The 1,25-Dihydroxy-vitamin D_3 Receptor Is Phosphorylated in Response to 1,25-Dihydroxy-vitamin D_3 and 22-Oxacalcitriol in Rat Osteoblasts, and by Casein Kinase II, in Vitro†

Peter W. Jurutka, Christopher M. Terpening,‡ and Mark R. Haussler*

Department of Biochemistry, College of Medicine, The University of Arizona, Tucson, Arizona 85724

Received February 11, 1993; Revised Manuscript Received May 12, 1993

ABSTRACT: We analyzed the endogenous nuclear 1,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$) receptor (VDR) in rat osteosarcoma (ROS 17/2.8) cells and present biochemical evidence that it is a phosphoprotein. When ROS 17/2.8 cells are labeled metabolically with [^{35}S]methionine, treatment with 10^{-8} M $1,25(OH)_2D_3$ elicits a decrease in the electrophoretic mobility of immunoprecipitated VDR in denaturing polyacrylamide gels, a property characteristic of phosphorylated proteins. Similar labeling of cells with [^{32}P]orthophosphate results in a rapid (≤30 min), $1,25(OH)_2D_3$-dependent incorporation of ^{32}P into a 54-kDa VDR species that comigrates with the slower migrating receptor species extracted from [^{35}S]methionine-labeled ROS 17/2.8 cells that have been exposed to $1,25(OH)_2D_3$. Alkaline phosphatase treatment of immunoprecipitated VDR from $1,25(OH)_2D_3$-treated cells converts the form of the VDR with reduced mobility to the faster migrating species present in $1,25(OH)_2D_3$-deficient cells. Incubation of ROS 17/2.8 cells with the non-hypercalcemic $1,25(OH)_2D_3$ analog, 22-oxacalcitriol (OCT), produces a level of VDR phosphorylation similar to that elicited by $1,25(OH)_2D_3$ treatment. Transient transfection of osteosarcoma cells with a reporter vector containing a vitamin D responsive element derived from the rat osteocalcin gene yields equivalent transcriptional activation in the presence of either $1,25(OH)_2D_3$ or OCT. Further experiments performed at various $1,25(OH)_2D_3$ concentrations to assess the relationship between receptor phosphorylation and transcriptional activity in intact cells showed a positive correlation between these two parameters, indicating that the $1,25(OH)_2D_3$ hormone stimulates VDR phosphorylation and transcriptional activation in parallel. Finally, highly purified casein kinase II (CK-II) phosphorylates the VDR in a $1,25(OH)_2D_3$-independent, in vitro reaction. Comparison of the migration in denaturing gel electrophoresis of in vivo and in vitro phosphorylated VDR reveals a different electrophoretic mobility of the CK-II-phosphorylated receptor, suggesting that CK-II-mediated phosphorylation may be distinct from that occurring in response to $1,25(OH)_2D_3$ or OCT. We speculate that hormone-dependent phosphorylation of the VDR may be required for transcriptional activation, while hormone-independent, CK-II-mediated phosphorylation may play a role in the modulation of receptor activity.

The 1,25-dihydroxy-vitamin D_3 receptor (VDR)[1] is a rare, labile protein which is a member of a large superfamily of receptors, including those for steroid hormones, thyroid hormone, and retinoic acid (Evans, 1988; Haussler et al., 1988a). The receptor binds 1,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$) with high affinity, localizes to and becomes tightly associated with the nucleus, and regulates the activity of target genes such as intestinal calcium binding protein (Christakos et al., 1989) and osteocalcin (Pike, 1990).

A number of members of the steroid/thyroid hormone receptor superfamily are reported to be phosphoproteins, including the progesterone receptor (Dougherty et al., 1982), glucocorticoid receptor (Housley & Pratt, 1983), thyroid hormone receptor (Goldberg et al., 1988), estrogen receptor (Auricchio et al., 1987), and androgen receptor (Goueli et al., 1984), as well as the VDR itself (Haussler et al., 1988b; McDonnell et al., 1989; Brown & DeLuca, 1990; Jones et al., 1991). Several studies have examined the phosphorylation of steroid hormone receptors, in vivo, and have found, in the case of the glucocorticoid receptor (Ortí et al., 1989), progesterone receptor (Logeat et al., 1985), estrogen receptor (Denton et al., 1992), and VDR (Pike & Sleator, 1985), that a component of the phosphorylation is hormone-dependent. These observations suggest that phosphorylation may play a functional role in the hormone-elicited actions of these receptors.

Other studies have focused on which kinases may catalyze receptor phosphorylation by examining phosphorylation reactions in vitro. The progesterone (Weigel et al., 1981), glucocorticoid (Singh & Moudgil, 1985), and $1,25(OH)_2D_3$ receptors (Jurutka et al., 1993a) can be effectively phosphorylated in vitro by cAMP-dependent protein kinase (PK-A). Another kinase of potential significance in terms of steroid hormone receptor phosphorylation is casein kinase II (CK-II). This predominantly nuclear-localized kinase (Krek et al., 1992) is present in a wide variety of eukaryotic cells (Tuazon & Traugh, 1991) and is known to phosphorylate nuclear oncogene products and a number of proteins involved

† This work was supported in part by National Institutes of Health Grants DK-33351 and AR-15781. This research constitutes a portion of the Ph.D. dissertation submitted to the Department of Biochemistry, The University of Arizona, by P.W.J.

* Correspondence should be addressed to this author.

‡ Present address: Department of Biochemistry, Colorado State University, Fort Collins, CO 80523.

[1] Abbreviations: VDR, 1,25-dihydroxy-vitamin D_3 receptor; $1,25(OH)_2D_3$, 1,25-dihydroxy-vitamin D_3; PK-A, cAMP-dependent protein kinase; CK-II, casein kinase II; ROS 17/2.8, a clonal rat osteosarcoma osteoblast-like cell line; rVDR, rat 1,25-dihydroxy-vitamin D_3 receptor; OCT, 22-oxa-$1,25(OH)_2D_3$ (22-oxacalcitriol); DMEM/F-12, Dulbecco's modified Eagle medium and Ham's F-12; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; mAb, monoclonal antibody; VDRE, vitamin D responsive element; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; hVDR, human 1,25-dihydroxy-vitamin D_3 receptor.

0006-2960/93/0432-8184$04.00/0

in gene expression, including RNA polymerases I and II (Dahmus, 1981; Stetler & Rose, 1982), topoisomerases I and II (Durban et al., 1985; Ackerman et al., 1988), c-myc (Lüscher et al., 1989), c-myb (Lüscher et al., 1990), and max (Berberich & Cole, 1992). The c-erbAα-encoded thyroid hormone receptor also appears to be a substrate for CK-II in vitro (Glineur et al., 1989).

The functional significance of steroid and thyroid hormone receptor phosphorylations is unknown, but may involve any of the following: (i) modulation of hormone binding; (ii) nuclear localization; (iii) specific DNA binding; (iv) association with a receptor auxiliary factor; (v) transcriptional control; and (vi) receptor recycling/processing. In an effort to characterize this post-translational modification biochemically, we have investigated phosphorylation of the VDR utilizing osteoblast-like rat osteosarcoma (ROS 17/2.8) cells (Majeska et al., 1980). Earlier studies of VDR phosphorylation examined the mouse (Pike & Sleator, 1985), chicken (Brown & DeLuca, 1990), and human (McDonnell et al., 1989; Jones et al., 1991) receptors. These previous reports demonstrated 1,25$(OH)_2D_3$ hormone-dependent phosphorylation of the endogenous mouse VDR in cultured fibroblasts (Pike & Sleator, 1985) and of the endogenous chicken VDR in embryonic chick duodenal organ culture (Brown & DeLuca, 1990), the latter post-translational modification taking place prior to significant 1,25$(OH)_2D_3$-induced calcium binding protein (M_r 28 000) mRNA accumulation. We demonstrate here that the endogenous rat VDR (rVDR), present in relatively high concentrations in ROS 17/2.8 cells, is rapidly phosphorylated in vivo by a hormone-dependent mechanism and that in vivo phosphorylation induced by either the natural 1,25$(OH)_2D_3$ hormone or the analog 22-oxacalcitriol (OCT) is correlated positively with the transcriptional activity of VDR. Also, rVDR is a substrate for CK-II, in vitro, but this reaction is apparently independent of the presence of 1,25$(OH)_2D_3$. We hypothesize that multisite phosphorylation of VDR may be an obligatory and/or regulatory step in the pathway leading to VDR-mediated transcriptional regulation of target genes.

