Overexpression of the Human Vitamin D_3 Receptor in Mammalian Cells using Recombinant Adenovirus Vectors

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The human vitamin D_3 receptor (hVDR) cDNA was cloned into the E1 region of the adenovirus genome to generate recombinant viruses which were used to infect 293 (adenovirus-transformed human fetal kidney) cells. High salt extracts from cells infected with the recombinant viruses were subjected to immunoblot analysis using a monoclonal antibody to chicken VDR and were shown to contain large quantities of a protein of approximately 50 kDa with a migration identical to that of the hVDR in T47D (human mammary adenocarcinoma) cells. Scatchard analysis showed that the infected cells express approximately 100-fold more receptor than T47D cells and that this receptor binds 1,25-dihydroxyvitamin D_3 with high affinity. The overexpressed hVDR also binds to DNA-cellulose and is eluted with a KCl concentration similar to that determined for fully active endogenous VDR. Nuclear extracts from cells infected with the hVDR-expressing adenoviruses contain an activity that specifically binds an oligonucleotide with sequences from the rat osteocalcin vitamin D_3 response element, as determined by gel mobility shift. This interaction can be inhibited by the presence of an anti-VDR antibody, but not by nonspecific immunoglobulins. We conclude, therefore, that the overexpressed receptor has the ligand- and DNA-binding characteristics defined for endogenous VDR and that adenoviruses can be used to efficiently express large quantities of functional hVDR in a human cell line. Finally, a second binding activity, specific for the vitamin D response element, but distinct from the VDR, has been identified in extracts from uninfected cells. (Molecular Endocrinology 5: 867–878, 1991)

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INTRODUCTION

The vitamin D_3 receptor (VDR) belongs to the steroid receptor superfamily and is one its smaller members by virtue of its very short amino-terminal region, which precedes the DNA-binding domain (1–5). The DNA-binding domain consists of two zinc fingers (6) and has greater homology to the DNA-binding domains of the estrogen and thyroid hormone receptors than to those of the glucocorticoid and progesterone receptors (7, 8). The hormone-binding domain is at the carboxy-terminus (6) and binds 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] with very high affinity ($K_d = 10^{-10}$-10^{-11} M) (9–12). The unoccupied receptor can bind to DNA-cellulose, but is eluted at a lower KCl concentration than occupied receptor (12, 13), implying a ligand-induced increase in its affinity for DNA. Recently, specific sequences in the osteopontin and osteocalcin genes have been shown to confer vitamin D responsiveness on associated promoters (14–18). These vitamin D response elements (VDRE) consist of at least two half-palindromes which resemble those found in other steroid-responsive genes. The VDR in crude nuclear extracts has been shown to bind specifically to the VDRE of the osteocalcin gene by gel mobility shift analysis and footprinting (19–22).

To further characterize the mechanism by which the VDR acts on the osteocalcin gene as well as other less

characterized target genes, *in vitro* analysis using purified VDR will be necessary. However, whereas other transcription factors have been purified and used to explore their function *in vitro*, functional steroid receptors have been difficult to purify even in moderate quantities. Because these proteins are generally of low abundance in cells, tissues have been traditionally used as sources for receptor purification, requiring large numbers of animals and resulting in low total yields. Therefore, various means of overexpression have been attempted since the cloning of cDNAs for the steroid receptors. Expression and purification of full-length receptors in bacteria have been difficult, although domains of various receptors have been successfully overexpressed to yield functional peptides (23–25). Yeast has been used successfully to express moderate quantities of various receptors, including the hVDR (11, 26, 27). In addition, baculovirus has been used to express glucocorticoid and estrogen receptors in infected *Spodoptera frugiperda* (Sf9) cells (28, 29). However, since none of these systems is mammalian, there is some uncertainty about whether receptors expressed in these cells are appropriately processed and modified. The processing of the VDR is not well understood, but it is known to be phosphorylated (30, 31).

Ideally, mammalian cells could be used to provide a source of correctly processed steroid receptors. There are several reports of high level (~10^6 receptors/cell) expression of glucocorticoid and estrogen receptors in Chinese hamster ovary (CHO) cells (32, 33). However, we have chosen to use a viral system for overexpression in mammalian cells rather than select for and stably maintain a cell line. Several groups have used adenovirus to express foreign proteins in mammalian cells (34–37). Adenoviruses efficiently infect cultured mammalian cells; take over their replication, transcription, and translation machinery; and produce large quantities of progeny viruses that lyse the cells within 48 h (38). This system is particularly attractive for overexpression, because large amounts of virally encoded proteins are expressed in a short period of time. Late in infection host protein synthesis is shut down (38), which may be advantageous in purification of the overexpressed protein. We have, therefore, inserted the hVDR cDNA into the adenovirus genome to create two recombinant viruses which differ in the choice of promoters driving the expression of the receptor. Infection of 293 cells (adenovirus-transformed human fetal kidney cells) with these viruses leads to the production of large quantities of functional hVDR.

RESULTS AND DISCUSSION

Construction of hVDR-Expressing Adenovirus

Adenovirus has been used previously to express large quantities of foreign proteins (34–37). Heterologous genes are generally inserted in place of the E3 or E1 gene regions of the adenovirus genome (38). Since E1 gene products are necessary for growth and replication, viruses without a functional E1 region have been propagated in 293 cells, which have the E1 region integrated into their genome and express the E1A and E1B gene products constitutively (39). A human adenovirus-5 (Ad5)-derived mutant, dl309 (40), which behaves like wild-type virus in cell culture, has a unique *Xba*I site in its E1 region which can be used to insert foreign genes. Using the method of Stow (41), we have inserted hVDR expression cassettes in place of the E1 region of dl309 by homologous DNA recombination between specially designed vectors containing the expression cassettes and *Xba*I-digested dl309.

The two hVDR expression plasmids generated to make the recombinant viruses are shown at the *top* of Fig. 1. In both plasmids the first 353 basepairs (bp) of the Ad5 genome lie to the right of the *Eco*RI and *Cla*I sites and contain the adenovirus origin of replication. This is followed by the major late promoter from Ad5 and the tripartite leader from an Ad2 cDNA. In pCV4hVDR a portion of the hVDR cDNA containing the entire reading frame lies between the tripartite leader and polyadenylation sequences from simian virus-40 (denoted SV40 A_n). In contrast, the polyadenylation sequences follow the tripartite leader in pCV5hVDR. The hVDR cDNA sequences are in the opposite orientation of that in pCV4hVDR and lie between the polyadenylation sequence and the cytomegalovirus immediate early enhancer/promoter (CMViee). We chose the CMV promoter because it is highly efficient (42) and independent of adenovirus replication and transcription factors. In both constructs the expression cassette is followed by sequences from the Ad5 genome between 9–15 map units. This part of the vector is necessary for recombination with the homologous region of dl309 inside the host cells.

