TRIIODOTHYRONINE (T$_3$) ENHANCES THE STIMULATORY EFFECT OF 1, 25-DIHYDROXYVITAMIN D$_3$ ON CALBINDIN-D$_{28K}$ mRNA EXPRESSION IN THE KIDNEY AND INTESTINE BUT NOT IN CEREBELLUM OF THE CHICK

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Summary

The present study was conducted to investigate the role of thyroid hormones in the regulation of gene expression of calbindin-D$_{28k}$ (CaBP-D28K) in the chicken. By employing slot blot and RIA analyses, levels of CaBP-D28K mRNA and CaBP-D28K protein in the intestine, kidney, cerebellum and liver were measured 6 and 12 h after i.m. injection of 1, 25-dihydroxyvitamin D$_3$ [1, 25 (OH)$_2$D$_3$; 250 ng/chick] and 3, 5, 3'-triiodothyronine (T$_3$; 500 ng/chick) in one-day-old chicks. The abundant messages of CaBP-D28K mRNA were detected in the intestine, kidney and cerebellum while there was little message in the liver. After 1, 25 (OH)$_2$D$_3$ treatment (6 + 12 hours), levels of CaBP-D28K mRNA increased in the intestine, but there was no change in the mRNA levels in the kidney and cerebellum. Although T$_3$ alone had no effect on CaBP-D28K mRNA levels, simultaneous administration of T$_3$ enhanced the 1, 25 (OH)$_2$D$_3$ effect on levels of CaBP-D28K mRNA in the intestine both 6 and 12 h post-treatment, and in the kidney 12 h post-treatment. At a protein level, co-treatment with 1, 25 (OH)$_2$D$_3$ and T$_3$ elicited a significant increase in CaBP-D28K expression in the intestine 12 h post-treatment, as compared to treatment with only 1, 25 (OH)$_2$D$_3$, whereas no differences were observed in the CaBP-D28K protein levels in the kidney and cerebellum. These results suggest that thyroid hormones may play a synergistic role with 1, 25 (OH)$_2$D$_3$ for CaBP-D28K gene expression in the intestine and kidney in chicks.
(**Key Words** : Calbindin-D$_{28K}$, Triiodothyronine (T$_3$), 1, 25-Dihydroxycholecalciferol, Gene Expression, Chick)

Introduction

In has been established that 1, 25 (OH)$_2$D$_3$ plays an important role in the regulation of CaBP-D28K gene expression (for review, Gross and Kumar, 1990; Walters, 1992). However, some recent investigations have shown that other steroid hormones might be involved in the regulation of CaBP-D28K gene expression. Hall et al. (1987) demonstrated *in vivo* that dexamethasone, a synthetic glucocorticoid, can elevate the content of CaBP-D28K mRNA in a vitamin D-deficient duodenum. Corradino and Fulmer (1991) reported that the preexposure of the duodenum to low concentrations of dexamethasone *in vitro* rapidly enhanced 1, 25 (OH)$_2$D$_3$-dependent CaBP-D28K mRNA biosynthesis. Moreover, Corradino (1993) showed synergistic stimulatory effect of estradiol and 1, 25 (OH)$_2$D$_3$ on CaBP-D28K production in the egg shell gland of the chicken.

Cross and Peterlik (1988, 1991) and Cross et al. (1990) have studied the influence of 1, 25 (OH)$_2$D$_3$ and 3, 5, 3'-triiodothyronine (T$_3$) on intestinal calcium absorption in organ cultures of embryonic chick intestine. They reported that 1, 25 (OH)$_2$D$_3$ increases calcium transport, and that T$_3$ acts synergistically with 1, 25 (OH)$_2$D$_3$. It is possible that the T$_3$ action is mediated via CaBP-D28K biosynthesis. Although the role of 1, 25 (OH)$_2$D$_3$ on the CaBP-D28K mRNA concentration in the chick intestine and kidney has been extensively studied (Clemens et al., 1988; Enomoto et al., 1992), neither the effect of T$_3$ alone

nor the synergistic effects of T_3 and 1, 25 $(OH)_2D_3$ have been demonstrated. Accordingly, this study was conducted to examine the possibility of a genomic action of T_3 in the regulation of CaBP-D28K production in the chick intestine, kidney and cerebellum. Using Northern and slot blot analyses, the present paper reports that T_3 can enhance the stimulatory effect of 1, 25 $(OH)_2D_3$ on gene expression of CaBP-D28K in the intestine as well as in the kidney.