MATERIALS AND METHODS

Reagents. All reagents were of electrophoresis and/or ultrapure molecular biology grade. Purified CK-II isolated from bovine testis was a gift from Drs. D. W. Litchfield and E. G. Krebs (Department of Pharmacology and Biochemistry, Howard Hughes Medical Institute, University of Washington). OCT was kindly provided by Drs. Y. Nishii and J. Abe, Chugai Pharmaceutical Co., Ltd. (Toshima-ku, Tokyo, Japan).

Cell Culture. ROS 17/2.8 cells were cultured in Dulbecco's modified Eagle medium and Ham's F-12 (DMEM/F12, 1:1) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 units/mL penicillin. The cells were incubated in 60-mm culture dishes in a humidified incubator at 37 °C.

Metabolic Labeling. ROS 17/2.8 cells (7×10^5/60 mm plate) were incubated for 16 h as described above. The cells were then washed twice with 5 mL of methionine-free or phosphate-free DMEM/F12 followed by incubation in 2.0 mL/plate of either methionine-free or phosphate-free DMEM/F12 supplemented with 2% dialyzed FBS, 0.5 mCi of [^{35}S]methionine (New England Nuclear, Boston, MA, translation grade, 1100 Ci/mmol), or 0.5 mCi of [^{32}P]orthophosphate (New England Nuclear, Boston, MA, carrier-free, 8500–9120 Ci/mmol) and various concentrations of 1,25$(OH)_2D_3$, 10^{-8} M OCT, or ethanol (vehicle) control. The labeling was carried out for up to 4 h at 37 °C. The cells were then washed twice in 5 mL of Tris-buffered isotonic saline, pH 7.5, in preparation for processing.

Preparation of Cellular Lysates. Cells were lysed directly on plates by incubation in 1.0 mL of high-salt lysis buffer (0.3 M KCl, 10 mM Tris-HCl (pH 7.4), 1.0 mM EDTA, 0.3 mM $ZnCl_2$, 0.5% Triton X-100, and 5 mM DTT) for 5 min. The lysates from ^{35}S-labeled cells were cleared by centrifugation at 170000g for 30 min, while lysates from ^{32}P-labeled cells were cleared by centrifugation at 16000g for 30 min.

Immunoprecipitation and Gel Electrophoresis. Cell lysates were immunoprecipitated with anti-VDR 4A5γ monoclonal antibody (mAb) linked to Sepharose beads (Pike et al., 1983). Typically, 500-μL aliquots of lysate were incubated with 10 μL of 4A5γ–Sepharose ($\approx$1 mg of mAb/mL) overnight at 4 °C with gentle shaking. Control reactions to identify nonspecific binding to 4A5γ–Sepharose contained a severalfold excess of free 4A5γ mAb. The Sepharose beads were then washed extensively with detergent-based wash buffers, essentially as described previously (Mangelsdorf et al., 1987). The washed beads were either further utilized for in vitro reactions (kinase or phosphatase) or resuspended in 30 μL of 2 × FSB (4% SDS, 10% β-mercaptoethanol, 0.125 M Tris-HCl (pH 6.8), and 20% glycerol), boiled for 4 min, and electrophoresed on 10% SDS–polyacrylamide gels as described by Laemmli (1970). The gels were fixed in 30% methanol/10% trichloroacetic acid/10% acetic acid, washed in water, impregnated with fluor (1 M sodium salicylate), dried, and fluorographed at −70 °C on Kodak X-OMAT AR film.

In Vitro Kinase and Alkaline Phosphatase Reactions. Cells (2×10^6) grown in the absence of 1,25$(OH)_2D_3$ were lysed and immunoprecipitated as described above. After extensive washing, the beads were washed twice in KTMD-0.15 (0.15 M KCl, 50 mM Tris-HCl (pH 7.4), 15 mM $MgCl_2$, 5 mM DTT), resuspended in 35 μL of KTMD-0.15, and incubated for 2 h with 10^{-8} M 1,25$(OH)_2D_3$ or ethanol, followed by incubation with 40 μCi of [γ-^{32}P]ATP (ICN, Irvine, CA, 25 Ci/mmol, 8 μCi/μL) and 6×10^{-4} unit of highly purified CK-II for 30 min at 30 °C. One unit of CK-II is defined as the amount of enzyme that catalyzes the transfer of 1.0 μmol of phosphate per min to a synthetic peptide substrate at 30 °C (Litchfield et al., 1990).

Alkaline phosphatase reactions utilized immunoprecipitated, [^{35}S]methionine-labeled VDR. The washed beads were resuspended in 40 μL of water, 5 μL of 10× phosphatase buffer (Promega Corp., Madison, WI), and 50 units calf intestinal alkaline phosphatase (Promega Corp.) and incubated for various times at 37 °C. The beads were washed again and subjected to electrophoresis as described above.

Cell Transfections and Transcription Assays. Transcriptional activity of the VDR was measured in ROS 17/2.8 cells (5×10^5 cells/60 mm plate) which had been transfected by calcium phosphate–DNA coprecipitation (Kingston, 1990) with a reporter vector (5 μg/plate) containing the vitamin D responsive element (VDRE) within approximately 1100 base pairs of the 5′-flanking DNA from the rat osteocalcin gene (rBGPGH), linked upstream of the human growth hormone gene (Terpening et al., 1991). As a control, a similar reporter vector containing only the osteocalcin core promoter and 146 base pairs of 5′-flanking DNA (without the VDRE) was utilized (P2AGH). Cells were treated for 24 h following transfection with either various concentrations of 1,25-$(OH)_2D_3$, 10^{-8} M OCT, or ethanol vehicle. Medium was then assayed for human growth hormone by radioimmunoassay using a kit from Nichols Institute Diagnostics (San Juan Capistrano, CA).

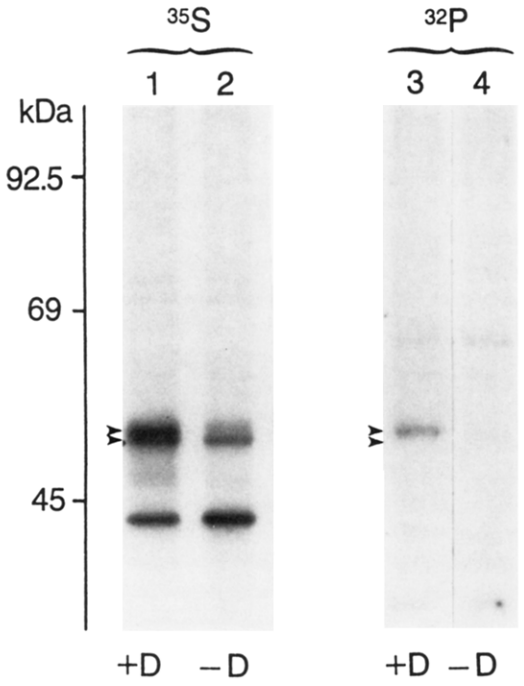

FIGURE 1: Hormone-dependent, in vivo phosphorylation of VDR isolated from ROS 17/2.8 cells. ROS 17/2.8 cells were labeled metabolically with 0.5 mCi of [^{35}S]methionine (lanes 1 and 2) or 0.5 mCi of [^{32}P]orthophosphate (lanes 3 and 4) and treated with 10^{-8} M $1,25(OH)_2D_3$ (lanes 1 and 3) or ethanol vehicle (lanes 2 and 4) for 4 h as described in Materials and Methods. Cell lysates were immunoprecipitated with mAb 4A5γ–Sepharose and subjected to 10% SDS–PAGE. The arrowheads indicate the positions of the hyperphosphorylated (upper arrow) and hypophosphorylated (lower arrow) forms of rVDR.