To generate the recombinant viruses, linearized expression vectors were transfected separately into 293 cells with the large fragment from *Xba*I digestion of dl309, as shown in Fig. 1. This large fragment does not contain the origin of replication and cannot replicate. It must recombine with the linearized expression vector to acquire the origin of replication. Viral plaques generated after transfection were expanded and screened, as described in *Materials and Methods*. Two recombinant viruses, CV4hVDR1 and CV5hVDR2, were plaque purified and characterized further. Their structures are shown in Fig. 1. In the former, expression of the hVDR is driven from the major late promoter, and transcripts contain the tripartite leader at their 5′ terminus. In the latter, the CMV enhancer/promoter drives the expression of the hVDR.

Immunoblot Analysis of Virally Expressed hVDR

To ensure that the immunoreactive proteins in extracts from 293 cells infected with CV4hVDR1 and CV5hVDR2 represent full-length hVDR and not other cellular or viral proteins, extracts isolated from T47D cells, mock-infected 293 cells, and 293 cells infected

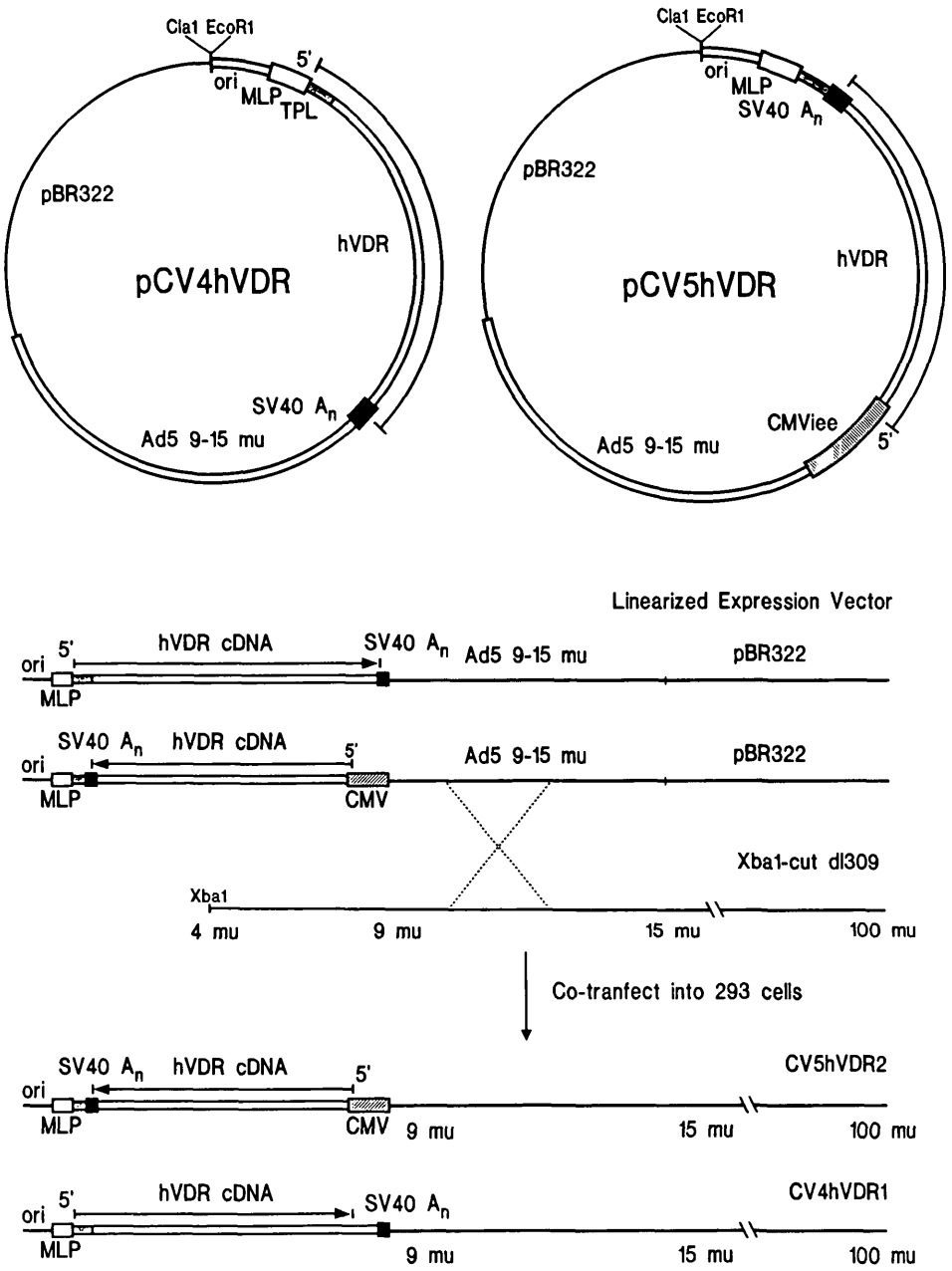

Fig. 1. Human VDR Expression Vectors and Viruses

The expression plasmids used to construct the recombinant adenoviruses are shown at the *top*. Shown *below* is the mechanism by which the viruses are generated by homologous recombination when the linearized expression vectors are cotransfected separately into 293 cells with the large *Xba*I fragment from dl309. The structures of viruses CV4hVDR1 and CV5hVDR2 appear at the *bottom*. ■, SV40 polyadenylation sequences; □, adenovirus tripartite leader sequences; ▨, the CMV immediate early enhancer. ori, Origin of replication; MLP, major late promoter; TPL, tripartite leader; mu, map units.

with dl309, CV4hVDR1, and CV5hVDR2 were subjected to immunoblot analysis, as shown in Fig. 2. Extracts from cells infected with CV4hVDR1 and CV5hVDR2 contain abundant quantities of an immunoreactive protein of approximately 50 kDa, which comigrates with an immunoreactive protein in T47D extracts. T47D cells are human mammary adenocarcinoma cells which express 15,000–20,000 VDR/cell (6). The difference in the quantity of VDR in the cells infected with CV4hVDR1 or CV5hVDR2 and that in T47D cells is striking considering that twice the amount of protein is contained in lane 7 and it is exposed twice as long as the rest of the lanes. In contrast, mock-infected and dl309-infected 293 cells contain very little of this protein species. Upon longer exposure a small amount of this protein can be detected in 293 cells, indicating that these cells express a low amount of endogenous VDR (data not shown). The virally expressed hVDR appears to be stable even in the absence of protease

