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$1,25(OH)_2D_3$-Dependent Regulation of Calbindin-D_{28k} mRNA Requires Ongoing Protein Synthesis in Chick Duodenal Organ Culture

Julia Meyer, Michael A. Galligan, Glenville Jones, Barry S. Komm, Carol A. Haussler, and Mark R. Haussler

Department of Biochemistry, The University of Arizona, Tucson, Arizona 85724 (J.M., M.A.G., C.A.H., M.R.H.); Department of Cell Biology, Wyeth-Ayerst Women's Health Institute, Radnor, Pennsylvania 19087 (B.S.K.); and Departments of Biochemistry and Medicine, Queen's University, Kingston, Ontario K7L3N6, Canada (G.J.)

Abstract Organ culture of 19-day-old chick embryo duodena was utilized to evaluate the mechanism of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)–dependent calbindin-D_{28k} (CaBP) expression. Duodenal CaBP and $1,25(OH)_2D_3$ receptor (VDR) expression were assessed by Western blot analysis, while CaBP and VDR mRNA levels were determined by Northern blot analysis. In untreated duodena, both VDR protein and mRNA were present, while CaBP protein and mRNA were undetectable. Treatment of cultured duodena with 25 nM $1,25(OH)_2D_3$ resulted in detectable CaBP mRNA after 4 h which continued to increase during a 24 h time period. Under these conditions, localization of [^{3}H-1β]1α,$25(OH)_2D_3$ in duodenal chromatin is rapid ($\leq$30 min). Thus, the delayed accumulation of detectable CaBP mRNA cannot be explained by slow nuclear binding of $1,25(OH)_2D_3$. The inclusion of 1.6 μM actinomycin D in the organ culture partially inhibited the $1,25(OH)_2D_3$-regulated increase in CaBP mRNA, which implies that there is a transcriptional component involved in the increased CaBP mRNA levels. Similarly, quantitative polymerase chain reaction studies allowed the detection of CaBP pre-mRNA and mRNA sequences 1 h after hormone treatment, suggesting that CaBP gene transcription is initiated rapidly. Treatment of cultures with 36 μM cycloheximide 1 h prior to $1,25(OH)_2D_3$ addition resulted in superinduction of VDR mRNA levels but sharply reduced CaBP steady-state mRNA levels. This dramatic reduction in CaBP mRNA reveals that $1,25(OH)_2D_3$-mediated CaBP expression is dependent on ongoing protein synthesis. Thus, we propose that a labile auxiliary protein or other cofactor, which may or may not be $1,25(OH)_2D_3$-dependent, is necessary for $1,25(OH)_2D_3$-mediated CaBP gene transcription in chick duodena.

Key words: calbindin-D_{28k}, 1,25-dihydroxyvitamin D_3, messenger RNA, organ culture, polymerase chain reaction

Calbindin-D_{28k} (CaBP) belongs to a family of intracellular proteins which bind calcium with high affinity [Christakos et al., 1989]. In the luminal epithelium of the avian intestine, the major response to 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is the induction of CaBP synthesis [Wasserman and Fullmer, 1982]. Therefore, CaBP has been implicated in the functional role of vitamin D to stimulate intestinal calcium transport [Chandler and Wasserman, 1987], although this remains to be elucidated at the molecular level. $1,25(OH)_2D_3$ exerts its effects on target tissues through the $1,25(OH)_2D_3$ receptor (VDR), which belongs to a superfamily of steroid/thyroid hormone receptors which are known to act as ligand-dependent transcription factors [Evans, 1988]. Based on this fact, the observed $1,25(OH)_2D_3$-mediated increase in CaBP expression has been attributed to transcriptional activation of the gene. In vivo studies support this hypothesis. Theofan et al. [1986], using nuclear run-off assays, demonstrated that duodenal CaBP gene transcription was elevated 15 min following administration of $1,25(OH)_2D_3$ to chicks raised in the absence of vitamin D_3. Similarly, Ferrari and Battini [1990] identified CaBP pre-mRNA, utilizing polymerase chain reaction (PCR) technology, 30 min after injection of vitamin D–deficient animals with $1,25(OH)_2D_3$.

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Address reprint requests to Mark R. Haussler, Department of Biochemistry, The University of Arizona, Arizona Health Sciences Center, Tucson, AZ 85724.

To date, defining a vitamin D–responsive element (VDRE) in the chicken intestinal CaBP gene has been unsuccessful. A DNA sequence (−320 to −305) in the CaBP promoter shows considerable homology to the VDRE from the rat and human osteocalcin genes [Kerner et al., 1989; Demay et al., 1990; Terpening et al., 1991]. However, in transient transfection studies, this sequence conferred only a modest increase on the $1,25(OH)_2D_3$-induced transcription rate [MacDonald et al., 1992]. Furthermore, studies employing gel shift [MacDonald et al., 1992] and DNase footprinting assays [Minghetti et al., 1991] have failed to definitively characterize a region of the CaBP promoter which interacts with VDR. More recent investigations illustrate that $1,25(OH)_2D_3$ controls the expression of several other genes posttranscriptionally [Tobler et al., 1988; Matthew et al., 1989], consistent with several reports that steroid hormone action is not restricted to transcriptional events [Nielsen and Shapiro, 1990]. Additionally, in vivo studies employing cycloheximide have implicated $1,25(OH)_2D_3$ as having both a transcriptional and posttranscriptional effect on CaBP mRNA accumulation [Clemens et al., 1988; Theofan and Norman, 1986].

In order to elucidate the influence of $1,25(OH)_2D_3$ on chick intestinal CaBP expression, we have utilized an organ culture system of 19-day-old chick embryo duodena. Corradino et al. [1969] originally demonstrated that the duodenal loop from the embryonic chick contains no detectable CaBP. Upon addition of $1,25(OH)_2D_3$ to the culture medium, CaBP synthesis is stimulated and calcium absorption is enhanced in the duodenal mucosa [Corradino and Wasserman, 1971; Franceschi and DeLuca, 1981]. In the present study inhibitors of translation and transcription, cycloheximide and actinomycin D, respectively, were utilized to evaluate the mechanism of $1,25(OH)_2D_3$-dependent CaBP gene expression. The data indicate that both ongoing transcription and translation are required for $1,25(OH)_2D_3$-dependent CaBP mRNA accumulation. However, the major effect of $1,25(OH)_2D_3$, presumably via its receptor and a putative labile protein coreceptor, is at the level of transcription and not CaBP mRNA stabilization.

MATERIALS AND METHODS

Materials

Waymouth's media was obtained from GIBCO (Grand Island, NY). $1,25(OH)_2D_3$ was provided by Hoffmann LaRoche (Nutley, NJ). [^{3}H-1β]1α,25$(OH)_2D_3$ (3 Ci/mmol) was synthesized as previously described [Makin et al., 1989]. Actinomycin D was purchased from Boehringer Mannheim (Indianapolis, IN). Cycloheximide was purchased from Sigma (St. Louis, MO). 5,6 dichloro-1-β-ribofuranosylbenzimidazole (DRB) was purchased from Calbiochem (La Jolla, CA).

