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Vitamin D and adaptation to dietary calcium and phosphate deficiencies increase intestinal plasma membrane calcium pump gene expression

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ABSTRACT **The effect of vitamin D and other variables on the synthesis of the chicken intestinal plasma membrane calcium pump (PMCA) mRNA was assessed. The DNA probe for Northern analysis was obtained by reverse transcription and PCR with intestinal poly(A)$^+$ RNA, using two 20-mer oligonucleotide primers homologous to the 3′ coding region of the human teratoma PMCA. An *Eco*RI restriction fragment of the PCR product was cloned into the pBluescript II KS(−) phagemid vector, and the chimeric plasmid was used to transform *Escherichia coli*. The amino acid sequence deduced from the nucleotide DNA sequence of the PCR product and the cloned DNA were 96% homologous with the teratoma sequence. Northern blots of intestinal poly(A)$^+$ RNA with ^{32}P-labeled DNA showed the presence of three major species of chicken PMCA mRNAs at about 6.6, 5.4, and 4.5 kb. Northern analysis with the chicken PMCA DNA indicated that repletion of vitamin D-deficient chickens with vitamin D increased PMCA mRNAs in the duodenum, jejunum, ileum, and colon. After injection of 1,25-dihydroxyvitamin D_3 intravenously into vitamin D-deficient chickens, duodenal PMCA mRNA tended to increase by 2 hr, reached a maximum at about 16 hr, and returned to baseline levels at 48 hr. Adaptation of chickens to either a calcium- or phosphorus-deficient diet resulted in a 2- to 3-fold increase in duodenal PMCA mRNA. These results indicate that vitamin D and specific variables that affect calcium absorption through the vitamin D–endocrine system increase intestinal PMCA gene expression.**

A major factor controlling the efficiency of calcium absorption is the vitamin D hormone, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] (1, 2). 1,25$(OH)_2D_3$ elicits both genomic (3) and nongenomic (4–10) effects on the intestinal cell. Among the former effects are the induction of the synthesis of intestinal calbindin-D_{9k} in mammalian species and intestinal calbindin-D_{28k} in avian species (3). In addition, we recently demonstrated another apparent genomic response, a vitamin D-dependent increase in the amount of calcium pump units in the basolateral membrane of the avian enterocyte (11). This finding is consistent with previous observations (12–16) showing that repletion of vitamin D-deficient animals with vitamin D or 1,25$(OH)_2D_3$ results in an enhancement of the ATP-dependent uptake of Ca^{2+} by isolated basolateral membrane vesicles. Further, animals fed diets deficient in calcium or phosphorus adapt to these deficiencies by an increase in the synthesis of 1,25$(OH)_2D_3$ (17–21), resulting in an increase in the efficiency of calcium and phosphorus absorption. These adaptations are associated with an increased synthesis of calbindin-D and its mRNA (17–20, 22–28) and, as recently shown, an increase in the net synthesis of the basolateral calcium pump (11).

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The present studies were undertaken to determine if the previously observed vitamin D-dependent increase in the amount of the chicken intestinal plasma membrane calcium pump (PMCA) is due to an effect of vitamin D on gene expression. The data indicate that the repletion of vitamin D-deficient chickens with vitamin D or 1,25$(OH)_2D_3$ increases intestinal PMCA mRNA. Zalinski *et al.* (29) previously reported a similar response of the rat intestinal calcium pump mRNA to 1,25$(OH)_2D_3$. An increase in calcium pump gene expression** as a consequence of the adaptation of animals to mineral-deficient diets is also shown.

MATERIALS AND METHODS

Animals and Diets. White Leghorn cockerels (Clock & DeCloux, Ithaca, NY) from day of hatching were fed a rachitogenic diet (30) for 4 weeks until the day of the experiment. In some studies, vitamin D-deficient chickens were given (orally) 500 units of vitamin D_3 72 hr prior to sacrifice. In other experiments, the vitamin D-deficient chickens were injected intravenously with 1,25$(OH)_2D_3$ (0.5 μg per chicken) at 0, 2, 8, 16, 24, and 48 hr before sacrifice.

For the mineral deficiency studies, 1-day-old chickens were fed a commercial chicken starter diet (Agway, Syracuse, NY) for about 20 days and then divided into three groups. Each group was then fed for 10 days a semisynthetic diet that varied in calcium and phosphorus content, as follows: group 1, the normal group, 1.2% calcium and 0.8% phosphorus; group 2, the low calcium group, 0.05% calcium and 0.8% phosphorus; and group 3, the low phosphorus group, 1.2% calcium and 0.3% phosphorus. The basal diet was from Teklad (TD90092; Madison, WI) and contained 1200 units of vitamin D_3 per kg of diet. All chickens were killed by decapitation.