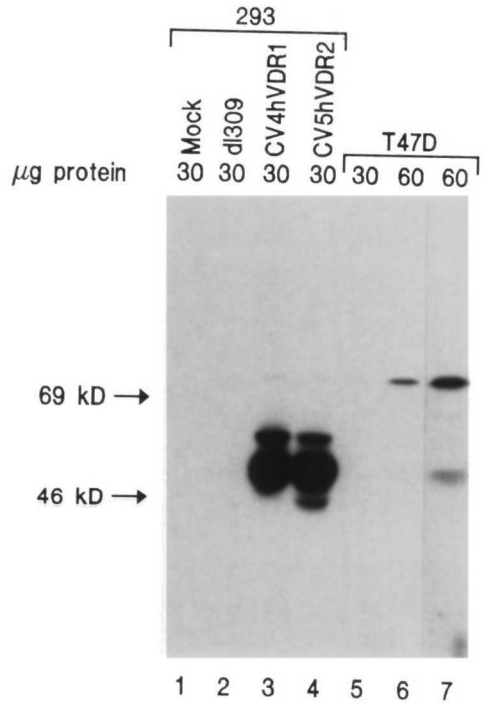

Fig. 2. Immunoblot Analysis of Infected Cell Extracts

Extracts were isolated from T47D cells at 70% confluency (lanes 5–7) and from 293 cells 20 h after infection with the viruses shown above lanes 1–4. Lanes 1–5 contain 30 μg protein and lanes 6 and 7 contain 60 μg protein. Lane 7 represents a longer exposure of lane 6.

inhibitors, with only a slight amount of degradation seen in CV5hVDR2-infected extracts.

Two higher mol wt proteins are visible on the immunoblot. The largest is present in all cell extracts tested, while the other, migrating only slightly slower than the major 50-kDa protein, is present only in the cells infected with the two recombinant viruses. This protein is probably VDR related, because it shows the same time course of expression as the 50-kDa protein (see Fig. 3). We do not know the nature of the larger protein, but its levels are always very low compared with those of the 50-kDa protein. The lower mol wt fragment in the extracts from CV5hVDR2-infected cells is most likely a degradation product, which has been observed previously (43).

Time Course of hVDR Expression

To determine the peak of hVDR expression in infected 293 cells, extracts were isolated at various times after infection and subjected to immunoblot and ligand binding analysis. The experiment shown in Fig. 3 is representative of multiple time-course assays. The *bar graphs* represent levels of ligand-binding activity and immunoreactive material as a function of total protein at 4-h intervals after infection. The *insets* show the immunoblots that were used to determine the amounts of immunoreactive material. In CV4hVDR1-infected 293 cells the levels of ligand-binding activity and immunoreactive material increase gradually as the infection proceeds (*upper panel*). This is consistent with the activity of the major late promoter, which drives the VDR in this virus and is more active at later times in adenovirus infection (38).

A different pattern of expression is observed in CV5hVDR2-infected cells (Fig. 3, *lower panel*). Ligand-binding activity peaks at 16 h and then declines somewhat before increasing again 32 h postinfection. The amount of immunoreactive material reaches its maximum level at 12 h and then declines gradually as infection proceeds. This pattern is a function of the CMV enhancer, which is not dependent on viral functions and can be activated immediately.

Scatchard Analysis

To estimate the number of ligand-binding receptors produced in infected 293 cells, we carried out saturation binding analysis on extracts from cells 16–20 h after infection. The results are shown in Fig. 4. As determined by Scatchard analysis, a single saturable high affinity binding site was detected in both extracts. The dissociation constants are 6.8×10^{-10} and 7.8×10^{-10} M for extracts from CV4hVDR1-infected and CV5hVDR2-infected cells, respectively. Previously reported values for the dissociation constants of the VDR range from approximately 4.5×10^{-10} to 3.8×10^{-11} M (4, 6, 11, 12). The values we have obtained are slightly out of this range. Expression of the VDR at these levels may cause a small decrease in overall affinity, although we have observed dissociation constants of $2–3 \times 10^{-10}$ M in other receptor preparations (data not shown). CV5hVDR2-infected cells express almost 2 times the amount of ligand-binding material as CV4hVDR1-infected cells. The observed maximum binding values indicate that CV5hVDR2-infected cells contain 3.6×10^6 sites/cell, while CV4hVDR1-infected cells contain 1.9×10^6 sites/cell. This represents the generation of approximately 1.6–3.0 μg ligand-binding material/10^7 cells. This level of expression is the highest reported for VDR and appears to be roughly equivalent to levels of human glucocorticoid receptor produced by baculovirus (29). Although we have carried out our experiments using 293 cells growing in monolayer, there is a 293-derived cell line, 293-N3S, which grows in suspension and can be infected with recombinant adenoviruses (44). A scale-up of infection using 293-N3S cells would allow for the capacity to produce large amounts of hVDR or other foreign proteins, giving this system a clear advantage over stable expression systems in CHO cells, which grow only in monolayer.

Binding of Overexpressed hVDR to DNA-Cellulose

The DNA-binding properties of the VDR have traditionally been tested and defined using DNA-cellulose chromatography with a KCl concentration gradient. Receptor occupied by ligand binds to DNA-cellulose and elutes at 0.2–0.22 M KCl. Recently, receptors with point