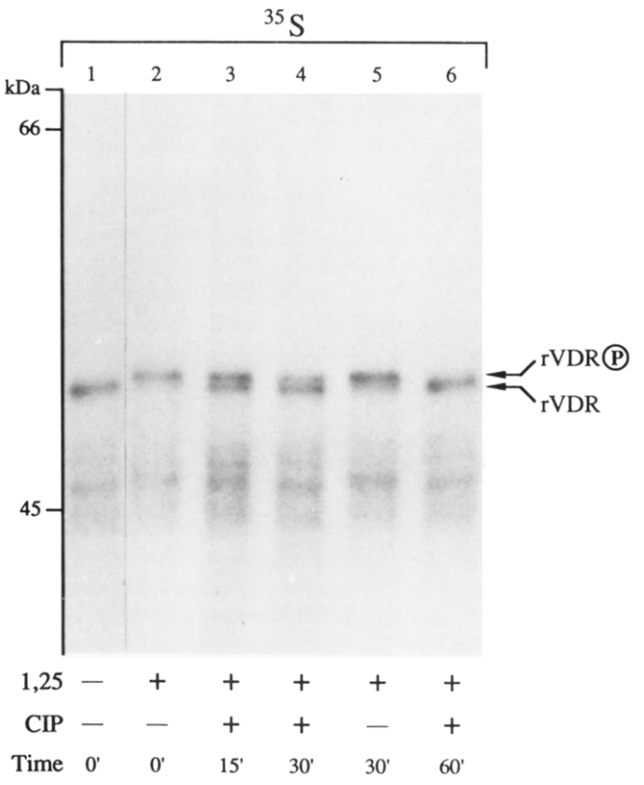

FIGURE 2: Phosphatase treatment of in vivo phosphorylated VDR. ROS 17/2.8 cells were labeled metabolically with 0.5 mCi of [^{35}S]methionine in the absence (lane 1) or presence (lanes 2–6) of 10^{-8} M $1,25(OH)_2D_3$ for 4 h. Cell lysates were immunoprecipitated with mAb 4A5γ–Sepharose and incubated in either the absence (lanes 1, 2, and 5) or presence (lanes 3, 4, and 6) of calf intestinal alkaline phosphatase (50 units) for 0–60 min at 37 °C, as described in Materials and Methods. The arrows indicate the positions of the hyperphosphorylated (upper arrow) and hypophosphorylated (lower arrow) forms of rVDR.

RESULTS

Hormone-Dependent Phosphorylation of VDR. The phosphorylation of VDR was examined in ROS 17/2.8. These cells express a relatively high level of endogenous receptor (≈15 000 receptors/cell) and therefore represent a useful system in which to study VDR phosphorylation. ROS 17/2.8 cells were labeled metabolically with [^{35}S]methionine in the presence and absence of $1,25(OH)_2D_3$ (Figure 1, left panel). When VDR from untreated cells was immunoextracted with anti-VDR mAb 4A5γ linked to Sepharose, the resulting autoradiogram displayed two prominent ^{35}S-labeled protein bands, one migrating at ≈54 kDa and the other band migrating at ≈43 kDa (lane 2). We have previously reported (Jones et al., 1991) that the 43-kDa protein is not VDR-related because its intensity is not reduced in the presence of excess free 4A5γ mAb during solid-phase immunoprecipitation. In contrast, the intensity of the 54-kDa species is greatly reduced when excess free anti-VDR antibody competes for binding with the solid-phase antibody [data not shown; see also Jones et al. (1991)], confirming that this species (and not the labeled protein at 43 kDa) is the rVDR. In the presence of 10^{-8} M $1,25(OH)_2D_3$ (lane 1), the 54-kDa band was partially converted to a slower migrating form (≈56 kDa), a characteristic observation for many phosphoproteins such as the retinoblastoma gene product p105 (Buchkovich et al., 1989; DeCaprio et al., 1989) and c-fos (Barber & Verma, 1987). In order to determine whether this observation represented a phosphorylation event, ROS 17/2.8 cells were similarly labeled with [^{32}P]orthophosphate in the absence and presence of 10^{-8} M $1,25(OH)_2D_3$. Immunoextraction of VDR revealed that the majority of ^{32}P incorporation into the receptor protein band was dependent on the hormone, as seen in the right-hand panel of Figure 1 (compare lanes 3 and 4). This phosphorylated VDR species also comigrated with the slower migrating VDR extracted from ^{35}S-labeled cells treated with the $1,25(OH)_2D_3$ hormone (compare lanes 1 and 3). Taken together, these observations indicate that the rVDR apparently is hyperphosphorylated in response to $1,25(OH)_2D_3$ and that this phosphorylation reduces the electrophoretic mobility of the receptor in denaturing polyacrylamide gels.

Additional Analysis of VDR Phosphorylation. In order to confirm biochemically that the hormone-dependent modification of the rVDR and subsequent gel retardation properties are coincident with phosphorylation of the receptor, a phosphatase experiment was performed. VDR was isolated by immunoprecipitation with 4A5γ–Sepharose from ROS 17/2.8 cells that were labeled metabolically with [^{35}S]methionine. The slower migrating, putatively hyperphosphorylated form of the receptor isolated from $1,25(OH)_2D_3$-treated cells was converted to a faster migrating, hypophosphorylated form by exposure to calf intestinal alkaline phosphatase for increasing incubation times (Figure 2). Thus, hyperphosphorylated rVDR isolated from cells exposed to $1,25(OH)_2D_3$ hormone can be transformed enzymatically to a hypophosphorylated form which comigrates with receptor isolated from untreated cells. These data unequivocally demonstrate that the hormone-dependent retardation of rVDR migration in denaturing polyacrylamide gels is the result of receptor phosphorylation.

We next examined the time frame in which hormone-dependent phosphorylation occurs, beginning with hormone incubations as early as 30 min which is the approximate interval required for $1,25(OH)_2D_3$ binding and receptor localization

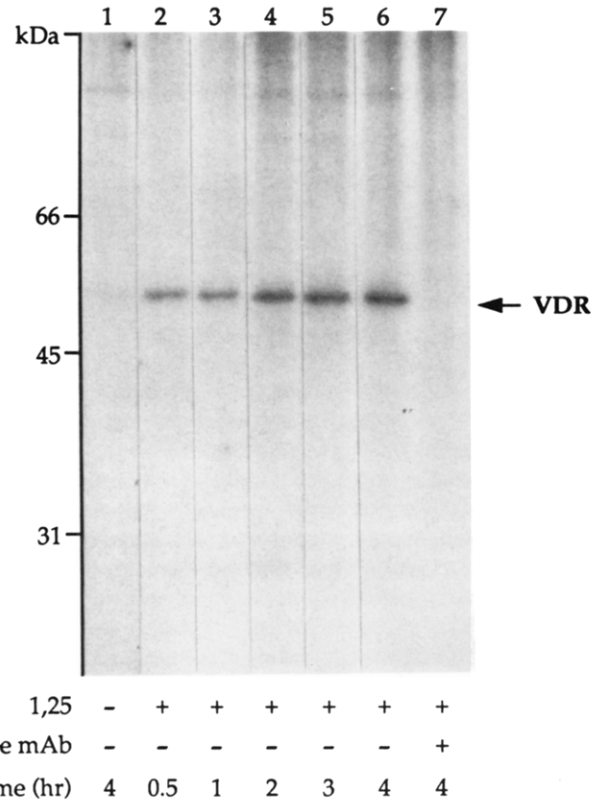

FIGURE 3: Time course of hormone-dependent, in vivo phosphorylation of VDR isolated from ROS 17/2.8 cells. ROS 17/2.8 cells were labeled metabolically with 0.5 mCi of [^{32}P]orthophosphate for 4 h followed by treatment with ethanol (lane 1) or 10^{-8} M $1,25(OH)_2D_3$ for 0.5, 1, 2, 3, and 4 h (lanes 2–7). Cell lysates were immunoprecipitated with mAb 4A5γ–Sepharose as described in Materials and Methods. Lane 7 included an excess of soluble 4A5γ mAb to assess nonspecific binding to the 4A5γ–Sepharose and should result in selective disappearance of the rVDR band. The arrow indicates the position of the rVDR.