Materials and Methods

Animals, treatment and collection of tissue samples

The studies were carried out on one-day-old male chicks of White Leghorn breed. During the time course of the experiment, the birds were provided with water, but without food to eliminate nutritional factor. Forty chicks were randomly divided into four experimental groups. the birds received i.m. injections of 1, 25 $(OH)_2D_3$ and T_3 shown in table 1.

TABLE 1. SCHEDULE OF THE EXPERIMENT

Group	N	Injection (i.m.) :	
		1, 25 $(OH)_2D_3$[a]	T_3[b]
Control	2×5	VH_1	VH_2
D_3	2×5	250 ng	VH_2
T_3	2×5	VH_1	500 ng
$D_3 + T_3$	2×5	250 ng	500 ng

[a] -dissolved in 100 μl of 50% ethanol : 50% saline;
[b] -dissolved in 100 μl 0.01 N NaOH in saline;
VH_1-vehicle 1 (as for 1, 25 $(OH)_2D_3$);
VH_2-vehicle 2 (as for T_3).

Twenty chicks (five of each group; n = 5) were decapitated at 6 hours, and the twenty other chicks at 12 hours after treatment, respectively. Doses of T_3 (500 ng/chick) and 1, 25 $(OH)_2D_3$ (250 ng/chick) were chosen according to Abdel-Fattah et al. (1990), and Clemens et al. (1988). After decapitation, tissues of the intestine (duodenum), kidney, cerebellum, and liver of each bird were collected, immediately placed in sterile microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at $-80°C$ until RIA and RNA extraction.

RNA extraction

The tissue of each bird was homogenized in a solution containing 0.5% Nonidet-P40 (Sigma), 10 mM Tris-HCl and 1 mM ethylenediamino tetraacetate (EDTA). Half of the homogenate of each tissue was taken into new Eppendorf tubes and stored at $-20°C$ for radioimmunoassay (RIA) of CaBP-D28K and protein estimation. Total RNA was extracted from the remaining aliquote according to the procedures described by Chomczynski and Sacchi (1987). The RNA samples prepared from each bird were then stored at $-30°C$ in 100% ethyl alcohol (1:2.5 v/v) until electrophoresis and Northern and slot blot analyses.

Preparation of [^{32}P] labelled Calbindin D_{28k} cDNA probe

A 767 bp fragment of chicken CaBP-D28K cDNA, which was subcloned into the EcoRI site of pUC18 plasmid (Mangelsdorf et al., 1987), was used in this study as a probe (kindly provided by Dr. Barry S. Komm). The cDNA was radiolabelled with deoxycytide 5'-triphosphate ([α^{32}P] dCTP 3000 Ci/mmol, New England Nuclear, Boston, MA) using a random primed labelling kit (Boehringer-Mannheim, Mannheim, Germany) to a specific activtiy $1-5 \times 10^8$ cpm/μg and used for both Northern and slot blot hybridization. The radioactive probe was separated from unincorporatd [^{32}P]-dCTP by gel filtration on a Sephadex G-50 column. Chicken β-actin cDNA was prepared similarly for hybridization analyses.