RNA Isolation. Intestinal segments were rapidly excised, slit lengthwise, rinsed with cold saline, and gently blotted. The mucosal tissue was scraped from the underlying musculature on a cold glass stage, and the pooled mucosa from three animals was immediately frozen in liquid nitrogen. Total RNA was prepared as described by Chirgwin *et al.* (31), and poly(A)$^+$ RNA was isolated by using the FastTrack poly(A)$^+$ RNA isolation kit (Invitrogen, San Diego).

Reverse Transcription and DNA Amplification by PCR. Reverse transcription of oligo(dT)-primed intestinal poly(A)$^+$ RNA and subsequent DNA amplification were performed by using the GeneAmp RNA PCR kit (Perkin–Elmer/Cetus)

Abbreviations: PMCA, plasma membrane calcium pump; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; BLMV, basolateral membrane vesicle.

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under conditions recommended by the supplier. The oligonucleotide primers used for DNA amplification were as follows: primer 11 (sense, "upstream"), 5'-GCCATCTTC-TGCACAATTGT-3'; and primer 12 (antisense, "downstream"), 5'-TCAGAGTGATGTTTCCAAAC-3'. These primers share sequence homology with nucleotides 3200–3219 (primer 11) and 3825–3844 (primer 12, reverse complement) of the human teratoma PMCA (32). This region (644 bp) encodes the carboxyl-terminal portion of the human protein. After amplification by PCR, the DNA reaction products were analyzed on 1% agarose gels containing ethidium bromide. The subsequent cloning procedures were performed as described by Maniatis *et al.* (33).

Cloning and Sequencing of the PCR Product. The major PCR product (644 bp) was recovered by elution following agarose gel electrophoresis. The DNA was prepared for cloning by blunt ending with T4 DNA polymerase (33) and subsequent restriction digestion with *Eco*RI restriction endonuclease. The resulting DNA fragment (550 bp) was ligated into the *Eco*RI/*Sma* I site of pBluescript II KS(−) phagemid vector (Stratagene). After transformation, selection, and amplification (*Escherichia coli* DH5α; GIBCO/BRL), recombinant phagemids were purified and used to prepare DNA hybridization probes for Northern blots or as templates for DNA sequencing.

Sequencing of the chimeric phagemid DNA was performed by the dideoxynucleotide chain termination method (34). The 3' terminal portion (86 bp) of the PCR product (deleted from the clone's DNA by *Eco*RI digestion) was sequenced directly (as above), using primer 12 and a modified procedure for template preparation and primer annealing (35). DNA sequences were analyzed using PCGENE software (IntelliGenetics).

Northern Analysis of Intestinal RNA. Poly(A)$^+$ RNA (2.5 μg) or total RNA (10 μg) was analyzed for the presence of the intestinal PMCA mRNA by hybridization analysis (35). All DNA probes were isolated from vector DNA by appropriate restriction endonuclease digestion and gel purification prior to nick-translation (36). The PCR product was also gel purified prior to nick-translation and used in hybridization analysis. Probe DNA at 5–10 ng/ml (probe specific activity was 10^7–10^8 dpm/μg of DNA) was used for hybridization. After hybridization for 18 hr at 42°C, the membranes were washed at high stringency, blotted dry, and exposed to Kodak XAR-5 film with an intensifying screen for 4–24 hr at −70°C. An actin cDNA probe (37) was used as a control for estimation of mRNA integrity and as a quantitative index of RNA sample loading among various preparations.

Densitometry. Densitometric analyses of the autoradiographs were performed with an UltraScan XL enhanced laser densitometer (Pharmacia LKB) and an image-analysis computer program (IMAGEPRO; Media Cybernetics, Silver Spring, MD). In each experiment, the density of the mRNA band of the control group was arbitrarily assigned the value of one. The density values of the mRNA bands of the treated groups are given as a function of the ratio of the treated group to the control group.

Statistical Analysis. Statistical analysis was by Student's *t* test or, when appropriate, by analysis of variance performed with the use of a computer program (Minitab, State College, PA). Duncan's multiple range test (38) was employed for multiple comparison of means.

