for these measurements. The level of receptor was determined to be nearly 1500 times greater in the baculovirus system than from the pig nuclear extract (1.35 pmol/mg) (Table 1). The average level of total 1,25-$(OH)_2D_3$ receptor in the extracts from DR-AcNPV-infected cells was 2300 pmol per mg of protein using the

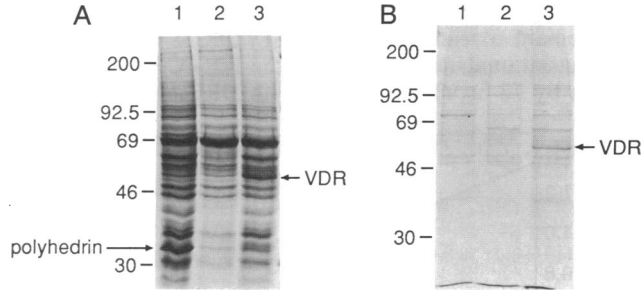

FIG. 2. SDS/polyacrylamide gel electrophoresis of protein extracts from sf21 cells. (*A*) Total protein extracts from wild-type baculovirus-infected (lane 1), uninfected (lane 2), or receptor recombinant baculovirus-infected (lane 3) sf21 insect cells electrophoresed on an SDS/9% polyacrylamide gel and stained with Coomassie brilliant blue. The positions of the recombinant 1,25-$(OH)_2D_3$ receptor and baculovirus polyhedrin proteins are noted. Molecular weights (shown as $M_r \times 10^{-3}$) corresponding to the following protein markers, from top to bottom, are shown: myosin, phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase. (*B*) Total soluble protein extracts from sf21 cells grown under the same conditions and depicted in the same lane designations as those described for *A*. The position of the recombinant 1,25-$(OH)_2D_3$ receptor is noted.

Table 1. Measurements of 1,25-$(OH)_2D_3$ receptor

Sample	Ligand binding assay,* pmol/mg of protein	IRMA, pmol/mg of protein
Recombinant DR-AcNPV/sf21 cytosol	2000 ± 1000	2300 ± 1000
Pig intestinal nuclear extract	1.35 ± 0.15	1.68 ± 0.12

Values represent an average of measurements performed in triplicate on six different cytosolic preparations.
*Obtained using the hydroxylapatite assay.

IRMA measurement. Nuclear extract from pig intestine contained an average level of 1.68 pmol/mg of protein.

Characteristics of the Recombinant 1,25-$(OH)_2D_3$ Receptor. A 1,25-$(OH)_2D_3$ saturation analysis of cytosol from DR-AcNPV-infected sf21 cells was plotted by the method of Scatchard (Fig. 3). The equilibrium dissociation constant (K_d), calculated by linear regression, was 1×10^{-11} M. The K_d value is consistent with the reported measurements of 10^{-10} to 10^{-11} M for the hormone–receptor complex in crude preparations (7).

Monoclonal antibodies directed against the 1,25-$(OH)_2D_3$ receptor protein were used in a Western blot analysis to confirm the authenticity of the recombinant receptor (Fig. 4). Monoclonal antibody IVG8C11, reported to cross-react with 1,25-$(OH)_2D_3$ receptor from pig, rat, monkey, human, and chicken (32), was used in Western analysis of extract from sf21 cells (Fig. 4*A*). A doublet at M_r 55,000 was detected in the recombinant extract when IVG8C11 was used as the primary antibody (Fig. 4*A*, lane 2). No bands were evident in either the sf21 cell extract or the wild-type AcNPV-infected sf21 cell extract on the same Western blot (Fig. 4*A*, lanes 3 and 4, respectively). The pig nuclear extract served as the 1,25-$(OH)_2D_3$25-OH-D_3 receptor-positive control with the receptor band present at M_r 55,000 (Fig. 4*A*, lane 1).

An identical blot was analyzed with the monoclonal antibody XVIE10B6A5 as the primary antibody (Fig. 4*B*). This antireceptor antibody reportedly reacts only with porcine-derived 1,25-$(OH)_2D_3$ receptor (32). The Western analysis using XVIE10B6A5 showed no reactivity with the extract from DR-AcNPV-infected sf21 cells (Fig. 4*B*, lane 2). Detection of the receptor protein from the pig nuclear extract was apparent (Fig. 4*B*, lane 1).