Duodenal Organ Culture

The procedure used to culture embryonic chick duodena has been described by Corradino [1984]. Nineteen-day-old chick embryos were obtained from the University of Arizona Poultry Farm (Tucson, AZ). The duodena were excised and the pancreas removed from each under sterile conditions. The tissue was mounted, mucosal side up, on steel grids in 60 mm cell culture dishes containing approximately 6–7 ml of Waymouth's medium supplemented as described [Corradino, 1984]. $1,25(OH)_2D_3$, [^{3}H-1β]1α,25$(OH)_2D_3$, actinomycin D, cycloheximide, or DRB was dissolved in ethanol and added to the media, such that the final concentration of ethanol never exceeded 0.3%. Tissues were incubated for up to 24 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2 with a medium change every 12 h. Each experiment was repeated two to three times, and six to nine duodena were used per treatment group (three duodena per dish).

Binding of [^{3}H-1β]1α,25$(OH)_2D_3$ to Chromatin

Duodena were homogenized in 10 mM Tris-HCl (pH 7.4) with 15 strokes on a motor-driven, glass-Teflon homogenizer. The homogenate was centrifuged at 1,000*g* for 10 min. The resulting crude nuclear pellet was resuspended by homogenization in lysis buffer (25 mM NaCl/8 mM EDTA) and centrifuged at 12,000*g* for 10 min. This nuclear pellet was mixed with Triton X-100 buffer (10 mM Tris-HCl (pH 7.4)/1% Triton X-100), and a crude chromatin pellet was recovered by centrifugation at 16,000*g* for 10 min. Further purification of the chromatin pellet was achieved by washing in 10 mM Tris-HCl (pH 7.4) followed by centrifugation at 60,000*g* for 1 h. The nuclear accumulation of radiolabeled sterol, [^{3}H-1β]1α,25$(OH)_2D_3$, was quantitated by homogenization of the chromatin pellet in 400 μl of extraction buffer (10 mM Tris-HCl (pH 7.4)/0.3 M KCl). An aliquot of this extract was used for protein determination by Bradford assay [Bradford, 1976], and another aliquot was

directly counted in Ready Value liquid scintillation cocktail (Beckman, Fullerton, CA).

Measurement of VDR Turnover

Chromatin pellets were prepared as described above. Protein extracts were prepared for Western blot analysis by resuspending the chromatin pellet in 500 μl 1× final sample buffer (FSB: 2% SDS/62.5 mM Tris-HCl/10% glycerol) [Laemmli, 1970] and heating at 65°C for 10–15 min followed by centrifugation at 16,000*g* for 10 min. Samples were stored at −20°C until further preparation for Western blot analysis.

Western Blot Analysis

Duodenal extracts were prepared by homogenizing three duodena in 2 volumes (w/v) of 1× FSB with a glass homogenizer. Insoluble debris was removed by heating briefly at 80°C for 5 min followed by centrifugation at 16,000*g* for 10 min. Protein concentrations were quantitated with a BCA protein assay kit (Pierce, Rockford, IL). Lysates were stored at −20°C until used. Samples were prepared for electrophoresis by addition of β-mercaptoethanol and bromphenol blue to final concentrations of 5% and 0.004%, respectively. Duodenal VDR and CaBP protein levels were assessed by immunoblotting techniques adapted from previous procedures utilizing the 9A7γ antibody and anti-CaBP antisera, respectively [Pike et al., 1987; Mangelsdorf et al., 1987b]. Briefly, protein samples were resolved on 5–20% linear gradient SDS-polyacrylamide gels, and then electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA) in transfer buffer (25 mM Tris-HCl/190 mM glycine/20% methanol/0.01% SDS). Membrane washes were carried out as outlined previously [Allegretto et al., 1987] with the exception that TBS replaced PBS in the wash solutions.

RNA Extractions and Northern Blot Analysis

At the time of harvest, tissue was removed from the culture medium, quickly frozen under liquid nitrogen, and stored at −80°C. Poly (A)$^+$ RNA was isolated using the Fast Track mRNA Isolation Kit (Invitrogen, San Diego, CA). RNA was fractionated by electrophoresis through a 1% agarose/3% (w/v) formaldehyde gel. Northern blotting was performed by electrotransfer onto Nytran membranes (Schleicher & Schuell, Keene, NH). Hybridization with DNA probes and wash conditions were described previously [Meyer et al., 1992]. To normalize for transfer and loading, we utilized either the cDNA probe for chicken β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes.

Test of Inhibitors of Protein and RNA Synthesis

Cycloheximide activity was verified by measuring the incorporation of [^{35}S]methionine (1,186 Ci/mmol) (New England Nuclear, Boston, MA) into newly synthesized protein. Tissues were cultured in the presence of 24 μCi/ml of [^{35}S]methionine and 36 μM cycloheximide for 0.25, 0.5, 1, 2, 4, and 8 h. [^{35}S]methionine incorporation was quantitated by counting the trichloroacetic acid–precipitable material. This concentration of inhibitor reduced [^{35}S]methionine incorporation into newly synthesized protein by 95% within 30 min. Actinomycin D and 5,6 dichloro-1-β-ribofuranosylbenzimidazole (DRB) activities were determined by measuring the incorporation of [^{3}H]uridine (42.4 Ci/mmol) (New England Nuclear) into total RNA. Briefly, the tissues were incubated in the presence of these transcription inhibitors for 0.5, 1, and 2 h. Medium was removed and replaced with medium containing 10 μCi/ml [^{3}H]uridine for 1 h. [^{3}H]-uridine incorporation was quantitated by counting the trichloroacetic acid–precipitable material. While 8.2 μM actinomycin D inhibited total RNA synthesis by 30–50% (data not shown), concentrations of 0.5 and 1.6 μM did not significantly inhibit the incorporation [^{3}H]uridine into total RNA. Media containing 500 μM DRB was determined to effectively block 80% of total RNA synthesis.

Measurement of CaBP mRNA Stability

Embryonic chick duodena (two plates with three duodena per plate) were incubated in the presence of 200 pM 1,25(OH)$_2$D$_3$ for 8 h to allow CaBP mRNA accumulation. This concentration of hormone was chosen because it is more near physiological and it is sufficient to induce CaBP mRNA accumulation within 8 h. Because it is the minimal effective concentration of 1,25(OH)$_2$D$_3$, the level of hormone in the organ culture presumably should be reduced to less than bioactive concentrations by incubations in the absence of added 1,25(OH)$_2$D$_3$. For this procedure, the medium was replaced three times for 20 min periods with prewarmed Waymouth's media plus 2% bovine serum albumin. Medium containing 500 μM DRB in the presence or

absence of 200 pM $1,25(OH)_2D_3$ was used for the remainder of the incubation period. At 0, 2, 4, and 8 h tissue was removed from the culture medium, quickly frozen under liquid nitrogen, and stored at −80°C. Poly $(A)^+$ RNA was isolated and analyzed by Northern blot as described above.