Northern blot analysis

Total RNA (20 μg/lane) was denatured by incubation with formaldehyde at 65°C for 15 min. The RNA was fractionated on 1.2% formaldehyde agarose gel and then transferred to Hybond N^+ membranes (Amersham Inc., UK) in $10 \times$ SSC (1.5 M sodium chloride, 150 mM sodium citrate, pH 7.0) using standard techniques (Maniatis et al., 1989). Membranes were rinsed in 0.05 M NaOH for 5 min, washed in $2 \times$ SSC solution and stored at 4°C until hybridization. After prehybridization, membranes were used for a standard hybridization procedure for about 20 h at 42°C. The membranes were washed twice in $1 \times$ SSC (0.1% SDS) for 15 min at room temperature, then at 42°C for 20 min, followed by a final wash, i.e., in $0.1 \times$ SSC (0.1% SDS), first at 50°C and then 60°C for 15 min. The washed membranes were exposed to X-ray film at $-70°C$ for 20 h. Autoradiograms were stripped and rehybridized to ^{32}P-β-actin cDNA to normalize loading of the gel and check the quality of the RNA (8 h exposure).

RNA slot blot hybridization assay

For slot blot analysis, total RNA (0.625, 1.25, 2.5 and 5 μg) was denatured (65°C, 15 min) in $10 \times$ SSC

containing 20% formaldehyde, and was transferred onto Hybond N⁺ membrane (Amersham Inc., UK) using a slot blot microfiltration apparatus (BioRad, Inc., USA). Procedures for immobilization of RNA and for the hybridization were identical to those for Northern blot hybridization. The intensity of the ^{32}P-DNA-RNA hybridization was quantified by scanning the radioactivity of the membranes using the AMBIS Radioanalytic Image System (AMBS Radioanalytic Image System, Inc., San Diego, CA, USA). Signals were normalized to the signal of β-actin mRNA after rehybridization to chicken ^{32}P-β-actin cDNA.

Radioimmunoassay and analysis of results

The concentrations of CaBP-D28K in tissues were determined in duplicate according to the method described by Tohmon et al. (1988). The chicken CaBP-28K standard and antisera were received as a kind gift from Dr. Tohmon (Kobe University). The intraassay coefficient of variation was 6.5% concentration of CaBP-D28K was then expressed in μg/mg protein. Protein concentrations in samples were measured by the method of Lowry et al. (1951). All data were analysed using the two-way analysis of variance followed by multiple range Duncan's test (Duncan, 1955).

Results

Characterization of CaBP-D28K mRNA in the intestine, kidney, cerebellum and liver of one-day-old chicks

Examples of autoradiographs of Northern blots of the CaBP-D28K mRNA of intestine, kidney, cerebellum and liver of one-day-old chicks are shown in Figs. 1A, 2A, 3A, 4A, respectively. When abundant messages were detected in the examined tissues, three species of mRNA were identified (a major one, approximately 2.0 kb and two closely migratory minor species of 3.0 kb) while there was little message in the liver. 1, 25 (OH)₂D₃ increased CaBP-D28K mRNA levels, in the intestine and kidney (figure 1A and figure 2A, respectively), but it did not change CaBP-D28K mRNA levels in the cerebellum and liver (figure 3A and figure 4A, respectively). T₃ with 1, 25 (OH)₂D₃ markedly enhanced the appearance of CaBP-D28K message in the intestine and kidney (figure 1A and 2A, respectively). Since the amounts of loading of total RNA into the gel were not necessarily equal (e.i., figure 1B), data of slot blot analyses for CaBP-D28K mRNA were expressed as the ratio to the levels of β-actin mRNA. For example, CaBP-D28K mRNA levels in the intestine of a T₃-treated chick increased when compared to

those of control (VH), but β-actin mRNA levels also was elevated. Hence, the ratio was unchanged in these birds.

The effect of 1, 25 (OH)₂D₃ and T₃ on the CaBP-D28K mRNA levels and CaBP-D28K protein concentration in specific tissues