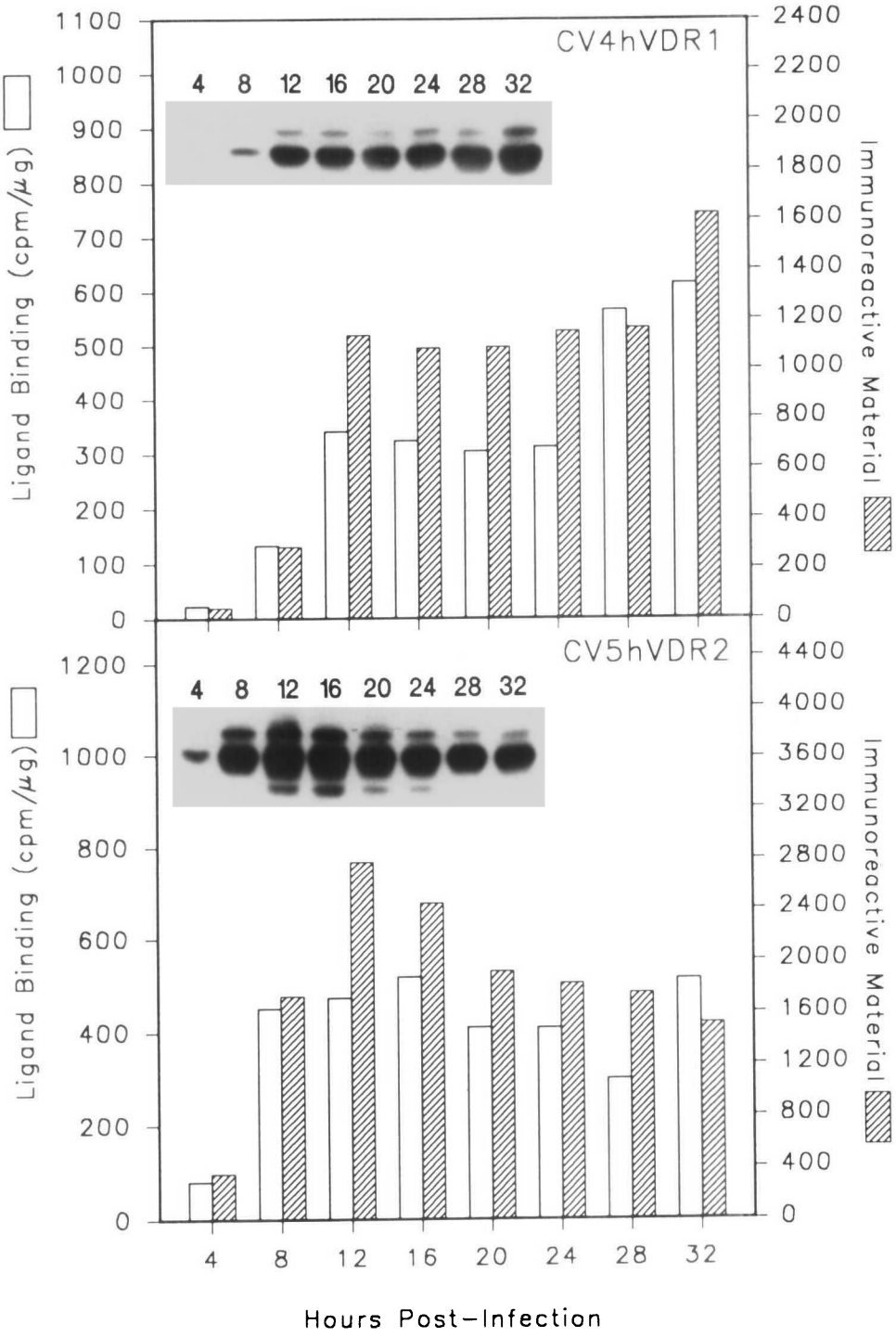

Fig. 3. Time-Course Analysis of Ligand Binding and Accumulation of Immunoreactive Material

Extracts were isolated from CV4hVDR1- and CV5hVDR2-infected 293 cells at 4-h intervals after infection. Aliquots of each extract were incubated with 1α,25-$(OH)_2$-[26,27-*methyl*-^{3}H]D_3 and subjected to hydroxylapatite assay to determine ligand-binding activity. The amount of immunoreactive material at each time point was determined by counting the VDR-containing portion of each lane in the immunoblots shown in the *insets*. These values represent [^{125}I]protein-A (counts per min) per μg protein. Each lane of the immunoblots contains 15 μg protein. *Upper graph*, Results from CV4hVDR1-infected cell extracts. *Lower graph*, Results from CV5hVDR2-infected cell extracts.

mutations in the zinc finger region of the VDR have been defined in patients with hereditary vitamin D-dependent rickets type II. These receptors also bind DNA-cellulose, but with a significantly lower affinity, eluting at 0.1 M KCl (45, 46). Figure 5A shows the DNA-cellulose column profiles of the overexpressed occupied receptors. The hVDR from both CV5hVDR2- and CV4hVDR1-infected cells elutes from the column as a