to the nucleus. When ROS 17/2.8 cells were labeled metabolically with [^{32}P]orthophosphate and treated with 10^{-8} M $1,25(OH)_2D_3$ for varying times, the immunoextracted VDR was phosphorylated within 30 min, with maximum ^{32}P incorporation occurring at 2–3 h (Figure 3). Interestingly, mRNA levels for osteocalcin, a bone matrix protein synthesized by osteoblasts, are elevated 4–8 h following exposure to $1,25(OH)_2D_3$ in ROS 17/2.8 cells (data not shown) and in ROS 17/2 cells (Fraser & Price, 1990); nuclear runon analysis has revealed that a $1,25(OH)_2D_3$-mediated increase in osteocalcin transcription can be detected in as little as 1 h and is maximally stimulated at 3 h in ROS 17/2 cells (Pan & Price, 1986). Thus, phosphorylation of rVDR in vivo is an early event which precedes $1,25(OH)_2D_3$-induced osteocalcin mRNA accumulation and correlates temporally with the activation of osteocalcin gene transcription, suggesting that receptor phosphorylation may play a functional role in the biological actions of $1,25(OH)_2D_3$ in bone cells.

Phosphorylation of and Transcriptional Activation by VDR. Employing a novel $1,25(OH)_2D_3$ analog, plus dose–response studies with the native hormone, we then determined whether there was more than a mere temporal relationship between in vivo phosphorylation of the rVDR and the ability of the receptor to activate transcription. VDR was first immunoextracted from ROS 17/2.8 cells labeled metabolically with [^{32}P]orthophosphate (Figure 4A) in the presence of ethanol control (lanes 1 and 2), 10^{-8} M $1,25(OH)_2D_3$ (lanes 3 and 4), or 10^{-8} M OCT (lanes 5 and 6), a potent synthetic analog of $1,25(OH)_2D_3$ with in vivo bioeffects that differ from those of

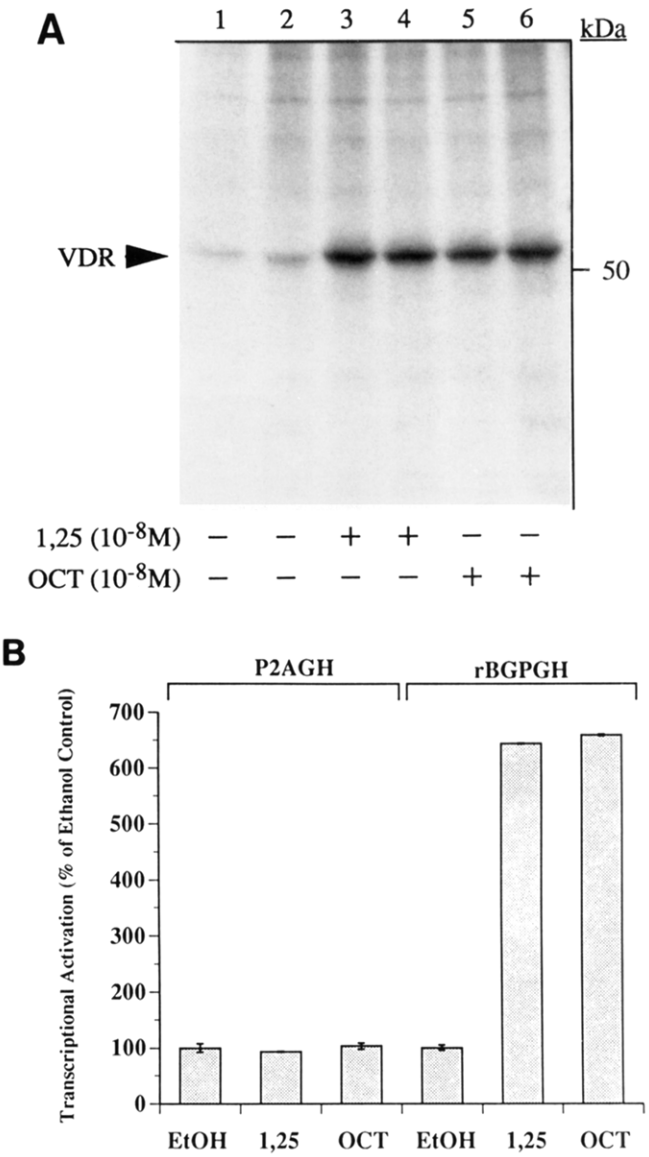

FIGURE 4: (A) Phosphorylation of the VDR in ROS 17/2.8 cells treated with $1,25(OH)_2D_3$ or OCT. ROS 17/2.8 cells were labeled metabolically with 0.5 mCi of [^{32}P]orthophosphate and treated with ethanol (lanes 1 and 2), 10^{-8} M $1,25(OH)_2D_3$ (lanes 3 and 4), or 10^{-8} M OCT (lanes 5 and 6) for 4 h as described in Materials and Methods. Cell lysates were immunoprecipitated with mAb 4A5γ–Sepharose and subjected to 10% SDS–PAGE. The arrowhead indicates the position of rVDR. (B) VDRE-mediated transcriptional activation of a reporter gene in transfected ROS 17/2.8 cells in response to $1,25(OH)_2D_3$ or OCT. ROS 17/2.8 cells (5×10^5 cells/plate) were transfected by calcium phosphate–DNA coprecipitation with a $1,25(OH)_2D_3$-responsive reporter vector (5 μg/plate) containing 1100 base pairs of the 5′-flanking DNA from the rat osteocalcin gene (rBGPGH) or only the rat osteocalcin core promoter as a control (P2AGH) linked upstream of the human growth hormone gene. After transfection, the cells were treated with ethanol, 10^{-8} M $1,25(OH)_2D_3$, or 10^{-8} M OCT for 24 h. The medium was then assayed for human growth hormone by radioimmunoassay. The values (mean ● SD) represent three independent experiments, each with triplicate samples in the various treatment groups.

$1,25(OH)_2D_3$ (Abe et al., 1989). Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) clearly demonstrated that the level of phosphorylation of rVDR and its migratory position in the denaturing polyacrylamide gel were nearly identical upon binding to and activation by either the $1,25(OH)_2D_3$ hormone or its OCT analog (Figure 4A, compare lanes 3 and 4 to lanes 5 and 6), while very little phosphorylation was detectable in the absence of either ligand (lanes 1 and 2).