Quantitation of CaBP Pre-mRNA and mRNA by the Polymerase Chain Reaction (PCR)

The method for quantifying transcripts by means of PCR amplification with an internal control is essentially that described by Wang et *al. [1989]*. A synthetic gene, pCaBP, was constructed for the purposes of preparing complementary RNA (cRNA) to utilize as an internal control. pCaBP is a cassette of linked PCR primers ligated into the commercial vector pSP64(poly A) (Promega Biotec, Madison, WI) between the SP6 polymerase promoter and a poly(A) tail. SP6 polymerase was employed to synthesize pCaBP cRNA according to the procedure recommended by Promega Biotec. The cRNA (1327 nucleotides) was purified by oligo (dT)-cellulose (Boehringer Mannheim) affinity chromatography and quantitated by spectrophotometric analysis of the absorbance at 260 nm.

Total RNA was extracted from the duodenal tissue utilizing previously described methods [Chirgwin et al., 1979] and reagents supplied in the Total RNA Isolation Kit purchased from Invitrogen. The RNA pellet was dissolved in sterile water, quantified by absorbance at 260 nm, and stored at −80°C. Aliquots of the total RNA were mixed with a known amount of cRNA, and RNA-based PCR was performed using the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, CT) and oligonucleotide primers (21 mers) as described previously [Ferrari and Battini, 1990]. Reverse transcriptase reactions (20 μl) were carried out at 42°C for 30 min in the presence of 3 pmol of 3′-primer and 1×10^6 cpm of ^{32}P-labeled 3′-primer. (The 3′-primer was end-labeled by T4 polynucleotide kinase (Promega Biotec) according to the instructions from the manufacturer.) Serial dilutions (1:3) of the reverse transcribed cDNA were mixed with 3 pmol of the appropriate 5′-primer and GeneAmp RNA PCR kit reagents. The mixture was overlaid with 50 μl of mineral oil, and thermal cycling was performed using an Eppendorf Microcycler. The thermal cycling profile was as follows: denaturation at 94°C for 1 minute, hold for 2 min at 78°C while ampliTaq polymerase is added, followed by 25 cycles of 95°C denaturation for 30 s, 58°C primer annealing for 1 min, and 72°C extension for 1 min. After amplification, 10 μl of the PCR reaction was added to 5 μl gel loading buffer (25% glycerol, 50 mM EDTA, 0.5% SDS, 0.2% bromphenol blue, 0.2% xylene cyanol), and the PCR products were separated in a 6% polyacrylamide gel. After electrophoresis, the gels were dried under vacuum and exposed to X-ray film (XAR, Kodak) overnight. The developed film was aligned with the gel, and the appropriate bands were cut out and quantified by Cerenkov counting.

RESULTS

Binding of [^{3}H-1β]1α,25$(OH)_2D_3$ to Duodenal Chromatin

Utilizing duodenal organ culture as an in vitro model system, we first examined the ability of $1,25(OH)_2D_3$ to localize to the chromatin where it presumably is specifically bound to VDR. Following exposure of embryonic duodena to [^{3}H-1β]1α,$25(OH)_2D_3$, chromatin-associated hormone is detectable within 30 min, and near maximal binding occurs within 1–2 h (Fig. 1). Saturable binding of $1,25(OH)_2D_3$ is reached between 2 and 4 h after the addition of labeled hormone. The time course localization of [^{3}H-1β]1α,$25(OH)_2D_3$ in the duodenal chromatin is very similar to that reported in previous in vivo studies measuring saturation of $1,25(OH)_2D_3$ binding to the nuclear [Theofan et al., 1986] or chromatin fractions [Brumbaugh and Haussler, 1974] from rachitic chick intestinal mucosa. In these studies significant amounts of hormone localized in the nucleus within 15–30 min and reached a plateau near 2–3 h [Theofan et al., 1986; Brumbaugh and Haussler, 1974]. The results of Figure 1 indicate that the duodenal organ culture is a valid system for investigating the early actions of $1,25(OH)_2D_3$ at the nuclear level. This rapid time course of $1,25(OH)_2D_3$ association to the chromatin is consistent with a receptor-mediated mechanism of action for the alteration of CaBP gene expression and other $1,25(OH)_2D_3$-dependent physiological responses.

Duodenal VDR in the Absence and Presence of $1,25(OH)_2D_3$

The relative concentrations of duodenal VDR after exposure to $1,25(OH)_2D_3$ were analyzed by Western blot and are shown in Figure 2. VDR, which in chickens is a doublet of molecular

weights 60 and 58 kDa, is present in the duodenal tissue prior to culture (data not shown) and when cultured in the absence of 1,25(OH)$_2$D$_3$ (Fig. 2). The molecular mass standards (kDa) are denoted on the right, and the control lane contains a partially purified preparation of chicken duodenal VDR. The lower molecular weight species represent a major proteolytic product of VDR and other abundant proteins that cross-react, nonspecifically, with the 9A7γ VDR antibody. As illustrated in Figure 2, treatment of the duodena with 1,25(OH)$_2$D$_3$ for increasing amounts of time (up to 24 h) produced a slight increase in VDR levels, especially at the 4 and 8 h time periods. These results demonstrate that duodenal VDR expression is not absolutely dependent on its ligand, but that the sterol hormone does upregulate or stabilize [Wiese et al., 1992] its receptor to some degree. Similar results have been obtained comparing rachitic to normal chickens, in vivo, where intestinal VDR mRNA levels were relatively independent of vitamin D intake [Meyer et al., 1992; Mangelsdorf et al., 1987a].

Time Course Appearance of CaBP Protein and mRNA After 1,25(OH)$_2$D$_3$ Treatment

CaBP protein concentration was examined in embryonic chick duodena cultured in the presence of 25 nM 1,25(OH)$_2$D$_3$ (Fig. 3). A trace amount of CaBP was detected by immunoblot in the control duodena that were maintained in the absence of 1,25(OH)$_2$D$_3$ (Fig. 3). The detection of CaBP protein prior to hormone exposure may be reconciled by observations from previous studies which determined that CaBP begins to appear in duodena of chicks at hatch (21 days) [Corradino et al., 1969]. Since the duodena used for these studies are from 19-day-old embryos, it is possible that an occasional sample in the pool of tissues is further along in development and therefore expressing CaBP. In the presence of 1,25(OH)$_2$D$_3$, CaBP synthesis was increased, a response which has been well established in previous in vivo studies [Theofan et al., 1986; Ferrari and Battini, 1990; Clemens et al., 1988; Theofan and Norman, 1986]. The first detectable increase in CaBP was observed at 4 h and continued to a final measurement at 24 h. Our data correlate well with those of Brown and DeLuca [1990], who utilized radioimmunoprecipitation to assay an induction of CaBP by 1,25(OH)$_2$D$_3$ between 4.5 and 6 h in duodenal organ culture. Also, in vivo observations show that levels of CaBP start to increase by 5–8 h when a single dose of 6.5 nmol of 1,25(OH)$_2$D$_3$ is administered to vitamin D–deficient chicks [Theofan et al., 1986].