RESULTS

Amplification of Chicken Intestinal Ca^{2+} Pump DNA. The strategy for analyzing chicken intestine for a PMCA-related mRNA was based on the high level of sequence conservation (i.e., homology) within this multigene family (39). By using primers derived from hPMCA1b, the human teratoma PMCA (32), we were able to amplify a distinct region of the poly(A)$^+$-containing RNA from chicken intestine by PCR following reverse transcription. The location of these primer sequences within hPMCA1b mRNA delimits a 644-bp region, which coincides with the size of the amplified chicken sequence (Fig. 1).

Characterization of the DNAs. To compare and characterize the PCR product (644 bp) and cloned 550-bp DNAs, each was purified, nick-translated, and used as a probe for hybridization analysis of chicken intestinal mRNA. The results, shown in Fig. 2, indicate that the PCR product (644 bp) and the DNA fragment (550 bp) recognize identical mRNA species and that vitamin D administration to vitamin D-deficient chickens results in a substantial increase in these mRNA species.

Further characterization of these DNAs was accomplished by complete nucleotide sequencing of the cloned DNA (550 bp) and the purified PCR product. The PCR product gave nucleotide sequence data for the 3' terminal region (*Eco*RI fragment) of the transcript that was deleted during subcloning. The deduced partial amino acid sequence of the chicken intestinal PMCA (cPMCA) was aligned with the comparable region of the human teratoma pump (Fig. 3), indicating that the DNA encodes the homolog of a splicing variant of the human calcium pump isoform 1b (39). Excluding the primer

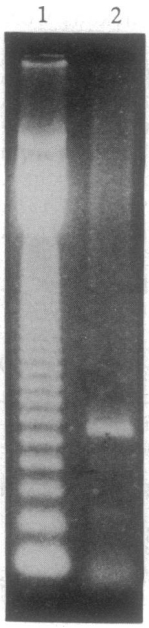

FIG. 1. Electrophoretic separation of PCR products derived from chicken intestinal poly(A)$^+$ RNA. Poly(A)$^+$ RNA (0.5 μg) of chicken intestine was subjected to a reverse transcription step followed by PCR in the presence of two synthetic probes based on the 3' coding region of the human teratoma PMCA mRNA (37). The PCR products (lane 2) and the 123-bp nucleotide ladder (lane 1) were electrophoretically separated on a 1% agarose gel, stained with ethidium bromide, and visualized by ultraviolet light. The band at about 640 bp (lane 2) was eluted from comparable gels for DNA cloning.

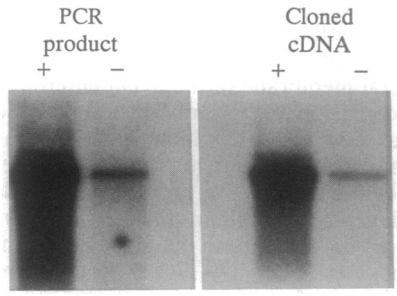

FIG. 2. Northern blot analysis of chicken intestinal poly(A)$^+$ RNA probed with the purified 644-bp PCR product and the 550-bp cloned cDNA. Denatured intestinal poly(A)$^+$ RNA (2.5 μg) samples from vitamin D-deficient (−) and vitamin D-repleted (+) chickens were separated electrophoretically on 1.2% agarose/formaldehyde gels and transferred to nitrocellulose membranes. The transferred RNAs were probed with ^{32}P-labeled purified PCR product (644 bp) or labeled, cloned cDNA (550 bp). ^{32}P-labeling was by nick-translation in the presence of [^{32}P]dCTP.

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                 **********************************************
cPMCA    AIFCTIVLGTFVVQIIIVQFGGKPFSCSKLLIEQWLWSVFLGMGTLLWGQ  -50
hPMCA1b  ----------------------------E-S-------I-----------  -1056
         **************************************************
cPMCA    LISTIPTSHLKFLKEAGHGTQKEEIPEEELAEDVEEIDHAERELRRGQIL  -100
hPMCA1b  --------R-------------------------------------------  -1106
         **************************************************
cPMCA    WFRGLNRIQTQIRVVNAFRSSLYEGLEKPETRSSIHNFMTHPEFRIEDSE  -150
hPMCA1b  ----------------------------S-------------------  -1156
         **********************************
cPMCA    PHIPLIDDTDAEDDAPTKRNSTPPPSPNKNNNAVDSGIHLTTDMNKSATS  -200
hPMCA1b  --------------------S-------------------IE-------  -1206