Intestine

1, 25 (OH)₂D₃ caused a significant increase in mRNA levels of CaBP-D28K in the intestine 6 and 12 h after the injection by 5.6- and 5.2-fold, respectively, when compared to those of the control group (figure 5A, p < 0.01). T₃, however, did not affect the CaBP-D28K mRNA levels. On the other hand, when T₃ was injected together with 1, 25 (OH)₂D₃, CaBP-D28K mRNA levels increased more than those with 1, 25 (OH)₂D₃ injection alone. The increases were 1.55- and 1.41-fold higher at 6 and 12 h, respectively (figure 5A, p < 0.01).
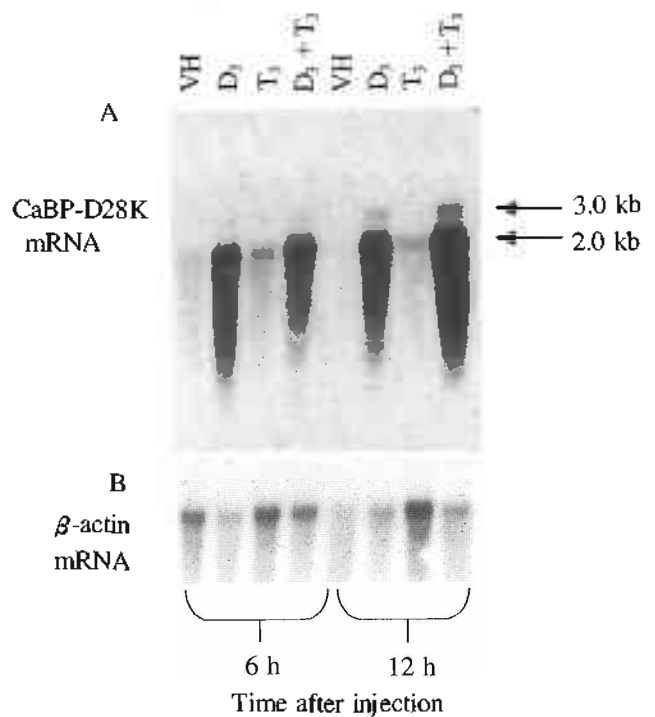

Figure 1. Northern blot analysis of CaBP-D28K mRNA in the intestine of one-day-old chicks. (A) Total RNA (20 μg/lane) collected from the intestine of vehicle (VH), 1, 25 (OH)₂D₃ (D₃), 3, 5, 3' -triiodothyronine (T₃) and 1, 25 (OH)₂D₃ + T₃ (D₃ + T₃) injected chicks was used for Northern blot analysis for CaBP-D28K mRNA. Numbers on the right indicate the position of mRNA species. (B) Autoradiograph for β-actin mRNA.
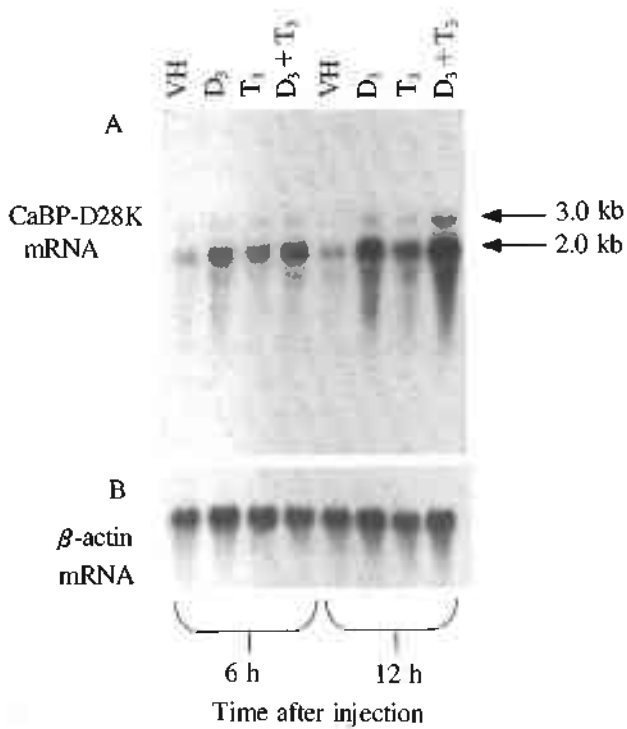
Figure 2. Northern blot analysis of CaBP-D28K mRNA in the kidney of one-day-old chicks. (A) CaBP-D28K mRNA. (B) β-actin mRNA (for other details see Figure 1).
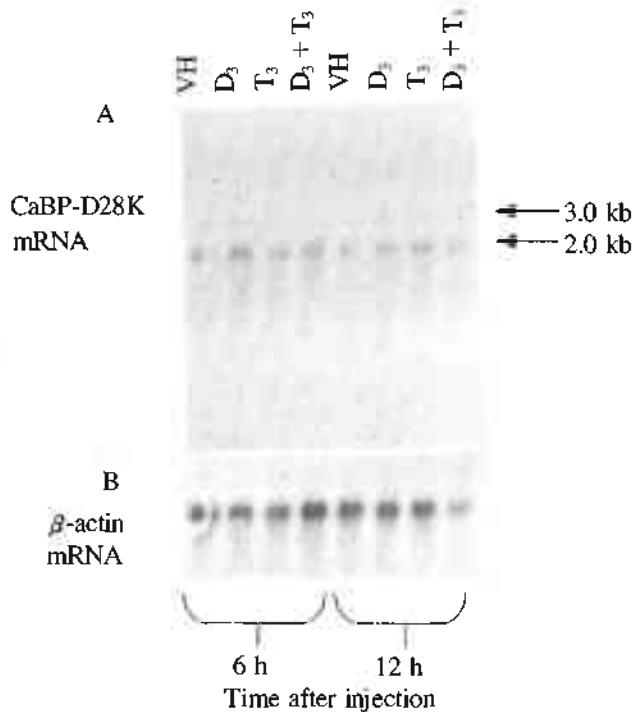