We next examined the temporal relationship between 1,25(OH)$_2$D$_3$-dependent CaBP mRNA accumulation and CaBP protein synthesis. The Northern blot in Figure 4 illustrates that CaBP mRNA was undetectable in duodena cultured in the absence of 1,25(OH)$_2$D$_3$. Treatment with 25 nM 1,25(OH)$_2$D$_3$ resulted in detectable levels of CaBP mRNA after 4 h which continued to increase through 24 h (Fig. 4). Thus, 1,25(OH)$_2$D$_3$-

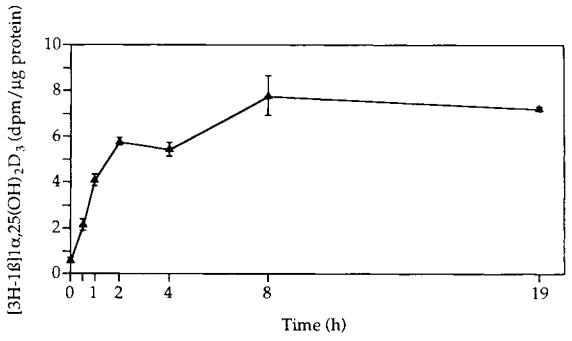

Fig. 1. Time-course localization of [^{3}H-1β]1α,25(OH)$_2$D$_3$ to duodenal chromatin after addition of hormone to the culture media. Chick duodena were cultured in Waymouth's media in the presence or absence of 25 nM [^{3}H-1β]1α,25(OH)$_2$D$_3$, exposing the tissue to hormone for the times indicated in the figure. The amount of [^{3}H-1β]1α,25(OH)$_2$D$_3$ in the duodenal chromatin per microgram of total protein was determined as described in Materials and Methods. Each data point is the average (±SE) of four chromatin extractions per time point, each extraction being performed on chromatin derived from three pooled duodena.

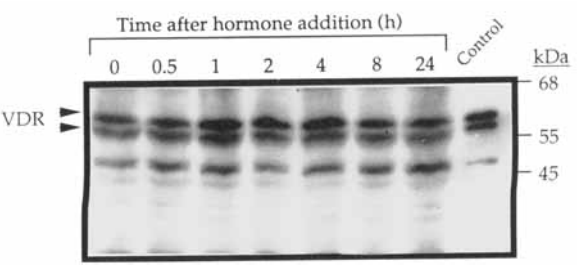

Fig. 2. Western blot analysis of VDR expression in cultured embryonic chick duodenum. Duodena were incubated in Waymouth's media, and at the times indicated 1,25(OH)$_2$D$_3$ was added to achieve a final concentration of 25 nM. VDR (arrows) appears as a doublet (at 58 and 60 kDa), and the positions of molecular mass standards (kDa) are denoted on the right. The control lane to the left of the molecular mass standards is a partially purified preparation of chicken VDR. The lower molecular weight species represent a major proteolytic product of VDR and other abundant proteins that cross-react nonspecifically with the 9A7γ VDR antibody.

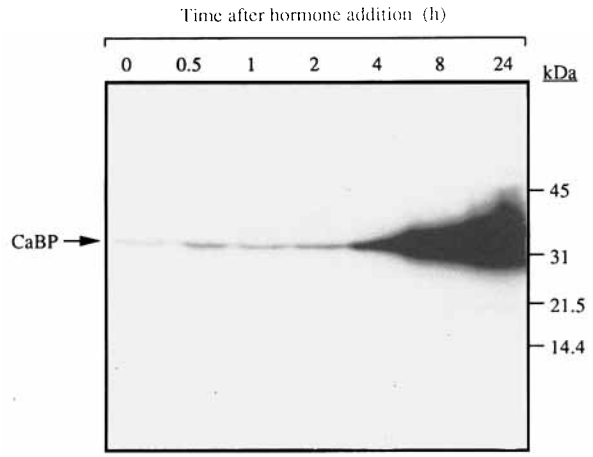

Fig. 3. Western blot analysis of $1,25(OH)_2D_3$-dependent CaBP expression in cultured embryonic chick duodenum. Duodena were incubated in Waymouth's media, and at the times indicated, $1,25(OH)_2D_3$ was added to achieve a final concentration of 25 nM. The protein extracts are identical to the preparations used in Fig. 2. CaBP is indicated by the arrow on the left, and the molecular mass standards (kDa) are shown on the right.

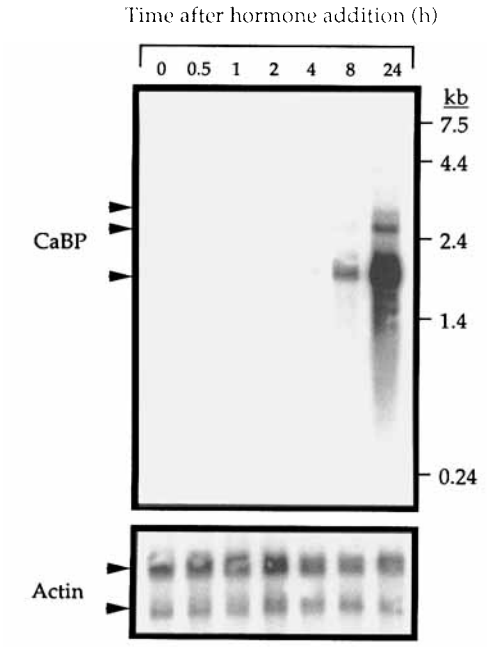

Fig. 4. Time course of $1,25(OH)_2D_3$-dependent CaBP mRNA induction in cultured embryonic chick duodenum: Northern blot. Duodena were cultured in Waymouth's media in the presence or absence of 25 nM $1,25(OH)_2D_3$ for the indicated times. Each lane contains equal amounts (5 μg) of poly $(A)^+$ RNA, determined by UV spectrophotometry. RNA was isolated from the tissues harvested during the experiment shown in Figs. 2 and 3. **Top:** The arrows indicate hybridization of the CaBP cDNA probe (at 2.0, 2.8, and 3.1 kb). **Bottom:** Rehybridization of the blot with a chicken β-actin cDNA after removal of the CaBP cDNA in order to demonstrate that approximately equal amounts of RNA were loaded in each lane.

dependent CaBP mRNA accumulation (Fig. 4) occurs subsequent to nuclear localization of hormone (Fig. 1) and coincides with the increase in CaBP protein accumulation (Fig. 3).