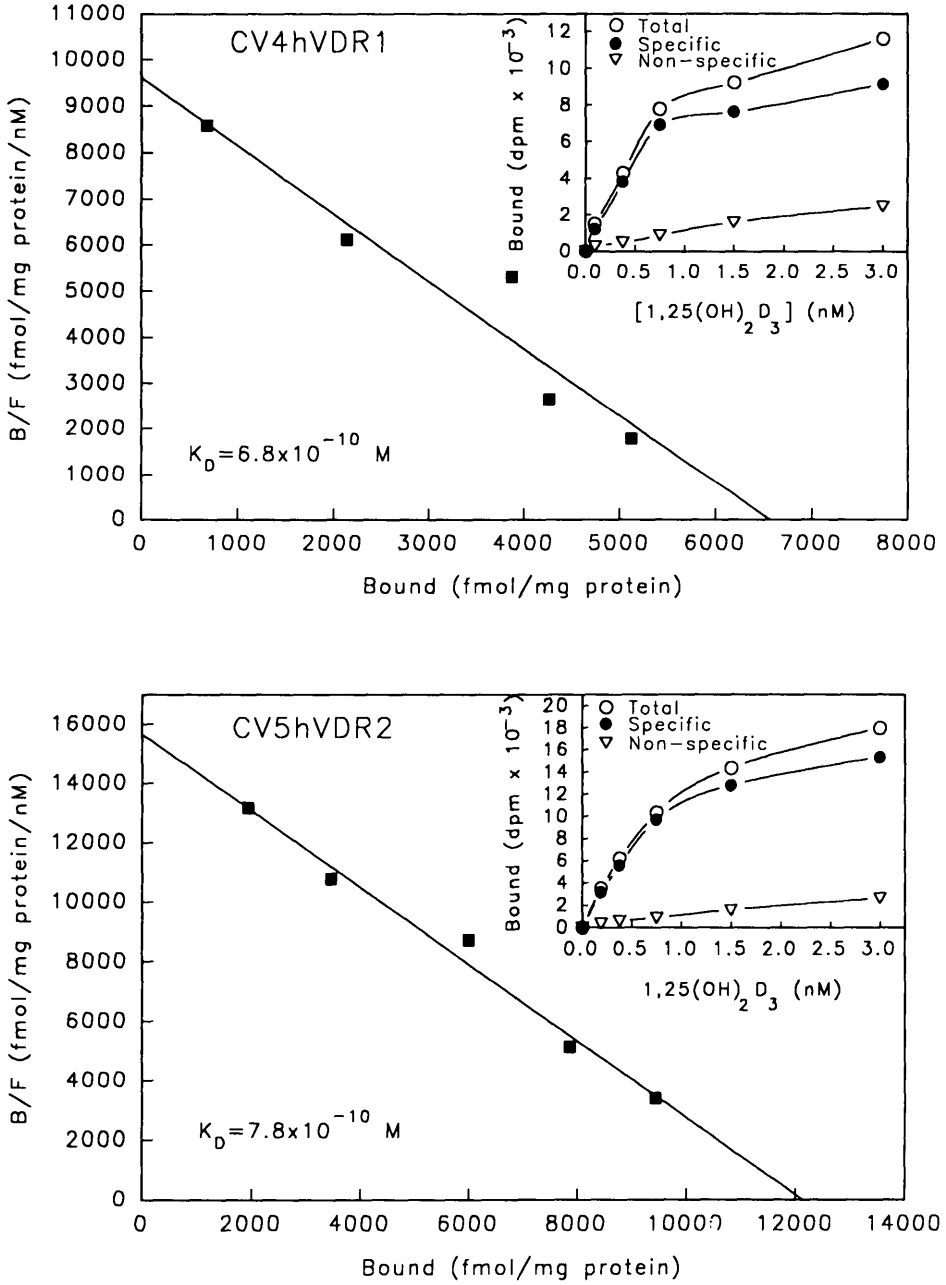

Fig. 4. Scatchard Analysis

Aliquots of extracts isolated 16–20 h after infection of 293 cells with CV4hVDR1 and CV5hVDR2 were incubated for 20 h with varying concentrations of tritiated 1,25-$(OH)_2D_3$ in the presence or absence of an excess of cold 1,25-$(OH)_2D_3$ and subjected to hydroxylapatite assay. The data were transformed by the method of Scatchard. The *insets* show the saturation binding curves. B/F, Bound/free ratio.

single peak at a KCl concentration of 0.2 M. Figure 5B shows sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis of extracts from CV5hVDR2-infected cells in various stages of the chromatography process. (Analysis of extracts from CV4hVDR1-infected cells gives nearly identical results.) The original cell extract was examined as well as the flow-through and pooled peak of tritiated 1,25-$(OH)_2D_3$ label. The samples (10 μg) were run in duplicate on one gel, which was cut in half after electrophoresis and stained with Coomassie blue or subjected to immunoblot analysis. In the Coomassie blue-stained gel (Fig. 5B, *leftmost panel*), a band at approximately 50 kDa (indicated by an *arrow on the left side of the panel*) is seen in all lanes, and its relative abundance in each fraction is mirrored by the immunoreactive material seen in the immunoblot (*rightmost panel*). It is interesting to note that the majority of the VDR remains undegraded even though molybdate was not included in any of the buffers used in this procedure. We have not yet carried out detailed experiments to determine the fraction of receptor in our preparation that is competent to bind

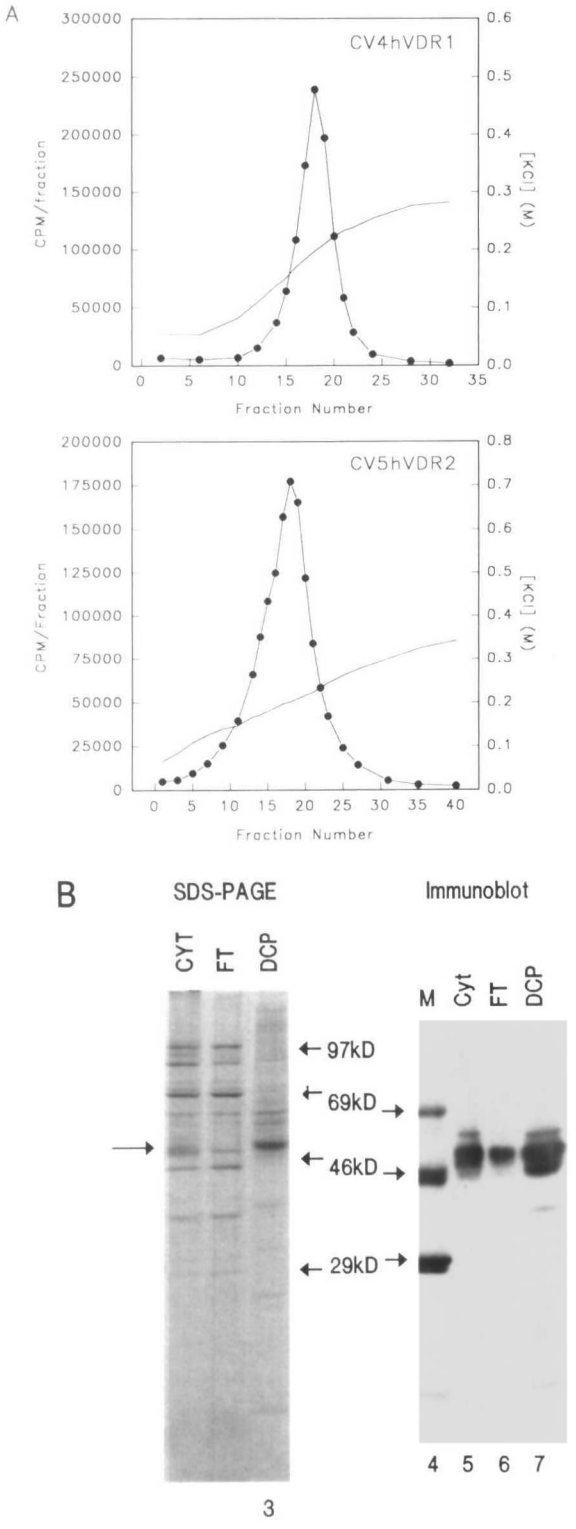

Fig. 5. DNA-Cellulose Chromatography

A, Elution profiles of occupied hVDR from CV4hVDR1- and CV5hVDR2-infected cells. Extracts from CV4hVDR1- and CV5hVDR2-infected cells were processed as described in *Materials and Methods*, mixed with a 50% DNA-cellulose slurry, and loaded into a column. The flow-through and wash were collected in one pool, and the hVDR receptor was eluted in a KCl gradient from 0.05–0.4 M. The presence of receptor was monitored by counting 50-μl aliquots of each fraction. B, Gel analysis of column fractions from CV5hVDR2-infected cells. Ten micrograms of protein from the cytosol before mixing with DNA-cellulose (lanes 1 and 5), from the column flow-through (lanes 2 and 6), and from a concentrate of the pooled peak fractions (lanes 3 and 7) were run in duplicate a sodium dodecyl sulfate-polyacrylamide gel. Half was stained with Coomassie blue and is shown on the *left*. The other half was subjected to immunoblot analysis and is shown on the *right*. CYT, Cytosol; FT, flowthrough; DCP, DNA-cellulose peak; M, protein mol wt standards.

hormone. In yeast expression systems only about 10–20% of expressed steroid receptors are able to bind ligand (21, 27, 47). Since only 5–10% of the ligand-binding material was found in the flow-through (data not shown), we estimate from the immunoblot in Fig. 5B that approximately 50% of the receptors in our system are able to bind hormone.