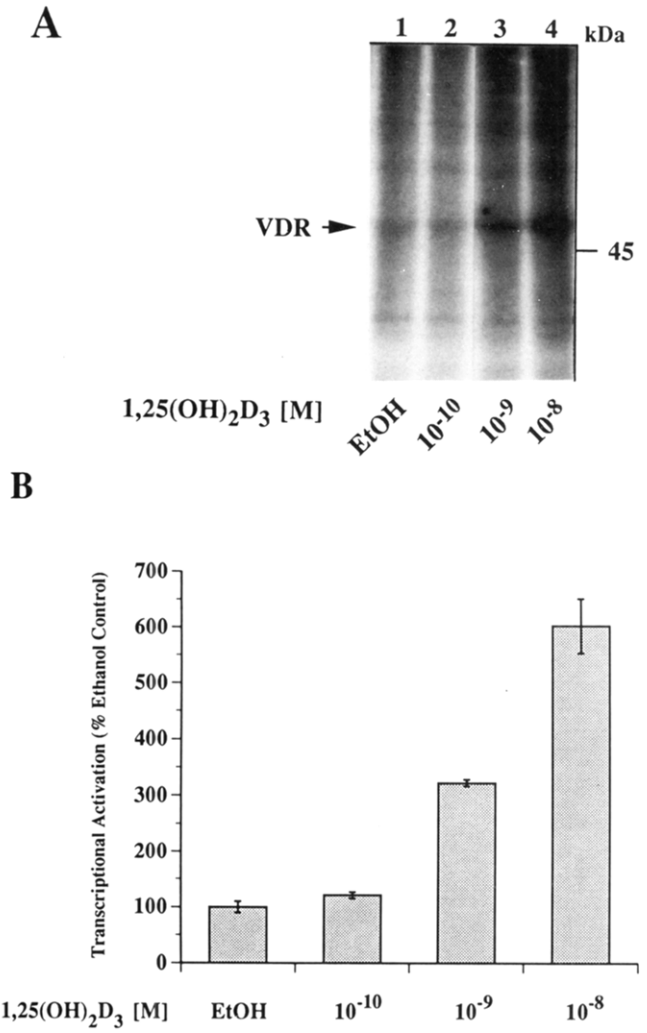

FIGURE 5: (A) Phosphorylation of the VDR in ROS 17/2.8 cells treated with increasing concentrations of $1,25(OH)_2D_3$. ROS 17/2.8 cells were labeled metabolically with 0.5 mCi of [^{32}P]orthophosphate and treated with either ethanol (lane 1) or the indicated concentration of $1,25(OH)_2D_3$ (lanes 2–4) for 4 h as described in Materials and Methods. Cell lysates were immunoprecipitated with mAb 4A5γ–Sepharose and subjected to 10% SDS–PAGE. The arrowhead indicates the position of rVDR. (B) VDRE-mediated transcriptional activation of a reporter gene in transfected ROS 17/2.8 cells in response to increasing concentrations of $1,25(OH)_2D_3$. ROS 17/2.8 cells (5×10^5 cells/plate) were transfected by calcium phosphate–DNA coprecipitation with a reporter vector (5 μg/plate) containing 1100 base pairs of the 5′-flanking DNA from the rat osteocalcin gene linked upstream of the human growth hormone gene. After transfection, the cells were treated with either ethanol or the indicated concentration of $1,25(OH)_2D_3$ for 24 h. Medium was then assayed for human growth hormone by radioimmunoassay. The values (mean ± SD) represent three independent experiments, each with triplicate samples in the various treatment groups.

In addition, ROS 17/2.8 cells were transfected with a reporter vector consisting of approximately 1100 base pairs of 5′-flanking DNA from the rat osteocalcin gene (Terpening et al., 1991), which contains a vitamin D responsive element (VDRE). Treatment of these transfected cells with either 10^{-8} M $1,25(OH)_2D_3$ or 10^{-8} M OCT resulted in equivalent levels of VDRE-dependent transcriptional activation of a human growth hormone reporter gene (Figure 4B), while cells treated with ethanol vehicle or cells transfected with a reporter vector lacking the VDRE displayed only basal levels of transcriptional activity (Figure 4B). In a similar set of experiments, the degree of phosphorylation of the rVDR (Figure 5A) and its ability to activate transcription (Figure 5B) were monitored in response to increasing doses of the natural $1,25(OH)_2D_3$ ligand. Both the level of phosphorylation in vivo and the ability of VDR to activate transcription of a reporter vector increased in parallel in response to higher concentrations of $1,25(OH)_2D_3$. Therefore, these results demonstrate a positive correlation between VDR phosphorylation and transcriptional stimulation in terms of activation by two chemically distinct vitamin D receptor ligands, as well as with respect to the presumed level of $1,25(OH)_2D_3$-occupied receptor.

Phosphorylation of VDR, in Vitro, by Casein Kinase II. An analysis of the deduced amino acid sequence of the rVDR (Burmester et al., 1988) for the presence of theoretical consensus recognition sites for several protein kinases revealed a number of minimal CK-II consensus recognition sites of the type (Ser/Thr)-X-X-(Asp/Glu)-X-(Asp/Glu) (Marin et al., 1986), where either the +3 or the +5 position possesses an acidic residue, while classical consensus sites for several other major protein kinases were not present. Most intriguing were two clusters of CK-II sites in the hormone binding domain, one consisting of Ser 178, 180, 188, and 198 and Thr 187 and the other cluster including Ser 261 and 273 and Thr 250 and 275. The more N-terminal group of theoretical sites overlaps a domain corresponding to one that has previously been reported to include a major phosphorylation region of the human VDR (hVDR) in transfected ROS 17/2.8 cells (Jones et al., 1991). Therefore, we tested the ability of the rVDR to serve as a substrate for a highly purified preparation of CK-II. The VDR was immunoextracted from non-hormone-treated ROS 17/2.8 cells, and the mAb–Sepharose–VDR complex was incubated with 10^{-8} M $1,25(OH)_2D_3$ for 2 h followed by the addition of 6×10^{-4} unit of CK-II. As shown in Figure 6, the rVDR is an efficient substrate for CK-II in vitro. The ^{32}P-labeled band that appears at ≈28 kDa represents autophosphorylation of the CK-II β-subunit (Hathaway & Traugh, 1982), and its intensity is not reduced by inclusion of excess free 4A5γ mAb in the immunoprecipitation reaction (data not shown). Quantitative densitometric scanning of the [^{32}P]VDR images depicted in Figure 6 and in repeat experiments indicates that the presence of $1,25(OH)_2D_3$ does not appear to significantly modulate VDR phosphorylation (Figure 6, compare lanes 3 and 4). Incubation of the rVDR with $1,25(OH)_2D_3$ prior to immunoprecipitation also reveals no significant effect of the hormonal sterol on rVDR phosphorylation by CK-II (data not shown). Furthermore, incubation of partially purified rVDR in solution, free from the mAb–Sepharose complex, with CK-II results in efficient, $1,25(OH)_2D_3$ hormone-independent phosphorylation (data not shown).

Finally, we addressed the potential relationship between rVDR phosphorylation in intact cells and that catalyzed by CK-II, in vitro, by examining the electrophoretic mobility of in vivo and in vitro phosphorylated receptor. ^{35}S-labeled VDR was immunoextracted from ROS 17/2.8 cells incubated in the absence or presence of hormone; as expected, the receptor phosphorylated in response to $1,25(OH)_2D_3$ in intact cells was retarded in its migration compared to hypophosphorylated VDR extracted from ethanol-treated cells (Figure 7A, lanes 1 and 2). However, the in vivo phosphorylated receptor species (lane 2) migrated slightly more rapidly in these denaturing polyacrylamide gels than did VDR phosphorylated by CK-II in vitro (lane 3), suggesting either a different distribution of target sites for CK-II or a more extensive phosphorylation of VDR by this enzyme in vitro. Because the perceived differences in migration were very small between the $1,25(OH)_2D_3$-dependent hyperphosphorylated receptor in

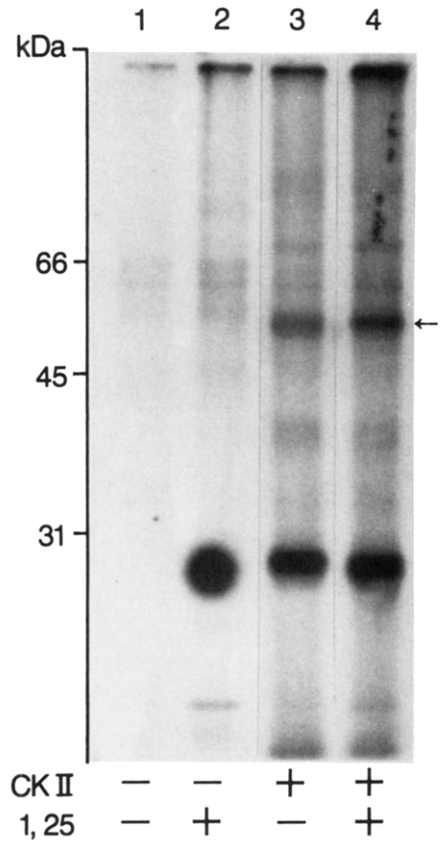

FIGURE 6: Phosphorylation of immunopurified rVDR by CK-II. Lysates were prepared from ROS 17/2.8 cells (2×10^6) grown in the absence of $1,25(OH)_2D_3$, and VDR was immunoprecipitated with mAb 4A5γ as described in Materials and Methods. The immunoprecipitates were resuspended in 35 μL of KTMD-0.15 (kinase buffer) and incubated with 10^{-8} M $1,25(OH)_2D_3$ (lanes 2 and 4) or ethanol (lanes 1 and 3) for 2 h, followed by incubation with 40 μCi of [γ-^{32}P]ATP in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 6×10^{-4} unit of purified CK-II for 30 min at 30 °C. The immunoprecipitates were then washed and subjected to 10% SDS–PAGE. The arrow indicates the position of the rVDR.

intact cells and that resulting from reaction with CK-II in vitro, an independent repeat experiment was carried out and the lanes were organized differently to allow clear visual resolution of the various species of receptor. Results from this additional experiment (Figure 7B) again revealed that the VDR phosphorylated by CK-II in vitro (lane 2) consistently displayed retarded migration as compared to the in vivo phosphorylated receptor species (lane 3). These observations suggest that the pattern of CK-II-catalyzed in vitro phosphorylation may be distinct from that occurring in response to $1,25(OH)_2D_3$ or OCT in vivo.