Effect of Transcription and Translation Inhibitors on $1,25(OH)_2D_3$-Dependent CaBP mRNA Accumulation

In initial experiments to discern the mechanism of $1,25(OH)_2D_3$-mediated CaBP expression, we evaluated the effect of the transcription inhibitor, actinomycin D, on CaBP mRNA accumulation. Figure 5 illustrates that exposure to 0.5 and 1.6 μM actinomycin D inhibits the $1,25(OH)_2D_3$-dependent accumulation of CaBP mRNA at 4 and 8 h. No effect of actinomycin D was observed on the nonregulated glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA probed on the same membrane, demonstrating that the effect of this drug is not general but targeted to specific mRNAs such as CaBP. The result of this experiment indicates that transcription is, in part, required for $1,25(OH)_2D_3$-mediated CaBP expression, although it is not necessarily the only mechanism responsible for increased CaBP mRNA steady-state levels.

To determine the role of ongoing protein synthesis in the $1,25(OH)_2D_3$-mediated CaBP expression, we employed the translation inhibitor, cycloheximide. Cycloheximide (36 μM) inhibits protein synthesis by 95% in the duodena culture system as quantitated by a decrease in $[^{35}S]$methionine incorporation (data not shown). Northern blot analysis of poly $(A)^+$ RNA extracted from tissues pretreated with cycloheximide for 30 min prior to addition of hormone demonstrated nearly a complete inhibition of $1,25(OH)_2D_3$-dependent CaBP mRNA accumulation at 4 and 8 h post–hormone treatment (Fig. 6A). Since cycloheximide treatment effected a concurrent superinduction in VDR mRNA levels (Fig. 6B) and only modestly attenuated GAPDH mRNA levels (Fig. 6C), we conclude that the antibiotic treatment was not toxic to the tissue during the time course evaluated. Nevertheless, the visible reduction in GAPDH mRNA levels in the presence of cycloheximide that somewhat paralleled the inhibition of $1,25(OH)_2D_3$-induced CaBP mRNA led us to perform an additional control experiment. In this experiment (data not shown) we monitored β-actin mRNA concentrations in duodena treated

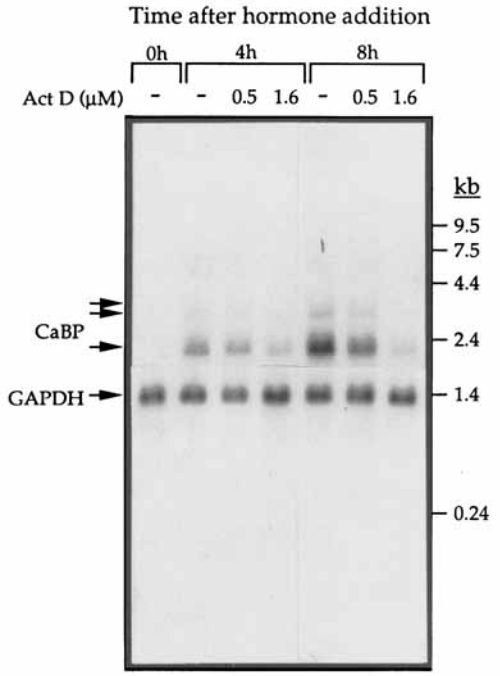

Fig. 5. Effect of actinomycin D treatment on 1,25(OH)$_2$D$_3$-dependent CaBP mRNA expression in cultured embryonic chick duodenum: Northern blot. Duodena were cultured for a total of 24 h. Four or 8 hours prior to the termination of the culture period, 0, 0.5, or 1.6 μM actinomycin D (0.5 or 1.6 μM final concentration) was added simultaneously with 1,25(OH)$_2$D$_3$ at a final concentration of 25 nM. Each lane contains equal amounts (5 μg) of poly (A)$^+$ RNA. The upper three arrows indicate hybridization of the CaBP cDNA probe. To verify equal RNA loading per lane and transfer efficiency, the blot was rehybridized with GAPDH cDNA probe (lowest arrow).

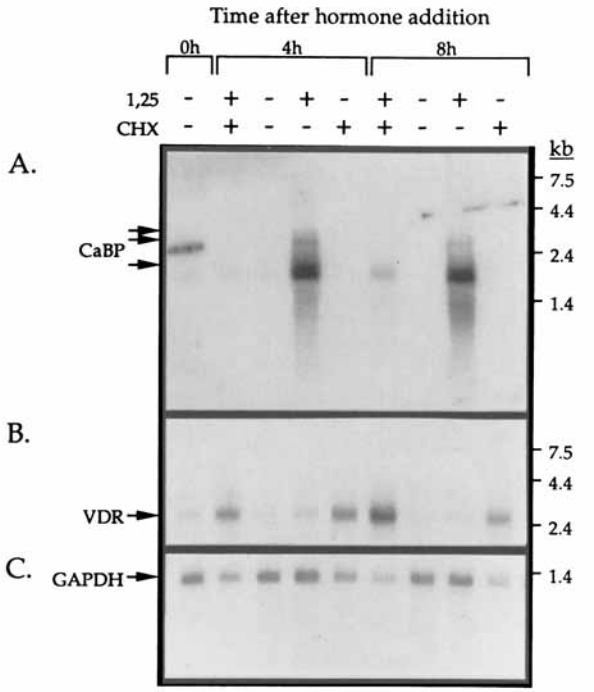

Fig. 6. Effect of cycloheximide treatment on 1,25(OH)$_2$D$_3$-dependent CaBP and VDR mRNA expression in cultured embryonic chick duodenum: Northern blot. Prior to termination of the culture period, the tissue was pretreated for 30 min with either a final concentration of 36 μM cycloheximide (CHX) or ethanol vehicle followed by incubations in the presence or absence of 25 nM 1,25(OH)$_2$D$_3$ (1,25) for 4 or 8 h. **A:** Hybridization of the CaBP cDNA probe (arrows). **B:** The blot was stripped and rehybridized with the VDR cDNA probe (arrow points to the 2.4 kb VDR RNA transcript). **C:** The same blot was stripped a second time and rehybridized with the GAPDH cDNA probe.

for 4 and 8 h with 1,25(OH)$_2$D$_3$ and cycloheximide and observed no effect on β-actin mRNA concurrent with the same dramatic inhibition of 1,25(OH)$_2$D$_3$-induced CaBP mRNA and superinduction of VDR mRNA that were seen in Figure 6A,B.

Although Figure 6B does show evidence that 1,25(OH)$_2$D$_3$ treatment of duodena upregulates VDR mRNA (compare, for instance, 8 h, + cycloheximide, ± 1,25(OH)$_2$D$_3$) and cycloheximide alone causes superinduction of VDR mRNA, one concern was that inhibition of protein synthesis by cycloheximide might attenuate VDR protein levels sufficiently to account for the inhibitory effects of the drug on CaBP mRNA induction by 1,25(OH)$_2$D$_3$. Previous evaluation of VDR stability in rat osteosarcoma cells (ROS 17/2) [Pan and Price, 1987] and pig kidney cells (LLC-PK$_1$) [Costa and Feldman, 1987] revealed the half-life of the 1,25(OH)$_2$D$_3$ receptor to be 2.7 h and 4.3 h, respectively. In order to verify that the incubation with cycloheximide did not lead to a complete elimination of VDR, chromatin-associated proteins were isolated from duodena exposed to cycloheximide for 0, 4, and 8 h. Western blot analysis of chromatin extracts showed some decrease in VDR levels in the presence of cycloheximide at each time point examined (Fig. 7). However, comparing CHX to the 0 h control, significant levels of VDR persist over the 4–8 h period where cycloheximide completely inhibits CaBP mRNA accumulation. Therefore, we argue that sufficient levels of VDR exist for 1,25(OH)$_2$D$_3$ to regulate CaBP gene expression, but continual synthesis of another protein is necessary for 1,25(OH)$_2$D$_3$-dependent CaBP expression.