Figure 3. Northern blot analysis of CaBP-D28K mRNA in the cerebellum of one-day-old chicks. (A) CaBP-D28K mRNA, (B) β-actin mRNA (for other details see Figure 1).
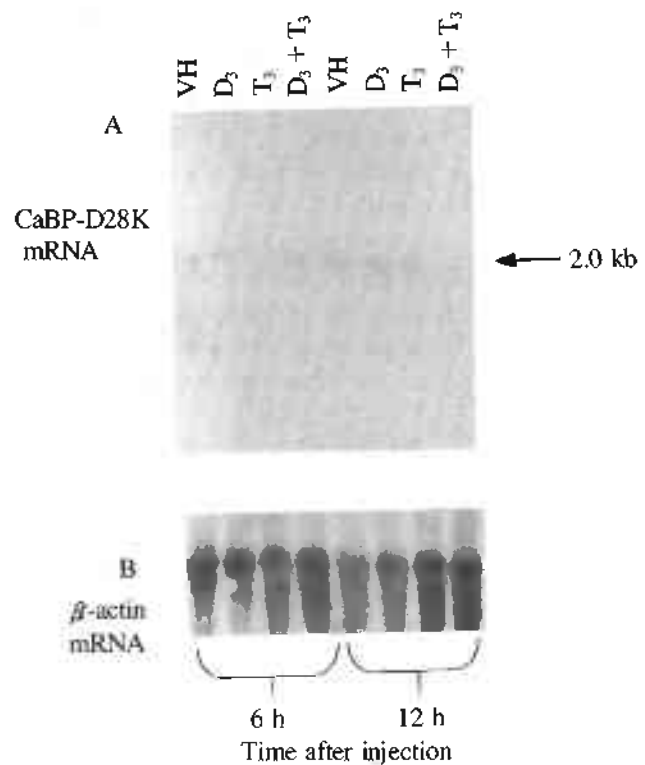

Figure. 4. Northern blot analysis of CaBP-D28K mRNA in the liver of one-day-old chicks. (A) CaBP-D28K mRNA (40-hour exposure), (B) β-actin mRNA (for other details see Figure 1).

There was no change in CaBP-D28K protein concentration 6 h after injections of 1, 25 $(OH)_2D_3$ or T_3, but injections of both 1, 25 $(OH)_2D_3$ and T_3 produced significant increase in CaBP-D28K concentrations 12 h after injection (figure 5B, $p < 0.05$).

Kidney

Neither 1, 25 $(OH)_2D_3$ nor T_3 alone induced any change in mRNA levels of CaBP-D28K 6 h after the injection. However, when these agents were injected simultaneously, the mRNA levels increased significantly 12 h after the injection (figure 6A, $p < 0.05$).