The doublet at M_r 55,000 that was detected when the recombinant extract reacted with IVG8C11 may represent posttranslational modifications of the 1,25-$(OH)_2D_3$ receptor protein by 72 hr after infection with DR-AcNPV (Fig. 4*A*, lane 2). A Western analysis was performed using IVG8C11 as the primary antibody that reacted with sf21 extracts harvested at various times after infection with DR-AcNPV (Fig. 4*C*). At 24 hr after infection with the recombinant virus, only a single receptor protein is evident in the cell extract (Fig. 4*C*, lanes 2–5) corresponding to the same molecular weight as the band detected in the pig nuclear extract (Fig. 4*C*, lane 1). By 48 hr after infection, the doublet was apparent (Fig. 4*C*, lanes 6–9) and at 72 hr after infection even higher levels of both bands were visible (Fig. 4*C*, lanes 10–13). Western analysis of an extract from cells harvested 36 hr after DR-AcNPV infection displayed only the lower molecular weight band, although at a higher level than detected at the 24-hr time point (data not shown).

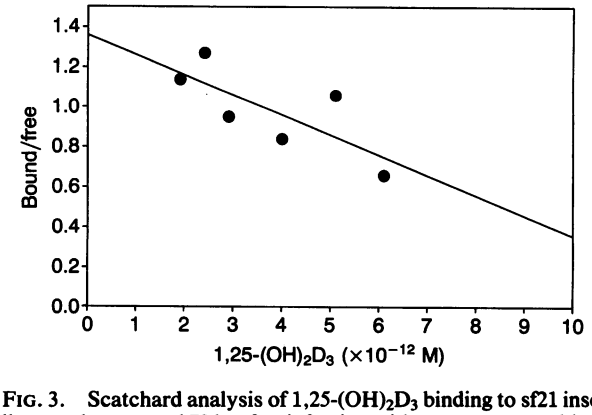

FIG. 3. Scatchard analysis of 1,25-$(OH)_2D_3$ binding to sf21 insect cell cytosols prepared 72 hr after infection with receptor recombinant baculovirus. The cytosol preparation was incubated with increasing concentrations of 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (160 Ci/mmol) in the presence or absence of a 100-fold excess of nonradioactive 1,25-$(OH)_2D_3$ for 4 hr at 4°C.

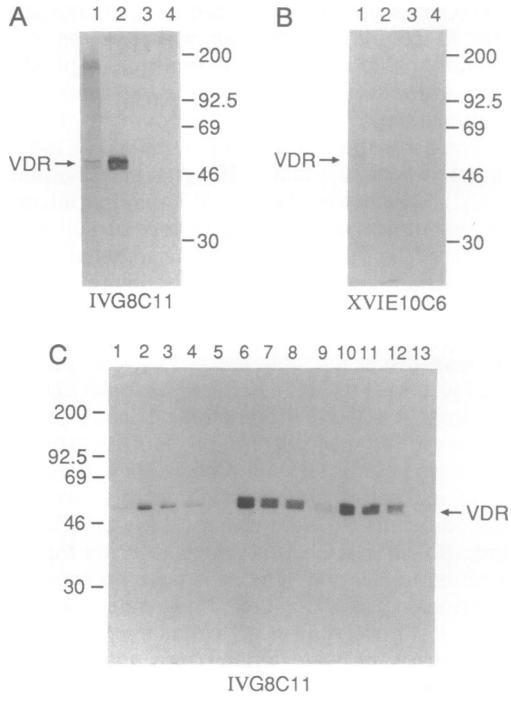

FIG. 4. Western blot of sf21 cell extracts with monoclonal antibodies against the 1,25-$(OH)_2D_3$ receptor. (*A*) Protein extracts from recombinant receptor baculovirus-infected (lane 2), uninfected (lane 3), or wild-type baculovirus-infected (lane 4) sf21 cells. The cross-reacting monoclonal antibody IVG8C11 was the primary antibody. Pig nuclear extract was the positive control for 1,25-$(OH)_2D_3$ receptor (lane 1). Molecular weights (shown as $M_r \times 10^{-3}$) corresponding to the following protein markers, from top to bottom, are shown: myosin, phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase. (*B*) Protein extracts are identical to those shown in *A*. The porcine-specific monoclonal antibody XVIE10B6A5 was the primary antibody. Molecular weight markers are those listed for *A*. (*C*) Protein extracts of sf21 cells prepared over a time course 24 hr (lanes 2–5), 48 hr (lanes 6–9), or 72 hr (lanes 10–13) after infection with 1,25-$(OH)_2D_3$ recombinant baculovirus. Each lane within the particular group of four lanes from each time point represents dilutions (i.e., 1:25, 1:50, 1:100, 1:500) of protein extract in ascending order of lane number. Pig nuclear extract was the positive control (lane 1). Molecular weight markers are those listed for *A*.