cPMCA    SSPGSPLHSLETSL   -214
hPMCA1b  --------------   -1220
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FIG. 3. Deduced amino acid sequence of the purified 644-bp PCR product and the 550-bp cloned DNA. Nucleotide sequencing of the 644-bp PCR product and the 550-bp cloned DNA of the chicken intestinal PMCA, designated cPMCA, was by the dideoxynucleotide chain termination method (39), and the deduced amino acid sequence was determined by the use of the PCGENE software (IntelliGenetics). The solid lines above residues 1–7 and 209–214 indicate the deduced amino acid sequences of the two 20-mer primers. The deduced amino acid sequence of the cloned DNA is designated by stars. The sequence of the human teratoma PMCA, designated hPMCA1b by Strehler (39), is that of Verma *et al.* (32). Only the residues of hPMCA1b different from those of cPMCA are indicated.

sequences (primers 11 and 12, residues 1–7 and 209–214, respectively), the sequence homology between the deduced amino acid sequence of cPMCA (residues 8–208) and hPMCA1b is 96%. Homologies with the deduced amino acid sequences of hPMCA1a, hPMCA2b, hPMCA4b, and rat PMCA3b (39, 40) were 72%, 81%, 59%, and 73%, respectively. This suggests, on the basis of the limited sequence information, that the chicken intestinal pump is more closely related to the hPMCA1b isoform than to the other isoforms examined.

By Northern blot analysis of total RNA of duodenal mucosa, the DNA probes recognized at least three RNA species of the PMCA, with mRNA transcript sizes of about 4.5, 5.4, and 6.6 kb (Fig. 4). In comparison, Greeb and Shull (40) and Zalinski *et al.* (29) identified two major mRNA transcripts of the rat intestinal Ca^{2+} pump at 5.5–6.0 and 7.6–7.8 kb and a minor band at 4.6–4.8 kb.

Northern Blot Analysis of cPMCA mRNA. Northern analysis of total RNA from mucosa of the duodenum, jejunum, ileum, and colon showed that vitamin D repletion of deficient chickens increased the amount of the Ca^{2+} pump mRNA in each of these segments (Fig. 5).

The temporal response of the Ca^{2+} pump mRNA to the intravenous injection of a single dose of $1,25(OH)_2D_3$ into vitamin D-deficient chickens was assessed, and the Northern blot data of one representative experiment are shown in Fig. 6. The results from several experiments are summarized graphically in Fig. 7. Significant differences ($P < 0.05$) occurred at 8, 16, and 24 hr with respect to the zero time control value. The mean response at 2 hr was greater than the zero time value, but the difference was only significant at $P = 0.076$. The value at 48 hr was not different from the control value.

We previously reported that the number of intestinal Ca^{2+} pump units is also increased in the adapted animal (11). The present data now demonstrate that the amount of the Ca^{2+} pump mRNA is also greater in the animals adapted to a low calcium or a low phosphorus diet as compared to the normally fed control group (Fig. 8). Densitometric analysis of Northern blots of four separate experiments showed that the ingestion of the low Ca and low P diet increased the density of the mRNA bands by factors of about 2.3 and 2.6, respectively, as compared to that of the normal diet.

DISCUSSION

The repletion of vitamin D-deficient chickens with vitamin D was previously shown, by immunoblotting methodology, to increase the number of PMCA units of chicken intestine (11). It was also reported that adaptation of vitamin D-repleted chickens to a calcium- or phosphorus-deficient diet likewise stimulated the net synthesis of this Ca^{2+} pump (11). The present study was undertaken to determine whether the increased levels of Ca^{2+} pump units were due to an increase in Ca^{2+} pump gene expression. To accomplish this objective, a DNA probe against the chicken intestinal PMCA was produced. The strategy to obtain a useful DNA probe was based on the assumption of a close homology of the nucleotide sequence of the known teratoma PMCA (32) with that of the chicken intestinal pump. The initial experiments showed that the two 20-mer primers derived from the 3′ coding region of the teratoma sequence yielded a PCR product of about the predicted size (i.e., 644 bp). A 550-bp

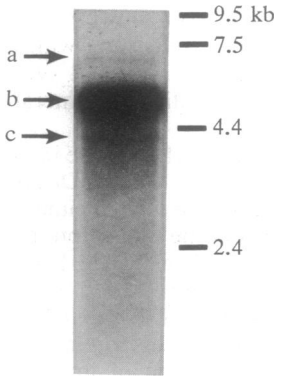

FIG. 4. Major isoforms of the cPMCA mRNAs determined by Northern blot analysis. Chicken intestinal poly(A)$^+$ RNA and a ^{32}P-labeled RNA ladder were electrophoretically separated on an agarose gel and transferred to GeneScreen*Plus* membranes. The poly(A)$^+$ RNA was probed with the cloned DNA. By reference to the standard RNA ladder, the estimated sizes of cPMCA, labeled a, b, and c, were 6.6, 5.4, and 4.5 kb, respectively.