Specific Binding of Overexpressed hVDR to the Rat Osteocalcin VDRE

Recently, a VDR-binding site has been characterized in the human and rat osteocalcin genes and the mouse osteopontin gene (14–18). We used an oligonucleotide representing sequences in the rat osteocalcin promoter from −462 to −428 bp as a probe in gel mobility shift analysis with nuclear extracts from infected 293 cells. The results are shown in Fig. 6A. In extracts from CV4hVDR1- and CV5hVDR2-infected cells, two strong shifted bands are present (indicated by 1 and 2), both of which are specifically competed away by an excess of unlabeled rat osteocalcin VDRE (rVDRE) oligonucleotide, but not by oligonucleotides containing either nuclear factor 1 (NF1)- or glucocorticoid receptor-binding sequences (GRE). In mock-infected and dl309-infected 293 cells, band 1 is present as well as a pair of faint bands in the vicinity of band 2. As in the other extracts, band 1 is specifically competed by cold rVDRE. The faint upper bands are competed for by cold rVDRE, but are also partially competed by the GRE and NF1 oligonucleotides. There is a very small amount of VDR in 293 cells (Smith, C. L., unpublished results), which could be responsible for the formation of either complex, but the protein(s) that forms band 1 seems to be present in approximately equivalent amounts in all the extracts. Figure 6B shows that the appearance of neither band 1 nor band 2 is dependent on preincubation of the extract with 1,25-$(OH)_2D_3$. The role of ligand binding in steroid receptor function is unclear. Ligand-modulated binding of the VDR to DNA in another expression system has recently been demonstrated (22). The ligand-independent binding we observe may be a reflection of the protein itself as expressed in our system or the way in which the extracts are prepared. This remains to be clarified. However, although there are several reports of ligand-dependent *in vitro* transcriptional activity for progesterone and estrogen receptors (48–50), ligand-independent DNA binding and *in vitro* transcriptional activity have been observed for steroid receptors in other cases (28, 51–54).

To determine which of the complexes contained the

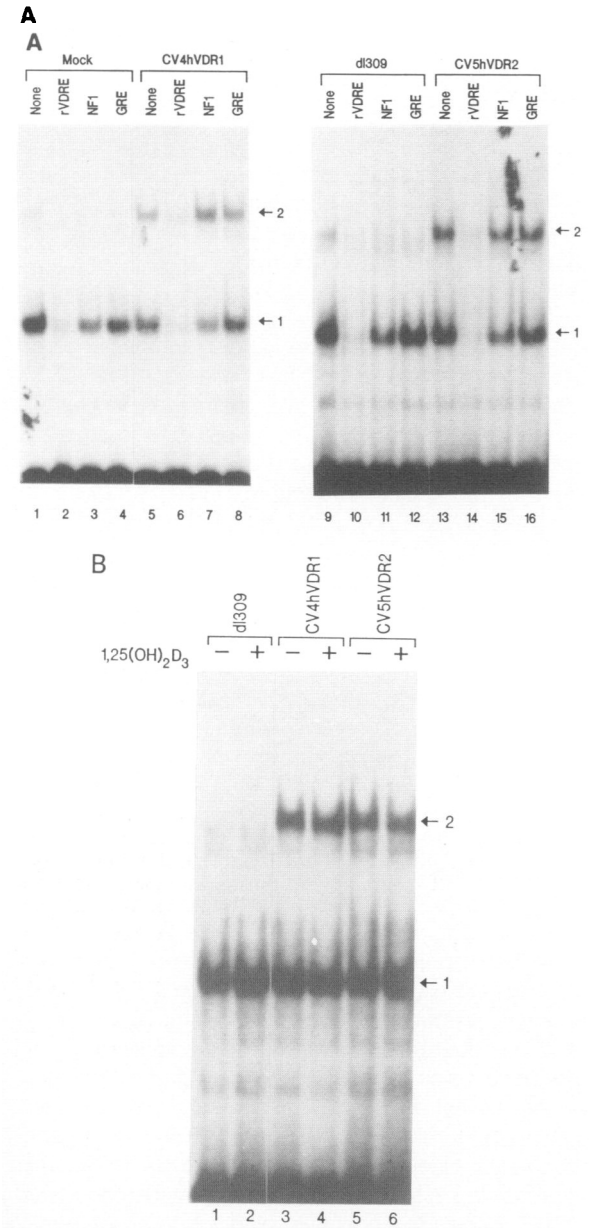

Fig. 6. Gel Mobility Shift Analysis using a Rat VDRE

Nuclear extracts from dl309-, CV4hVDR-, CV5hVDR2-, and mock-infected 293 cells pretreated with 1,25-$(OH)_2D_3$ were incubated with ^{32}P-labeled oligonucleotide containing the VDRE from the rat osteocalcin gene, as described in *Materials and Methods*. A, Two specific complexes are formed with the rVDRE. Fifteen micrograms of protein were run in each lane. Lanes 1, 5, 9, and 13 contain no unlabeled oligonucleotide competitors. Lanes 2, 6, 10, and 14 contain a 30-fold excess of unlabeled probe (rVDRE). Lanes 3, 7, 11, and 15 contain a 30-fold excess of an oligonucleotide with the binding sequence for NF1. Lanes 4, 8, 12, and 16 contain a 30-fold excess of an oligonucleotide with the GRE consensus sequence. B, Nuclear proteins from infected cells bind rVDRE independent of exposure to ligand. Each lane contains 15 μg extract that was preincubated with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) 1,25-$(OH)_2D_3$ for 1 h before addition of the labeled rVDRE probe. Nuclear extracts are from dl309-infected (lanes 1 and 2), CV4hVDR1-infected (lanes 3 and 4), and CV5hVDR-infected (lanes 5 and 6) 293 cells.

VDR, we used as a competitor monoclonal antibody 9A7, which recognizes an epitope just adjacent to the DNA-binding region of the VDR and inhibits the binding of the VDR to DNA. In Fig. 7 it is clear that band 2 alone is specifically competed away by 9A7 and not by nonspecific immunoglobulins, indicating that its formation is dependent on the VDR. The faint bands in the dl309 extracts which migrate slightly slower than band 2 (see Fig. 6B) are not affected by either, ruling out the possibility that they are formed as a result of the small amount of VDR in 293 cells. The identity of the protein(s) that binds specifically to the oligonucleotide probe to form band 1 is presently unknown. However, studies are currently in progress to better define it. These results clearly show that the hVDR generated by our recombinant viruses is able to bind specifically to a sequence that directs vitamin D_3-induced transcription of the osteocalcin gene.