Effect of 1,25(OH)$_2$D$_3$ on CaBP mRNA Stability in Cultured Embryonic Duodena

To determine whether the 1,25(OH)$_2$D$_3$-mediated increase in CaBP mRNA was the result of

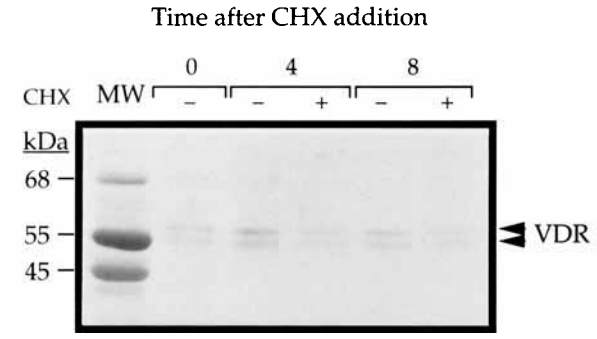

Fig. 7. VDR turnover in cultured embryonic chick duodenum: Western analysis of chromatin extracts. Duodena were incubated in the presence of 36 μM cycloheximide (+) or ethanol vehicle (−) for various intervals (0, 4, or 8 h) prior to harvest. Each lane contains equal amounts of total protein (400 μg) from chromatin extracts prepared as described in Material and Methods. VDR (arrows) appears as a doublet, and the positions of molecular mass standards (kDa) are denoted on the left.

an increase in CaBP mRNA half-life, RNA synthesis was blocked by 500 μM DRB. Three time-course experiments were carried out examining CaBP mRNA decay in the presence or absence of $1,25(OH)_2D_3$ as assessed by Northern blot (data not shown). Linear regression analysis of data from the three independent experiments (data not shown) revealed that CaBP mRNA decays with a half-life of 9.5 and 9.6 h in the absence or presence of $1,25(OH)_2D_3$, respectively. Statistical analysis verified that these half-lives are not significantly different and that no significant difference existed at any of the time points evaluated (2, 4, and 8 h).

Effect of $1,25(OH)_2D_3$ on CaBP Gene Transcription in Cultured Embryonic Chick Duodena as Assessed by Quantitative PCR

To evaluate whether the lag in $1,25(OH)_2D_3$-induced CaBP mRNA accumulation of 4 h (see Fig. 4) was the result of a delayed transcriptional response to the hormone, nascent CaBP gene transcription was analyzed by following the time-course accumulation of CaBP pre-mRNA and mRNA sequences utilizing quantitative PCR technology. Total RNA was prepared from duodena which had been treated with $1,25(OH)_2D_3$ for various lengths of time (0, 0.5, 1, 4, and 8 h). RNA was mixed with pCaBP cRNA, the internal control RNA, and CaBP-specific RNA species were reverse transcribed into cDNA utilizing a ^{32}P-labeled 3′-primer complementary to sequences in exon 2 of the CaBP gene (Fig. 8). CaBP pre-mRNA and mRNA sequences were amplified by PCR employing 5′-primers homologous to sequences in intron 1 and exon 1, respectively (Fig. 8). Analysis of the PCR products reveals that duodena treated with $1,25(OH)_2D_3$ begins to accumulate CaBP pre-mRNA sequences 1 h after hormone exposure (Fig. 9), suggesting that CaBP gene transcription is initiated within 1 h. CaBP mRNA sequences also accumulate at 1 h, indicating that there is no significant lag between CaBP transcription and CaBP mRNA processing. Inclusion of a known amount of the internal standard (pCaBP cRNA) provides a means to estimate the amount of accumulated CaBP mRNA and pre-mRNA transcripts at the various times after hormone addition (Fig. 10). After 1 h exposure to hormone, CaBP pre-mRNA and CaBP mRNA were elevated from the basal levels of 250 and 1,000 to 1,100 and 4,500 copies per microgram of total RNA, respectively. Figure 10 also illustrates a coincidental increase in CaBP pre-mRNA and CaBP mRNA, demonstrating no significant delay in mRNA processing.

DISCUSSION

The expression of CaBP in the chick duodenum exhibits an absolute requirement for $1,25(OH)_2D_3$, but it remains unclear whether the effect of $1,25(OH)_2D_3$ and its receptor is directly on CaBP gene transcription or requires an additional cooperative or mediator protein. Previous data have indicated that $1,25(OH)_2D_3$-mediated CaBP regulation is primarily transcriptional or posttranscriptional, but not the result of altered translation efficiency [Meyer et al., 1992]. This study was designed to elucidate whether $1,25(OH)_2D_3$ and its receptor mediate a transcriptional or posttranscriptional effect that is direct or one that requires protein synthesis which would suggest the necessity for a cooperative or intermediary protein(s). In order to address these questions we employed an organ culture system of embryonic chick duodena. The data presented herein correlate well with earlier in vivo studies, demonstrating the utility of the organ culture system for the study of $1,25(OH)_2D_3$-induced CaBP expression. First, $1,25(OH)_2D_3$ rapidly localizes to the nucleus (Fig. 1). Second, VDR is present in the duodena and is only modestly upregulated by the hormonal ligand (Fig. 2). Finally, Western and Northern blot analysis of CaBP protein (Fig. 3) and mRNA (Fig. 4) levels clearly demonstrate that the time course of $1,25(OH)_2D_3$-induced CaBP expression is very similar to that observed in studies in