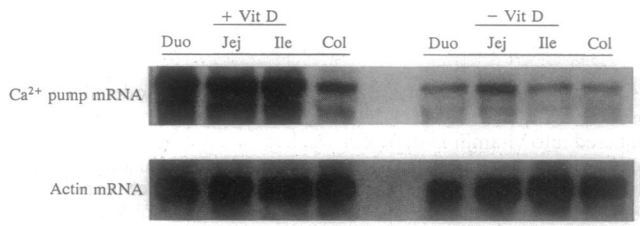

FIG. 5. Effect of vitamin D on cPMCA. Total RNA (10 μg) from intestinal segments of vitamin D-deficient (− Vit D) or vitamin D-repleted (+ Vit D) chickens was analyzed for the PMCA mRNA by Northern blot analysis, using the cloned DNA as the probe. Actin mRNA was also probed to indicate relative uniformity of sample loading. Note that each of the Ca^{2+} pump mRNAs was increased by vitamin D in each of the intestinal segments. Duo, duodenum; Jej, jejunum; Ile, ileum; Col, colon.

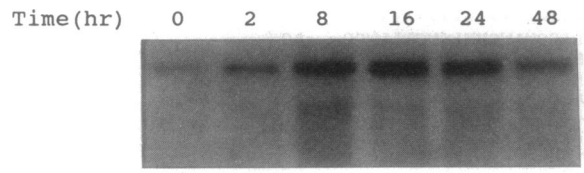

FIG. 6. Northern blot analysis of the intestinal PMCA mRNA as a function of time after $1,25(OH)_2D_3$ repletion of vitamin D-deficient chickens. $1,25(OH)_2D_3$ (0.5 μg) was injected intravenously into vitamin D-deficient chickens at zero time. At various times thereafter, total duodenal RNA (10 μg) was isolated and analyzed for the Ca^{2+} pump mRNA by Northern blot analysis, using the labeled, cloned DNA as the probe.

fragment resulting from the *Eco*RI digestion of the PCR product was successfully cloned into a plasmid vector. The deduced amino acid sequence of the PCR product and the cloned fragment both showed 96% identity with the region of the teratoma sequence defined by the synthesized primers. The above information establishes the usefulness of the cloned fragment as a DNA probe for the chicken PMCA mRNA.

The administration of vitamin D to vitamin D-deficient chickens increased the PMCA mRNA of each segment of the small intestine and the colon, and the administration of $1,25(OH)_2D_3$ to vitamin D-deficient animals yielded a time-dependent response of the Ca^{2+} pump mRNA. For comparison, previously reported data (41) on the temporal pattern of the $1,25(OH)_2D_3$ stimulation of the ATP-dependent uptake of Ca^{2+} by isolated BLMVs are also shown in Fig. 7. There is the expected time lag between the $1,25(OH)_2D_3$-dependent increase and decrease of the pump mRNA and the $1,25(OH)_2D_3$-enhanced uptake of Ca^{2+} by BLMVs. However, the precise quantitative time relationships between these two parameters require further study. Also requiring further investigation is the determination of the exact relationship between the amount of the Ca^{2+} pump protein and Ca^{2+} pump activity.

Two recent reports based on indirect biochemical evidence suggested that $1,25(OH)_2D_3$ does not effect an enhanced