DISCUSSION

Receptor phosphorylation by several distinct kinases is an important regulatory mechanism central to a number of signal transduction pathways. Many DNA binding proteins have, in recent years, been identified as important targets for regulation by phosphorylation (Hunter & Karin, 1992). The VDR belongs to this general class of DNA binding proteins, and in the present study, we present the first extensive biochemical evaluation of phosphorylation of this receptor isolated from the rat osteosarcoma osteoblast-like cell line, ROS 17/2.8. Furthermore, we have also evaluated the effects of OCT, an analog of $1,25(OH)_2D_3$ which contains an oxygen atom substituted for the methylene group at carbon 22, on VDR phosphorylation. While OCT binds to the VDR in ROS 17/2.8 cells with a K_d value that is indistinguishable from that of $1,25(OH)_2D_3$ (Pernalete et al., 1991) and can inhibit parathyroid hormone mRNA levels as effectively as the natural $1,25(OH)_2D_3$ hormone (Brown et al., 1989), this analog is at least 100 times less calcemic than $1,25(OH)_2D_3$ in mice (Abe et al., 1989). Interestingly, we have observed that both of these ligands induce a similar level of phosphorylation of the rVDR and can elicit a comparable increase in VDRE-dependent transcriptional activity by the receptor. Furthermore, both rVDR phosphorylation and the transcriptional activity of the receptor increase identically in response to escalating levels of $1,25(OH)_2D_3$. These observations suggest not only that hormone-dependent phosphorylation may be an important component of the molecular pathway leading to gene activation but also that VDR phosphorylation is probably not responsible for the differential biological actions of $1,25(OH)_2D_3$ and OCT. Thus, these studies confirm and extend those of Morrison and Eisman (1991), who reported that $1,25(OH)_2D_3$ and OCT similarly induce human osteocalcin transcription. Taken together, the data therefore point to other mechanisms for the selective actions of OCT, such as different binding characteristics for the serum vitamin D binding protein, rapid clearance from the circulation, and possibly differential target tissue metabolism and/or catabolism.

We have also clearly demonstrated that the rVDR is phosphorylated in vitro by CK-II, either in the absence or presence of $1,25(OH)_2D_3$ (Figure 6), and analysis of the in vitro phosphorylated receptor by SDS–PAGE (Figure 7) reveals that this hormone-independent phosphorylation may be distinct from that taking place in response to the $1,25(OH)_2D_3$ or OCT ligands. However, we cannot exclude the possibility that $1,25(OH)_2D_3$-dependent phosphorylation of VDR represents a subset of the total phosphorylation array which occurs as a result of CK-II-catalyzed hyperphosphorylation. To address this question, future studies will be required, including phosphopeptide mapping of VDR phosphorylated by CK-II in vitro and of phosphorylated receptor extracted from intact cells treated with $1,25(OH)_2D_3$.

Although our present data relate to VDR in ROS 17/2.8 cells, we have previously examined the human VDR in transfected ROS 17/2.8 cells and found that it is also a phosphoprotein. Deletion analysis of the hVDR in these transfected cells revealed that a major phosphorylation site(s) resides between residues Met 197 and Val 234 (Jones et al., 1991). One theoretical CK-II consensus recognition site, namely, the amino acid corresponding to Ser 203 in the human VDR (Ser 198 in rVDR), is located in this domain in all known VDR sequences, suggesting that the VDR may also be phosphorylated by CK-II in vivo. However, in a separate study (Jurutka et al., 1993b), we found that the major amino acid phosphorylated by CK-II in this domain of the hVDR is Ser 208, which is not conserved across all species and corresponds to an asparagine residue in the rVDR. In addition, when compared to rVDR (Figures 1 and 3), the phosphorylation of hVDR is less dependent on the presence of the $1,25(OH)_2D_3$ hormone (McDonnell et al., 1989; Jones et al., 1991), probably because of the more quantitatively significant, albeit hormone-independent, phosphorylation of Ser 208 in hVDR (Jurutka et al., 1993b). These observations suggest that the sites of rVDR phosphorylation may be at least partially distinct from those of the hVDR. We have also reported that the hVDR is phosphorylated on Ser 51, a conserved residue located between the two tetrahedrally coordinated zinc DNA binding fingers, by protein kinase C-β in vitro and in the