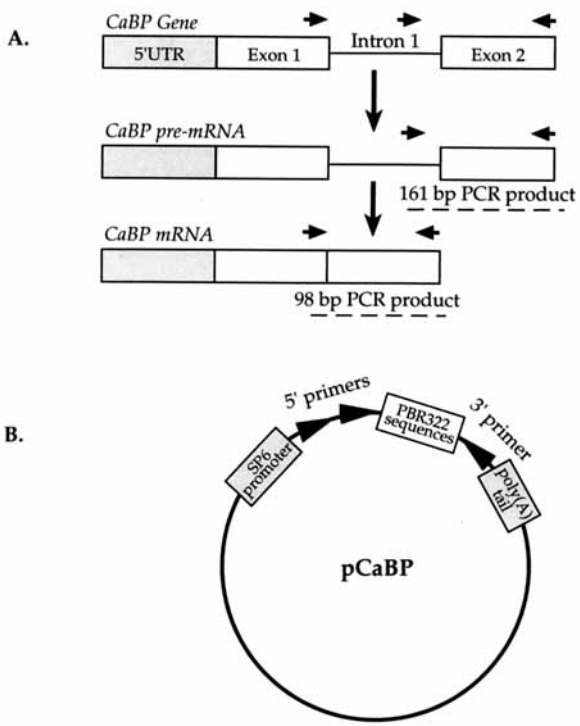

Fig. 8. **A:** Schematic map of 5'-region of the CaBP gene structure illustrating the exon/intron boundaries along with the location and direction of oligonucleotide primers (arrows) utilized in PCR amplification of CaBP pre-mRNA sequences (161 bp) and CaBP mRNA sequences (98 bp). **B:** Structure of the pCaBP synthetic gene utilized as an internal control in quantitative PCR. The plasmid was generated by ligating the oligonucleotide primer cassette between the SP6 polymerase promoter and polyadenylated sequence (stippled boxes) of the commercial vector pSP64(poly A). In vitro transcription from the SP6 promoter yields a 1,327 nucleotide cRNA.

vivo [Theofan et al., 1986; Brown and DeLuca, 1990].

The temporal relationship between hormone localization to the nucleus (Fig. 1), CaBP mRNA accumulation (Fig. 4), and CaBP protein synthesis (Fig. 3) suggests that the increase in CaBP mRNA can be attributed to a 1,25(OH)$_2$D$_3$ receptor-mediated transcriptional effect. Addition of actinomycin D to the culture media abrogates an increase of duodenal CaBP mRNA by hormone (Fig. 6), which supports the necessity of a transcriptional event. However, a significant increase in CaBP mRNA cannot be detected until 4 h after 1,25(OH)$_2$D$_3$ addition (Fig. 4), although hormone localizes to the nucleus within 30 min (Fig. 1). Several mechanisms could account for the delayed time course of 1,25(OH)$_2$D$_3$-dependent CaBP expression. The delayed response may suggest the possibility that the increase in CaBP mRNA is secondary to the initial accumulation of another hormone-regulated, primary gene product. This type of hormone regulation has been described in *Drosophila* where ecdysone directly activates the E74A promoter. Accumulated E74A, which is thought to be a nuclear regulatory protein that binds to specific DNA sites in the ecdysone-inducible puff genes, transduces the hormone signal [Urness and Thummel, 1990].

Our data reveal that pretreatment of the duodena with cycloheximide largely prevents CaBP mRNA accumulation (Fig. 6) without a corresponding dramatic decrease in VDR levels (*Fig.* 7). This suggests that protein synthesis is required either for CaBP gene transcription or CaBP mRNA stabilization. Transcriptional activation of some glucocorticoid-regulated genes exhibits cycloheximide sensitivity due to the requirement of a labile cooperative factor(s). For example, α_1-acid glycoprotein (AGP) RNA is induced severalfold in liver by glucocorticoids, and treatment with cycloheximide diminishes AGP mRNA steady-state levels [Klein et al., 1988]. In the AGP promoter, glucocorticoid responsiveness is conferred by a 15 base pair sequence, and inclusion of a downstream flanking sequence renders the hormone induction sensitive to cycloheximide [Chan et al., 1991]. Thus, a labile factor(s) is hypothesized to cooperate with glucocorticoid receptor (GR) to stimulate transcription. Unlike CaBP induction, there is no time delay in the induction of AGP by hormone. Hepatic rat α_{2u}-globulin (RUG) shows a delayed induction by glucocorticoids that can be blocked by an inhibitor of protein synthesis. A glucocorticoid responsive element (GRE)–like DNA segment, which differs from the consensus GRE, confers a delayed glucocorticoid induction which is cycloheximide sensitive, mimicking the expression of RUG, in vivo [Chan et al., 1991]. Chan et al. [1991] speculate that properties intrinsic to the unusual GRE and its interaction with the GR and other transcription factors are responsible for the delayed response. Tryptophan oxygenase (TO) is another gene which shows delayed activation by glucocorticoids and cycloheximide sensitivity. In this case, induction of TO by glucocorticoids requires interaction of the GR with the GRE [Nakamura et al., 1987] and a short-lived, constitutive protein, which is also regulated by glucocorticoids [Danesch et al., 1987]. These examples support the possibility that an uncharacterized protein is also involved in CaBP gene expression.

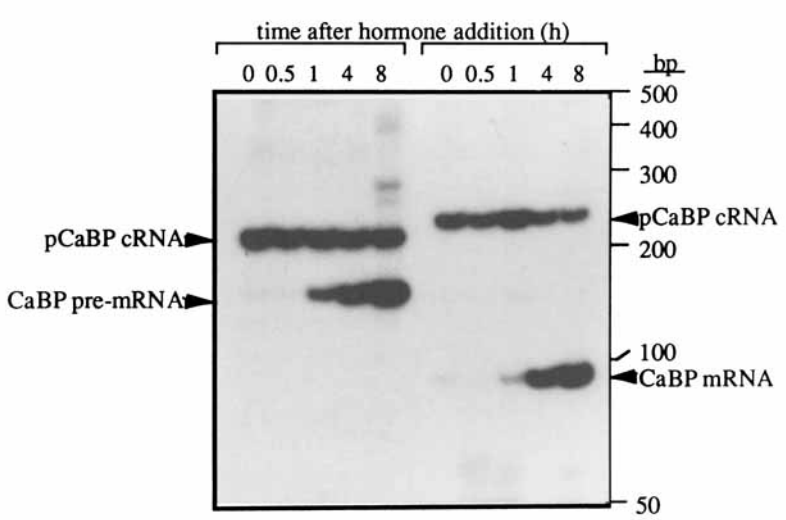

Fig. 9. PCR amplification of CaBP pre-mRNA and mRNA sequences. Duodena were exposed to 25 nM $1,25(OH)_2D_3$ for the indicated times (0, 0.5, 1, 4, and 8 h). Total RNA (20–200 ng) was mixed with pCaBP cRNA (1 pg) and reverse-transcribed into cDNA utilizing the 3′-primer located in exon 2 (Fig. 8). This reaction was followed by 25 cycles of PCR amplification employing either the 5′-primer from intron 1 (the five leftmost lanes) or the 5′-primer located in exon 1 (the five rightmost lanes), thereby amplifying CaBP pre-mRNA or mRNA sequences, respectively (Fig. 8).