DISCUSSION

Although it is likely that 1,25-$(OH)_2D_3$ hormonal activity occurs through its receptor to control the expression of particular genes, the interaction between the steroid and its receptor has not been examined at the molecular level. Study of the physical properties of the 1,25-$(OH)_2D_3$ receptor have been hampered because of the lack of an abundant source from which large quantities can be purified. Described herein

is a baculovirus system exploited for the production of 1,25-$(OH)_2D_3$ receptor that will provide the source required for large-scale purification of this protein. Foreign genes have been expressed in many different systems, including both prokaryotic (38) and eukaryotic (39, 40); however, the baculovirus expression system offers very high expression levels of recombinant protein that, due to the system's efficient processing machinery, are nearly identical to their authentic equivalent (41).

It has been proposed that the mechanism of vitamin D action involves the binding of 1,25-$(OH)_2D_3$ to its receptor, producing a conformational change in the receptor. The structural change in receptor allows for the interaction of the complex with chromatin to alter gene expression (7). To determine the validity of this theory, it is necessary to elucidate the three-dimensional structure of the receptor protein with, and without, bound hormone. In addition, analogs of 1,25-$(OH)_2D_3$, upon interaction with receptor, apparently cause conformational changes in the protein that bring about a discrimination in activities (42). For example, the homolog-bound receptor causes the expression of genes related to the differentiative process in human promyelocytic HL-60 cells, while concurrently reducing the expression of genes whose products are involved in the calcium mobilization process. Therefore, it will also be important to determine the three-dimensional structure of the receptor protein with these synthetic derivatives of 1,25-$(OH)_2D_3$ bound, so that a comparison can be made to the conformation of the protein with bound 1,25-$(OH)_2D_3$.

The mammalian (43) and avian (44) 1,25-$(OH)_2D_3$ receptors undergo rapid phosphorylation in the presence of 1,25-$(OH)_2D_3$. The physiological relevance of 1,25-$(OH)_2D_3$ receptor protein phosphorylation in steroid–receptor interaction and/or receptor–DNA binding may be important for the mechanism of 1,25-$(OH)_2D_3$ action. It is possible, but unlikely, that the apparent heterogeneity we observed with recombinant 1,25-$(OH)_2D_3$ receptor from the baculovirus system is due to a phosphorylation event. Cytosolic preparations must be examined over the time course described (Fig. 4*C*) for any differences in phosphorylation between the preparation with the doublet and the preparation with the single receptor band.

Another unanswered question lies with the molecular interaction between 1,25-$(OH)_2D_3$ receptor and DNA. Control exerted over the expression of the osteocalcin gene by 1,25-$(OH)_2D_3$ has been examined by several groups. The identification of DREs from several species (14–17) has provided a tool by which receptor protein–DRE interaction may be studied. An accessory factor(s) from mammalian cells has been shown to be required for recombinant receptor to bind a segment of DNA consisting of a DRE (45). Our analyses of the recombinant 1,25-$(OH)_2D_3$ receptor from the baculovirus system confirm the report that a mammalian-derived nuclear accessory factor(s) is necessary for receptor–DRE interaction (unpublished data). The positive band-shift analysis provides further evidence that the 1,25-$(OH)_2D_3$ receptor from insect cells is a completely functional recombinant protein.

We are grateful to Dr. Paul Friesen and Ms. Julie Dickson for assistance with the baculovirus expression system. We thank Dr. D. H. L. Bishop for providing us with baculovirus transfer vector pAcYM1. This work was supported by Program Project Grant DK-14881 from the National Institutes of Health and by the Harry Steenbock Research Fund of the Wisconsin Alumni Research Foundation. T.K.R. is a National Institutes of Health Postdoctoral Fellow (DK-08424).

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