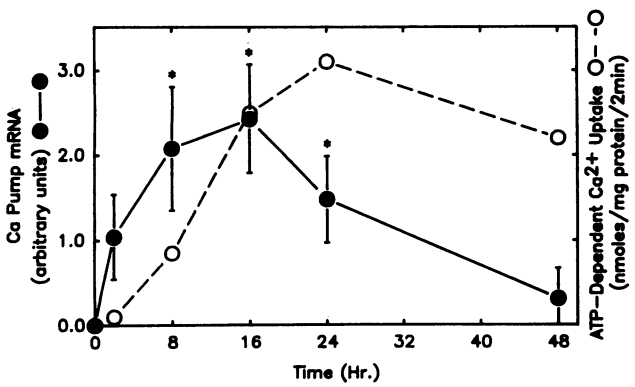

FIG. 7. Temporal pattern of response of the duodenal PMCA mRNA to $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ (0.5 μg) was intravenously injected into vitamin D-deficient chickens at zero time. The number of replicate experiments for the time periods 0, 2, 8, 16, and 24 hr was 8 and that for 48 hr was 6. Each time point in each experiment represents the Northern blot analysis of total duodenal RNA (10 μg) from a pool of three animals. The probe was the cloned DNA. Values at 8, 16, and 24 hr by densitometric analysis were greater than the zero time control value at $P < 0.05$. The P value for the 2-hr time point was 0.076; at 48 hr, $P > 0.5$. For comparison, previously published data (41) on the ATP-dependent uptake of ^{47}Ca by isolated basolateral membrane vesicles (BLMVs) from a similar protocol are shown. The values, for comparative purposes, are given as [(density at time t)/(density at zero time)] −1. ●, Ca^{2+} pump mRNA; ○, BLMV Ca^{2+} uptake.

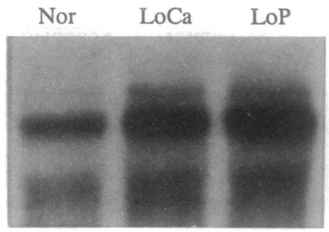

FIG. 8. Effect of dietary calcium or phosphorus deficiencies on the duodenal PMCA mRNA. Normal chickens at age 3 weeks were fed the deficient diets for 10 days before total duodenal RNA (10 μg) was analyzed for Ca^{2+} pump mRNA by Northern blot analysis with the cloned DNA. The normal (Nor), low calcium (LoCa), and low phosphorus (LoP) diets contained 1.2% calcium and 0.8% phosphorus, 0.05% calcium and 0.8% phosphorus, and 1.2% calcium and 0.3% phosphorus, respectively. All diets were adequate in vitamin D (1200 units/kg of diet). The illustrated Northern blot is representative of four experiments.

synthesis of the intestinal plasma membrane Ca^{2+} pump. In one study, Takito *et al.* (42) examined phosphorylated intermediates of the Ca^{2+} pump by autoradiographic analysis. In the other report, Timmermans *et al.* (43) examined the effect of $1,25(OH)_2D_3$ on the number of intestinal PMCA, employing a ^{125}I-labeled calmodulin binding assay by a gel overlay procedure.

The reasons for the discrepancies between the findings from the above biochemical studies (42, 43) and our studies are unknown at present. Although the anti-Ca^{2+} pump antibody (5F10) used in the previous immunological study (11) was produced against the human red cell membrane Ca^{2+} pump, this antibody cross-reacts with the PMCA proteins of rat intestine (44), rat and human kidney (45, 46), and human placenta (47); and, in our previous study (11), the 5F10 antibody immunohistochemically cross-reacted almost exclusively with a protein resident to the chicken intestinal basolateral membrane. Further, the molecular weights of the immunoreactive proteins of *purified* chicken intestinal basolateral membranes were nearly the same as those of the human erythrocyte Ca^{2+} pump protein. In the present study, the DNA was sufficiently well characterized to serve as a valid probe of the Ca^{2+} pump mRNA. Thus, our evidence (ref. 11; this report) points to the stimulation by $1,25(OH)_2D_3$ of the synthesis of the Ca^{2+} pump protein and, as also observed by Zalinski *et al.* (29), the $1,25(OH)_2D_3$-dependent enhancement of Ca^{2+} pump gene expression.††

Adaptation of chickens to calcium-deficient or phosphorus-deficient diets was also shown to increase Ca^{2+} pump gene expression. This is consistent with our previous demonstration of an increase in the net synthesis of the Ca^{2+} pump protein in the intestine of mineral-deficient animals (11).

Yet to be determined is whether the increase in the amount of Ca^{2+} pump mRNA in the vitamin D-repleted chickens and the animals adapted to the low calcium or low phosphorus diets is due to events at the transcriptional or posttranscriptional level or both. Of relevance here are the previous reports proposing that the control of the gene expression of intestinal (49, 50) and renal (51) calbindin-D by $1,25(OH)_2D_3$ occurs at both the gene transcriptional and posttranscriptional levels. It will also be of importance to determine whether the plasma membrane Ca^{2+} pump gene contains a vitamin D response element similar to that of the gene promoter of other vitamin D-transcribed proteins (52, 53).

††At a recent meeting, Armbrecht *et al.* (48) confirmed the enhancement of rat intestinal PMCA gene expression by $1,25(OH)_2D_3$. Also reported was an age-dependent decline in the Ca^{2+} pump mRNA.

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