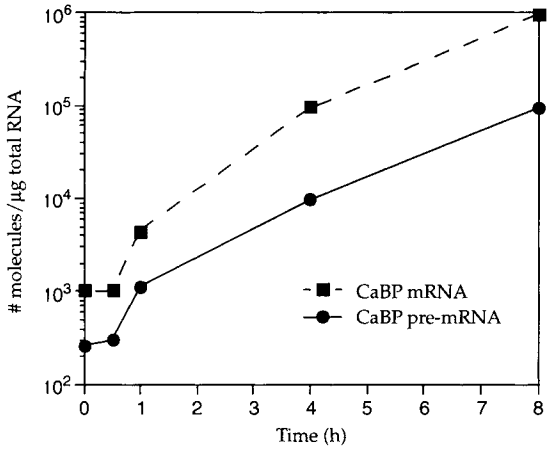

Fig. 10. Quantitative PCR analysis of CaBP pre-mRNA and mRNA sequences in cultured embryonic chick duodena at various times after $1,25(OH)_2D_3$ treatment. A dilution series of the reverse transcriptase reactions was PCR amplified. The inclusion of a known amount of internal control RNA (pCaBP cRNA) allowed the quantitation by extrapolation of the amount of CaBP pre-mRNA (circles) or CaBP mRNA (squares) transcripts per microgram of total RNA (see Materials and Methods). The same total RNA samples shown in Fig. 9 were employed for this analysis.

Alternatively, hormone-dependent mRNA stabilization is another potentially important facet of the delayed hormone-response and the requirement for ongoing protein synthesis that characterize CaBP expression. The β-casein gene demonstrates a lag in glucocorticoid induction and is sensitive to cycloheximide. Poyet et al. [1989] present evidence for a hormone-dependent β-casein mRNA stabilization which requires ongoing protein synthesis. Some of the previous studies of CaBP in vivo point to a similar mechanism for $1,25(OH)_2D_3$-dependent CaBP expression [Clemens et al., 1988; Theofan and Norman, 1986]. In vitamin D–replete chick intestine (vs. $1,25(OH)_2D_3$-induced CaBP), α-amanitin treatment had no effect on CaBP mRNA levels up to 4 h; however, cycloheximide treatment caused a significant reduction within 1 h [Theofan and Norman, 1986]. Thus, for chronic CaBP expression, transcription appears to play a very modest role, and perhaps mRNA stabilization is responsible for CaBP accumulation. In another study, treating vitamin D–deficient chicks intravenously with 500 ng of $1,25(OH)_2D_3$ after pretreatment with actinomycin D (50 μg/100 g) showed little effect on CaBP mRNA levels, and pretreatment with cycloheximide (25 μg/100 g) blunted the acute phase induction [Clemens et al., 1988]. Both of these in vivo studies demonstrate the necessity for continual protein synthesis and argue against the involvement of transcription. Finally, only a relatively inactive putative VDRE has been identified [MacDonald et al., 1992] in the chicken CaBP gene, consistent with the contention that direct transcriptional regulation serves a minor role in CaBP expression. In contrast, a moderately ac-

tive VDRE upstream of the promoter apparently occurs in the rat calbindin-D_{9k} gene [Darwish and DeLuca, 1992]. Moreover, a recent study reports that $1,25(OH)_2D_3$-induced CaBP gene transcription is associated with local changes at the chromatin level. Cancela et al. [1992] showed that $1,25(OH)_2D_3$ elicits the appearance of tissue-specific and hormone-dependent DNase-I hypersensitive sites in the 5′-flanking DNA of the CaBP gene and in the large second intron, implicating these DNA sequences as candidate VDREs or possibly control regions for the interaction of $1,25(OH)_2D_3$-regulated factors.

To clarify the questions raised above, we have determined in this study that CaBP mRNA decays with a half-life of approximately 9.5 h in the absence or presence of $1,25(OH)_2D_3$, indicating that $1,25(OH)_2D_3$ does not mediate directly the stabilization of CaBP mRNA. A caveat of this experiment is that $1,25(OH)_2D_3$ may regulate the expression of an intermediate protein which is involved in CaBP mRNA stabilization. Because we utilized the transcription inhibitor, DRB, in this experiment, this drug would abrogate the synthesis of the postulated intermediate protein. Therefore, we cannot absolutely rule out the possibility that $1,25(OH)_2D_3$ regulates the turnover of CaBP mRNA secondarily. Nevertheless, our data suggest that the delayed time course of $1,25(OH)_2D_3$-dependent CaBP expression may be attributed to an effect on transcription or other posttranscriptional mechanisms. These results are consistent with the recent report that $1,25(OH)_2D_3$-mediated CaBP expression in primary chicken kidney cells is dependent primarily on posttranscriptional mechanisms and to a lesser extent on CaBP gene transcription [Enomoto et al., 1992]. These studies demonstrate that a large increase in $1,25(OH)_2D_3$-mediated CaBP mRNA expression (ten- to twentyfold), which required ongoing protein synthesis, was accompanied by only marginal increases in CaBP gene transcription and no detectable effect on the measured CaBP mRNA half-life of 12 h. The present studies utilized PCR to detect CaBP pre-mRNA sequences 1 h after vitamin D hormone treatment, demonstrating $1,25(OH)_2D_3$-mediated de novo transcription of the CaBP gene (Fig. 9). Furthermore, the concurrent increase in CaBP pre-mRNA and mRNA sequences argues against a $1,25(OH)_2D_3$-mediated regulation of CaBP pre-mRNA processing (Figs. 9, 10) but does not rule out an effect on CaBP pre-mRNA turnover, a mechanism of demonstrated importance in eIF-2α gene expression [Cohen et al., 1990]. The rapid induction of CaBP pre-mRNA and mRNA sequences (Figs. 9, 10) suggests that the $1,25(OH)_2D_3$-receptor complex has a direct effect on CaBP gene transcription. However, further evaluation of CaBP gene transcription and its sensitivity to cycloheximide would be required in order to extend this conclusion. In addition, CaBP pre-mRNA stabilization is perhaps a posttranscriptional mechanism by which the $1,25(OH)_2D_3$-receptor complex enhances CaBP expression and should be evaluated by future PCR studies.

In conclusion, our data provide evidence that ongoing protein synthesis is required for the transcriptional or posttranscriptional control of CaBP by $1,25(OH)_2D_3$. It is apparent from these studies that the mechanism is more complicated than the conventional hormone receptor/transcriptional activation model which explains the regulation of osteocalcin and osteopontin genes by $1,25(OH)_2D_3$ [Kerner et al., 1989; Demay et al., 1990; Terpening et al., 1991; Noda et al., 1990]. We speculate that synthesis of an intermediary or cooperative protein is required for the $1,25(OH)_2D_3$-dependent CaBP transcription or pre-mRNA processing, as has been cited for other steroid hormone–regulated genes [Chan et al., 1991; Danesch et al., 1987; Klein et al., 1987, 1988; Nakamura et al., 1987; Poyet et al., 1989; Urness and Thummel, 1990]. Since it is now known that retinoid X receptors (RXRs) function as coreceptors with VDR in controlling the transcription of such genes as osteocalcin [MacDonald et al., 1993, 1994], it is tempting to speculate that isoforms of RXR or perhaps an intestine-specific transcription factor that binds to a crucial cis-element in the CaBP gene could represent the unknown protein for which evidence is reported herein. In fact, Ferrari et al. [1994] have detected VDRE binding activity in avian intestine that cooperates with RXR but apparently is not related to VDR.

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