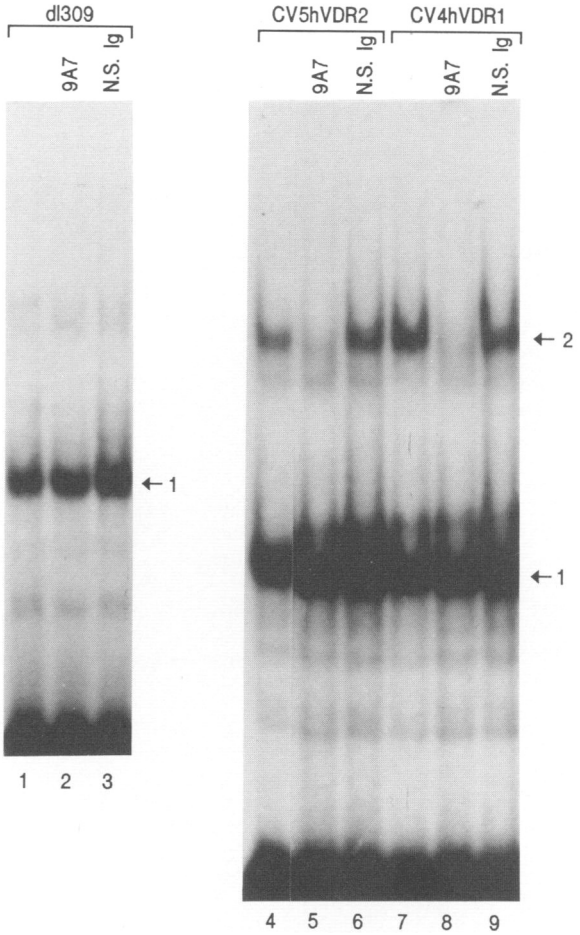

Fig. 7. Antibody Inhibition of hVDR Binding to Rat Osteocalcin VDRE

Nuclear extracts from dl309-infected (lanes 1–3), CV4hVDR-infected (lanes 4–6), and CV5hVDR2-infected (lanes 7–9) cells were preincubated with either monoclonal antibody 9A7 (lanes 2, 5, and 8) or nonspecific antirat immunoglobulins G and M (lanes 3, 6, and 9) in the presence of 1,25-$(OH)_2D_3$ for 30 min before the addition of labeled rVDRE oligonucleotide. Extracts in lanes 1, 4, and 7 were not incubated with any antibodies before the assay.

Summary

We have successfully overexpressed the human VDR using an adenovirus vector in human 293 cells. These receptors appear to be functional by all of the criteria that have traditionally been used to define endogenous VDR in various tissues and cultured cells. The overexpressed receptors are functionally stable even in the absence of molybdate and protease inhibitors, and large quantities of ligand-binding receptor are produced in 293 cells within 32 h after infection. A closely related cell line, 293-N3S, can be grown in suspension, allowing for scale-up of VDR production. These attributes make this system very attractive for large scale purification of the VDR for crystallography and *in vitro* studies of function.

MATERIALS AND METHODS

Cell Culture

The 293 cells (39) (obtained from Dr. Barrie Carter, NIDDK) were used as a host in all experiments. These cells are derived from human fetal kidney transformed with adenovirus and express the E1A and E1B proteins constitutively (39). They were maintained in Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY) with 10% fetal bovine serum (Gibco) and 50 μg/ml gentamicin. All adenovirus infections were carried out, as previously described (55), when the cells were 60–70% confluent.

Plasmid Constructions

The vector MpCV2 has been previously described (35). A derivative of this vector, MpCV3, was used to construct hVDR expression plasmids. MpCV2 was digested with *Bgl*II and *Xho*I, end filled with Klenow, and religated after the addition of *Bam*HI linkers to form MpCV3. This unique *Bam*HI site just down-stream from the major late promoter and tripartite leader was used to insert an oligonucleotide containing nine unique restriction sites (*Hin*dIII, *Xba*I, *Xho*I, *Kpn*I, *Bcl*I, *Hpa*I, *Mlu*I, *Bam*HI, and *Bgl*II). Two plasmids were derived, MpCV4 (5′-*Hin*dIII to *Bgl*II-3′) and MpCV5 (5′-*Bgl*II to *Hin*dIII-3′), which differ in the orientation of the inserted polylinker. A *Bam*HI/*Hpa*I fragment containing the SV40 polyadenylation signal (bp) was inserted between the *Hpa*I and *Bam*HI sites of MpCV4 to form pCV4SVA. The unique *Xba*I site in pCV4SVA was used to insert an *Xba*I fragment from phVDR2 which contains hVDR cDNA (+146 to +2140 bp) and includes the entire hVDR reading frame (phVDR2 contains hVDR cDNA cloned into the *Eco*RII site of pGEM7Zf+). The resulting plasmid, pCV4hVDR, was used to make the adenovirus recombinant CV4hVDR1. MpCV5 was digested with *Hin*dIII and *Hpa*I, and a *Hin*dIII/*Sma*I fragment from pCM524 (42) was inserted, which contains the cytomegalovirus immediate early enhancer/promoter (CMViee), the chloramphenicol acetyltransferase (CAT) gene, and the SV40 polyadenylation signal. The resulting construct, pCV5CMCT, was digested with *Xba*I and *Hpa*I to remove the entire CAT gene, and an *Xba*I/*Pvu*II fragment from phVDR2 containing the hVDR cDNA (+146 to +2061 bp) was inserted to generate pCV5hVDR. This expression plasmid was used to generate the recombinant virus CV5hVDR2.

Virus Construction and Screening

Human adenovirus mutant dl309 (40) was used as the parent virus in our viral constructs. Viral DNA was isolated from dl309-infected 293 cells, as previously described (55), and digested at the unique *Xba*I site in the E1 region of the genome, resulting in two fragments, 1.3 and 35 kilobases. These fragments were separated in a 5–20% sucrose gradient, as previously described (41). The large fragment (1 μg) was then cotransfected by Lipofectin (Bethesda Research Laboratories, Gaithersburg, MD) (56) into 293 cells (60-mm dishes) with a 5-fold molar excess of either pCV4hVDR or pCV5hVDR that had been linearized with *Cla*I. The transfected cells were covered with agar overlay (0.9% Noble agar in 1-fold concentrated Modified Eagle's Medium with 5% fetal bovine serum) and allowed to incubate for 10 days before staining with 0.1% neutral red to visualize any plaques. Individual plaques were isolated, and minilysates from the plaques were used to infect 60-mm dishes of 293 cells. When viral infection was apparent (4–7 days) lysates were prepared and used to infect 100-mm dishes of 293 cells. Cells from these plates were collected and used to make viral stocks and lysates for viral DNA analysis by the method of Hirt (57). Viral DNA was digested with *Bam*HI, electrophoresed on 1% agarose gels, transferred to a Nytran membrane, and hybridized with random primed hVDR cDNA, as previously described (58). Viral isolates containing hVDR sequences were expanded, and extracts from cells infected with these isolates were collected to examine for the presence of the hVDR protein by immunoblotting. One isolate from each of the two original transfections expressing large amounts of hVDR protein was used to infect 60-mm plates of 293 cells at various dilutions to plaque purify two viruses, CV4hVDR1 and CV5hVDR2.