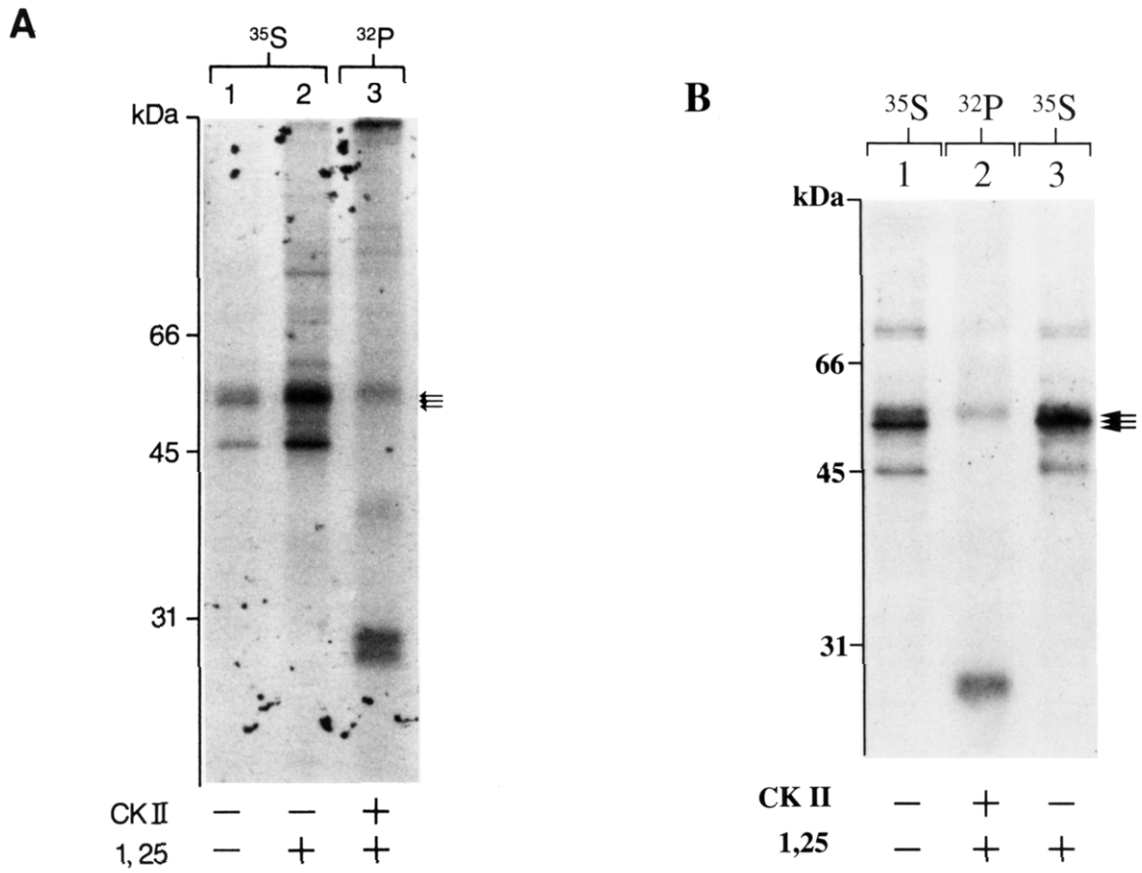

FIGURE 7: (A) Comparison of in vivo and in vitro phosphorylation of rVDR. VDR from [^{35}S]methionine-labeled ROS 17/2.8 cells treated with ethanol (lane 1) or 10^{-8} M 1,25$(OH)_2D_3$ (lane 2) for 4 h was immunoprecipitated as in Figure 1 or phosphorylated by CK-II, in vitro, as in Figure 6 (lane 3). The resulting immunoprecipitates were subjected to 10% SDS–PAGE as described in Materials and Methods. The three arrows indicate the position of in vivo hypophosphorylated (lane 1), in vivo hyperphosphorylated (lane 2), or in vitro CK-II-phosphorylated VDR (lane 3). (B) Independent analysis of in vivo and in vitro phosphorylation of VDR. VDR was isolated and analyzed as in A. The order of lanes 2 and 3 in A was intentionally reversed in B to permit a more definitive visual evaluation of the relative migration of the receptor species. The three arrows indicate the position of in vivo hypophosphorylated (lane 1), in vitro CK-II-phosphorylated (lane 2), or in vivo hyperphosphorylated VDR (lane 3).

presence of activators of protein kinase C in vivo (Hsieh et al., 1991). The effect of protein kinase C activators on the phosphorylation of rVDR is currently under investigation in our laboratory. Thus, while there are differences in the deduced amino acid sequence of the rat (Burmester et al., 1988), chicken (Haussler et al., 1988b), and human (Baker et al., 1988) VDRs, the ability of these receptors to be phosphorylated by both hormone-dependent and -independent mechanisms has clearly been retained, as demonstrated by the present study and previous work from our laboratory (Hsieh et al., 1991; Jones et al., 1991; Jurutka et al., 1993a,b) and from two different laboratories (Brown & DeLuca, 1990; McDonnell et al., 1989).

In the case of other members of the steroid/thyroid hormone receptor superfamily, both the glucocorticoid and progesterone receptors are phosphorylated in vivo in the absence of their ligands, while addition of ligand results in hyperphosphorylation (Hoeck et al., 1989; Ortí et al., 1989; Logeat et al., 1985; Sullivan et al., 1988). Examination of the residues phosphorylated in the mouse glucocorticoid (Bodwell et al., 1991) and chicken progesterone receptors (Denner et al., 1990), in vivo, reveals that four of these seven sites in the glucocorticoid receptor and all three of the sites in the progesterone receptor fit the criteria of sites for either the p34^{cdc2} catalytic subunit (Draetta, 1990; Hall et al., 1991) or the mitogen-activated protein kinases (Boulton et al., 1991; Rossomando et al., 1991). In addition, phosphorylation of the progesterone and thyroid hormone receptors in vitro by PK-A is independent of the presence of the cognate hormone (Hurd et al., 1989; Goldberg et al., 1988). These studies suggest that ligand-dependent and -independent phosphorylation may be a common feature of several members of the steroid/thyroid hormone receptor superfamily, including the VDRs, and they imply that a functional role may be associated with these post-translational modification events.

Relatively few studies have established a direct link between steroid hormone receptor phosphorylation and function. However, recent reports have suggested that phosphorylation of the human thyroid β1 (Lin et al., 1992) and glucocorticoid receptors (Rangarajan et al., 1992) increases their DNA binding activity, while phosphorylation of the human estrogen receptor may influence DNA binding (Denton et al., 1992) or transcriptional activation (Ali et al., 1993). Furthermore, it is intriguing to note that with the glucocorticoid, progesterone, and estrogen receptors, which possess a ligand-induced component of total phosphorylation, almost all of the residues which are hyperphosphorylated in response to the cognate hormone are localized to regions defined previously as transcriptional activation domains (Hoeck & Groner, 1990; Bodwell et al., 1991; Denner et al., 1990; Ali et al., 1993).

Given the following observations, (i) a temporal relationship exists within osteoblasts between hormone-dependent phosphorylation of the rVDR (Figure 3) and the induction of transcription of the rat osteocalcin gene (Fraser & Price, 1990), (ii) ligand-induced phosphorylation is localized to transcriptional activation domains of other steroid hormone receptors,

(iii) ligands that are chemically and biologically distinct induce phosphorylation of the rVDR in concert with their activation of osteocalcin transcription (Figure 4), and (iv) the extent of rVDR phosphorylation correlates well with the ability of the receptor to stimulate transcription (Figure 5), we speculate that phosphorylation of the VDR in response to $1,25(OH)_2D_3$ or OCT may be an integral element in the mechanism of VDR-dependent transcriptional activation. In contrast, CK-II-mediated phosphorylation, which is not linked to the presence of the $1,25(OH)_2D_3$ hormone (Figure 6) and is likely regulated by CK-II-associated signal transduction pathways, may play a role in signal transduction "cross-talk" modulation of receptor activity. In fact, our preliminary findings (Jurutka et al., 1992) indicate that CK-II-catalyzed phosphorylation of human VDR in COS-7 cells overexpressing CK-II elicits enhanced $1,25(OH)_2D_3$-dependent transcriptional activity of the receptor.

Further studies to identify the specific amino acids in rVDR that are phosphorylated by $1,25(OH)_2D_3$ hormone-dependent and -independent kinases, followed by site-directed mutational analysis of these residues, should result in a comprehensive understanding of the contribution of each of these phosphorylation events to VDR activity. The results reported in the present study should facilitate further evaluation and elucidation of the role of VDR phosphorylation and of its functional relevance to the mechanism of $1,25(OH)_2D_3$-mediated gene regulation in bone cells.

ACKNOWLEDGMENT

The authors are grateful to Drs. D. W. Litchfield and E. G. Krebs (Department of Pharmacology and Biochemistry, Howard Hughes Medical Institute, University of Washington) for their generous gift of purified bovine casein kinase II and to Drs. Y. Nishii and J. Abe (Chugai Pharmaceutical Co., Ltd., Toshima-ku, Tokyo, Japan) for providing 22-oxacalcitriol. We also thank Dr. G. Kerr Whitfield for critical reading of the manuscript.

REFERENCES

Abe, J., Takita, Y., Nakano, T., Miyaura, C., Suda, T., & Nishii, Y. (1989) *Endocrinology 124*, 2645–2647.

Ackerman, P., Glover, C. V. C., & Osheroff, N. (1988) *J. Biol. Chem. 263*, 12653–12660.

Ali, S., Metzger, D., Bornert, J., & Chambon, P. (1993) *EMBO J. 12*, 1153–1160.

Auricchio, F., Migliaccio, A., Di Domenico, M., & Nola, E. (1987) *EMBO J. 6*, 2923–2929.

Baker, A. R., McDonnell, D. P., Hughes, M., Crisp, T. M., Mangelsdorf, D. J., Haussler, M. R., Pike, J. W., Shine, J., & O'Malley, B. W. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3294–3298.

Barber, J. R., & Verma, I. M. (1987) *Mol. Cell. Biol. 7*, 2201–2211.

Berberich, S. J., & Cole, M. D. (1992) *Genes Dev. 6*, 166–176.

Bodwell, J. E., Ortí, E., Coull, J. M., Pappin, D. J. C., Smith, L. I., & Swift, F. (1991) *J. Biol. Chem. 266*, 7549–7555.

Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H., & Yancopoulos, G. D. (1991) *Cell 65*, 663–675.

Brown, T. A., & DeLuca, H. F. (1990) *J. Biol. Chem. 265*, 10025–10029.

Brown, A. J., Ritter, C. S., Finch, J. L., Morrissey, J., Martin, K. J., Murayama, E., Nishii, Y., & Slatopolsky, E. (1989) *J. Clin. Invest. 84*, 728–732.

Buchkovich, K., Duffy, L. A., & Harlow, E. (1989) *Cell 58*, 1097–1105.

Burmester, J. K., Wiese, R. J., Maeda, N., & DeLuca, H. F. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9499–9502.

Christakos, S., Gabrielides, C., & Rhoten, W. B. (1989) *Endocrinol. Rev. 10*, 3–26.