Preparation of Cell Extracts

For immunoblotting and the ligand binding assay used in the time-course experiments, infected cells were disrupted by sonication in TDKM-0.3 [10 mM Tris (pH 7.6), 5 mM dithiothreitiol (DTT), 0.3 M KCl, and 10 mM sodium molybdate]. For Scatchard analysis and DNA-cellulose binding, infected cells were disrupted by Dounce homogenization (Kontes Co., Vineland, NJ) in TDK-0.01 [10 mM Tris (pH 7.6), 5 mM DTT, and 0.01 M KCl]. The KCl concentration was adjusted to 0.3 M KCl after cell lysis. Sodium molybdate was added to extracts used for Scatchard analysis to a final concentration of 10 mM. All cell lysates were centrifuged at 4 C and 100,000 × *g* to generate high salt extracts.

Immunoblotting

Cytosolic proteins were separated on 10% mini sodium dodecyl sulfate-polyacrylamide gels (Mighty Tall, Hoeffer, San Francisco, CA) and transferred to Immobilon (Millipore, Bedford, MA) for 2 h at 200 mamp in 25 mM Tris (pH 8.3)-0.192 M glycine-20% methanol. Blots were blocked for 1 h at room temperature with TBS [20 mM Tris (pH 7.5) and 0.18 M NaCl] containing 0.5% nonfat dry milk, followed by overnight incubation with monoclonal antibody 9A7 (59) diluted 1:1000 in TBS-0.5% nonfat dry milk. The blots were washed three times for 15 min each time in TBST (TBS-0.05% Tween-20) and then incubated with second antibody (rabbit antirat immunoglobulins G and M, Zymed, San Francisco, CA) diluted 1:1000 in TBS-0.5% nonfat dry milk for 1–2 h at room temperature. After the blots were washed, as described above, they were incubated for 1 h at room temperature with [^{125}I]protein-A (Amersham, Arlington Heights, IL) in TBS-0.1% BSA at a concentration of 0.1 μCi/ml. Blots were then washed three times in TBST and exposed to film (XAR-5, Eastman Kodak, Rochester, NY) at −70 C.

Ligand Binding Assays

For time-course studies, cell extracts from each time point were diluted 1:50 and incubated at 0 C for 2 h with 1α,25-(OH)$_2$-[26,27-*methyl*-^{3}H]D$_3$ (Amersham and New England Nu-

clear, Boston, MA) at a final concentration of 2 nM in the presence or absence of a 1000-fold excess of unlabeled 1,25-$(OH)_2D_3$. Specific ligand binding to receptor was then determined by a hydroxylapatite binding assay (60). For Scatchard analysis, cell extracts were diluted with TDKM-0.3 to a protein concentration of 25–50 μg/ml, and 200-μl aliquots were incubated with 1α,25-$(OH)_2$-[26,27-*methyl*-^{3}H]D_3 at various concentrations for 16–20 h at 0 C. Specific binding was determined by a hydroxylapatite binding assay.

DNA-Cellulose Column Chromatography

DNA-cellulose was prepared by the method of Alberts and Herrick (61). Extracts from infected cells (5 ml) were incubated for 2 h with 1α,25-$(OH)_2$-[26,27-*methyl*-^{3}H]D_3 at a final concentration of 2 nM. They were then diluted 5-fold with TDK-0 [10 mM Tris (pH 7.6) and 5 mM DTT] and added to 10 ml of a 50% slurry of DNA-cellulose equilibrated with TDK-0.05 [10 mM Tris (pH 7.6), 5 mM DTT, 0.05 M KCl]. After stirring gently for 1 h at 4 C, the DNA-cellulose mixture was loaded into a column. The flow-through was collected, and the column was washed with 3 vol TDK-0.05. The receptor was eluted in a 100-ml gradient of 0.05–0.4 M KCl. Two-milliliter fractions were collected after the KCl gradient was started, and 50-μl aliquots of each were counted for ^{3}H content. The conductivity of each fraction was also determined.

Gel Mobility Shift Analysis

Nuclear extracts were prepared by a modified Dignam *et al.* protocol (62). Cells were disrupted by Dounce homogenization in buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT]. Nuclei were pelleted by centrifugation at 500 × *g*, washed once with buffer A, and pelleted again at 25,000 × *g*. Nuclear pellets were resuspended by gentle vortexing in TDK-0.3, incubated on ice for 15 min with occasional vortexing, and centrifuged at 25,000 × *g*. The supernatant was collected and used in gel shift analysis. After 1-h incubation with 200 nM 1,25-$(OH)_2D_3$, nuclear extracts were incubated for 20 min at room temperature with 2.7 μg polydIdC and 10 fmol labeled probe in the presence or absence of an excess of "cold" competitor oligonucleotides in a buffer of 10 mM Tris (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 1 mM EDTA, and 1 mM DTT. In the antibody inhibition study nuclear extracts were preincubated for 30 min on ice with monoclonal antibody 9A7 (59) or nonspecific purified antibodies to rat immunoglobulins at a ratio of 0.35 μg antibody protein/μg nuclear extract protein before being added to the other assay components (competitor oligonucleotides were not included). The samples were electrophoresed on 5% nondenaturing polyacrylamide gels in 0.5 × TBE at 6 V/cm. After electrophoresis, gels were dried and exposed to XAR-5 film at −70 C. A double stranded oligonucleotide containing sequences from the VDRE of the rat osteocalcin gene (5′-TCG AGCACTGGGTGAATGAGGACATTACTGACCGCTCC-3′) was used as a probe in all experiments and was labeled with [^{32}P]ATP by polynucleotide kinase. Competitor DNAs used were double stranded oligonucleotides containing the NF1 (5′-GATCCGATTGGAATTCATCCAAATCTTA-3′)-binding site from the mouse mammary tumor virus promoter and a consensus GRE (5′-GATCCAAAGTCAGAACACAGTG-TTCTGATCAAAGA-3′). All oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) 380B machine.

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