Dahmus, M. E. (1981) *J. Biol. Chem. 256*, 3332–3339.

DeCaprio, J. A., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Piwnica-Worms, H., Huang, C., & Livingston, D. M. (1989) *Cell 58*, 1085–1095.

Denner, L. A., Schrader, W. T., O'Malley, B. W., & Weigel, N. L. (1990) *J. Biol. Chem. 265*, 16548–16555.

Denton, R. R., Koszewski, N. J., & Notides, A. C. (1992) *J. Biol. Chem. 267*, 7263–7268.

Dougherty, J. J., Puri, R. K., & Toft, D. O. (1982) *J. Biol. Chem. 257*, 14226–14230.

Draetta, G. (1990) *Trends Biochem. Sci. 15*, 378–383.

Durban, E., Goodenough, M., Mills, J., & Busch, H. (1985) *EMBO J. 4*, 2921–2926.

Evans, R. M. (1988) *Science 240*, 889–895.

Fraser, J. D., & Price, P. A. (1990) *Calcif. Tissue Int. 46*, 270–279.

Glineur, C., Bailly, M., & Ghysdael, J. (1989) *Oncogene 4*, 1247–1254.

Goldberg, Y., Glineur, C., Gesquière, J.-C., Ricouart, A., Sap, J., Vennström, B., & Ghysdael, J. (1988) *EMBO J. 7*, 2425–2433.

Goueli, S., Holtzman, J., & Ahmed, K. (1984) *Biochem. Biophys. Res. Commun. 123*, 778–784.

Hall, F. L., Braun, R. K., Mihara, K., Fung, Y.-K. T., Berndt, N., Carbonaro-Hall, D. A., & Vulliet, P. R. (1991) *J. Biol. Chem. 266*, 17430–17440.

Hathaway, G. M., & Traugh, J. A. (1982) *Curr. Top. Cell. Regul. 21*, 101–127.

Haussler, M. R., Mangelsdorf, D. J., Komm, B. S., Terpening, C. M., Yamaoka, K., Allegretto, E. A., Baker, A. R., Shine, J., McDonnell, D. P., Hughes, M., Weigel, N. L., O'Malley, B. W., & Pike, J. W. (1988a) *Recent Prog. Horm. Res. 44*, 263–305.

Haussler, M. R., Terpening, C. M., Jurutka, P. W., Meyer, J., Schulman, B. A., Haussler, C. A., Whitfield, G. K., & Komm, B. S. (1988b) in *Progress in Endocrinology* (Imura, H., Shizume, K., & Yoshida, S., Eds.) Vol. 1, pp 763–770, Elsevier Science Publishers B. V., Amsterdam.

Hoeck, W., & Groner, B. (1990) *J. Biol. Chem. 265*, 5403–5408.

Hoeck, W., Rusconi, S., & Groner, B. (1989) *J. Biol. Chem. 264*, 14396–14402.

Housley, P., & Pratt, W. (1983) *J. Biol. Chem. 258*, 4630–4635.

Hsieh, J.-C., Jurutka, P. W., Galligan, M. A., Terpening, C. M., Haussler, C. A., Samuels, D. S., Shimizu, Y., Shimizu, N., & Haussler, M. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9315–9319.

Hunter, T., & Karin, M. (1992) *Cell 70*, 375–387.

Hurd, C., Nakao, M., & Moudgil, V. (1989) *Biochem. Biophys. Res. Commun. 162*, 160–167.

Jones, B. B., Jurutka, P. W., Haussler, C. A., Haussler, M. R., & Whitfield, G. K. (1991) *Mol. Endocrinol. 5*, 1137–1146.

Jurutka, P. W., MacDonald, P. N., Hsieh, J.-C., Haussler, M. R., & Whitfield, G. K. (1992) *J. Bone Miner. Res. 7* (Suppl. 1), S108.

Jurutka, P. W., Hsieh, J.-C., & Haussler, M. R. (1993a) *Biochem. Biophys. Res. Commun. 191*, 1089–1096.

Jurutka, P. W., Hsieh, J.-C., MacDonald, P. N., Terpening, C. M., Haussler, C. A., Haussler, M. R., & Whitfield, G. K. (1993b) *J. Biol. Chem. 268*, 6791–6799.

Kingston, R. E. (1990) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Siedman, J. G., Smith, J. A., & Struhl, K., Eds.) pp 9.1.1–9.1.4, Greene Publishing and Wiley-Interscience, New York.

Krek, W., Maridor, G., & Nigg, E. A. (1992) *J. Cell Biol. 116*, 43–55.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lin, K.-H., Ashizawa, K., & Cheng, S.-Y. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7737–7741.

Litchfield, D. W., Lozeman, F. J., Piening, C., Sommercorn, J., Takio, K., Walsh, K. A., & Krebs, E. G. (1990) *J. Biol. Chem. 265*, 7638–7644.
Logeat, F., Le Cunff, M., Pamphile, R., & Milgrom, E. (1985) *Biochem. Biophys. Res. Commun. 131*, 421–427.
Lüscher, B., Kuenzel, E. A., Krebs, E. G., & Eisenman, R. N. (1989) *EMBO J. 8*, 1111–1119.
Lüscher, B., Christenson, E., Litchfield, D. W., Krebs, E. G., & Eisenman, R. N. (1990) *Nature 344*, 517–522.
Majeska, R. J., Rodam, S. B., & Rodan, G. A. (1980) *Endocrinology 107*, 1494–1503.
Mangelsdorf, D. J., Pike, J. W., & Haussler, M. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 354–358.
Marin, O., Meggio, F., Marchiori, F., Borin, G., & Pinna, L. A. (1986) *Eur. J. Biochem. 160*, 239–244.
McDonnell, D. P., Scott, R. A., Kerner, S. A., O'Malley, B. W., & Pike, J. W. (1989) *Mol. Endocrinol. 3*, 635–644.
Morrison, N. A., & Eisman, J. A. (1991) *J. Bone Miner. Res. 6*, 893–899.
Ortí, E., Mendel, D., Smith, L., & Munck, A. (1989) *J. Biol. Chem. 264*, 9728–9731.
Pan, L. C., & Price, P. A. (1986) *J. Bone Miner. Res. 1* (Suppl. 1), Abstr. 20.
Pernalete, N., Mori, T., Nishii, Y., Slatopolsky, E., & Brown, A. J. (1991) *Endocrinology 129*, 778–784.
Pike, J. W. (1990) in *Calcium Regulation and Bone Metabolism* (Cohn, D. V., Glorieux, F. H., & Martin, T. J., Eds.) Vol. 10, pp 127–136, Elsevier Science Publishers, Amsterdam.
Pike, J. W., & Sleator, N. M. (1985) *Biochem. Biophys. Res. Commun. 131*, 378–385.
Pike, J. W., Marion, S. L., Donaldson, C. A., & Haussler, M. R. (1983) *J. Biol. Chem. 258*, 1289–1296.
Rangarajan, P. N., Umesono, K., & Evans, R. M. (1992) *Mol. Endocrinol. 6*, 1451–1457.
Rossomando, A. J., Sanghera, J. S., Marsden, L. A., Weber, M. J., Pelech, S. L., & Sturgill, T. W. (1991) *J. Biol. Chem. 266*, 20270–20275.
Singh, V. B., & Moudgil, V. K. (1985) *J. Biol. Chem. 260*, 3684–3690.
Stetler, D. A., & Rose, K. M. (1982) *Biochemistry 21*, 3721–3728.
Sullivan, W., Madden, B., McCormick, D., & Toft, D. (1988) *J. Biol. Chem. 263*, 14717–14723.
Terpening, C. M., Haussler, C. A., Jurutka, P. W., Galligan, M. A., Komm, B. S., & Haussler, M. R. (1991) *Mol. Endocrinol. 5*, 373–385.
Tuazon, P. T., & Traugh, J. A. (1991) *Adv. Second Messenger Phosphoprotein Res. 23*, 123–164.
Weigel, N., Tash, J., Means, A., Schrader, W., & O'Malley, B. (1981) *Biochem. Biophys. Res. Commun. 102*, 513–519.