The kidney concentrations of CaBP-D28K protein did not increase 6 and 12 h after the injection (figure 6B).

Cerebellum

Injection of 1, 25 $(OH)_2D_3$ and T_3, either alone or in combination, did not produce changes in mRNA levels of CaBP-D28K either 6 or 12 h after the treatment (figure 7A).

No difference was observed in CaBP-D28K protein concentrations 6 and 12 h after any treatment (figure 7B).
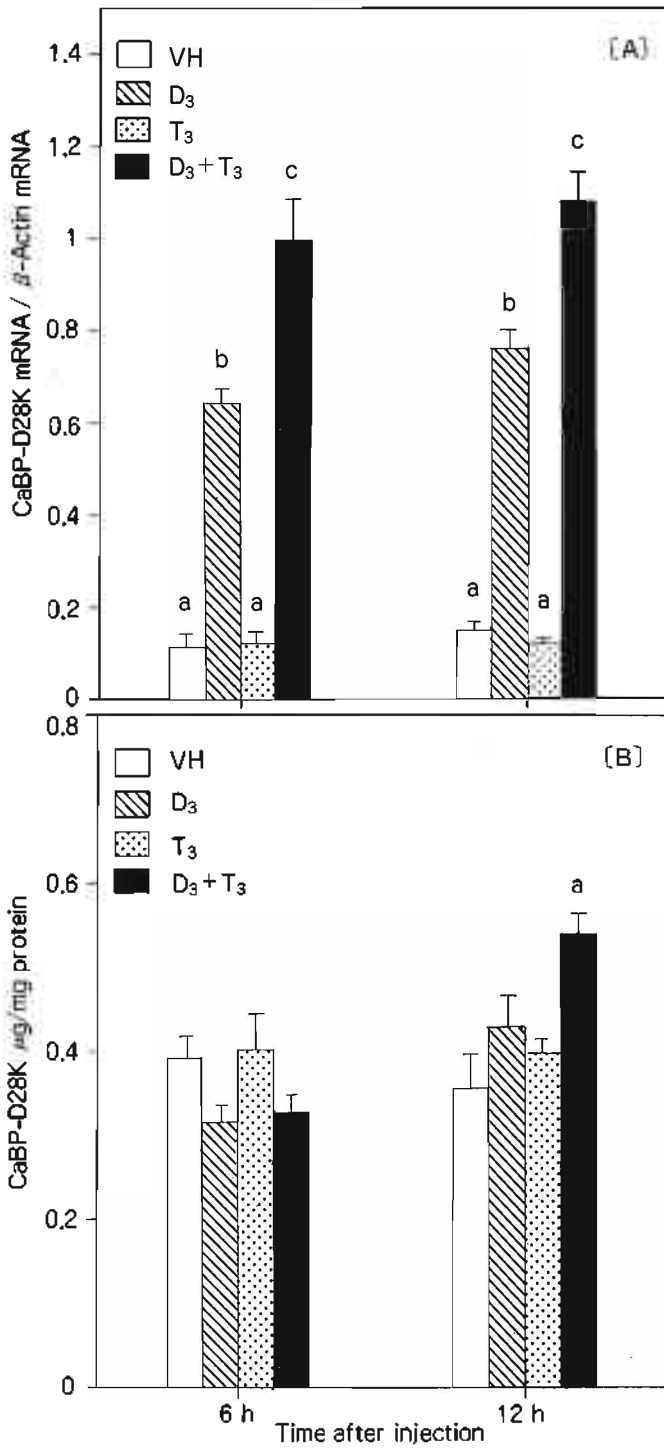
Figure 5. Levels of CaBP-D28K mRNA (A) and CaBP-D28K protein (B) in the intestine of one-day old chicks, 6 and 12 h after i.m. treatment with VH, D₃, T₃ and D₃ + T₃. Panel A: values with different superscripts differ significantly (p < 0.05). Panel B: a − p < 0.05, when compared with other groups (mean ± SEM; n = 5).
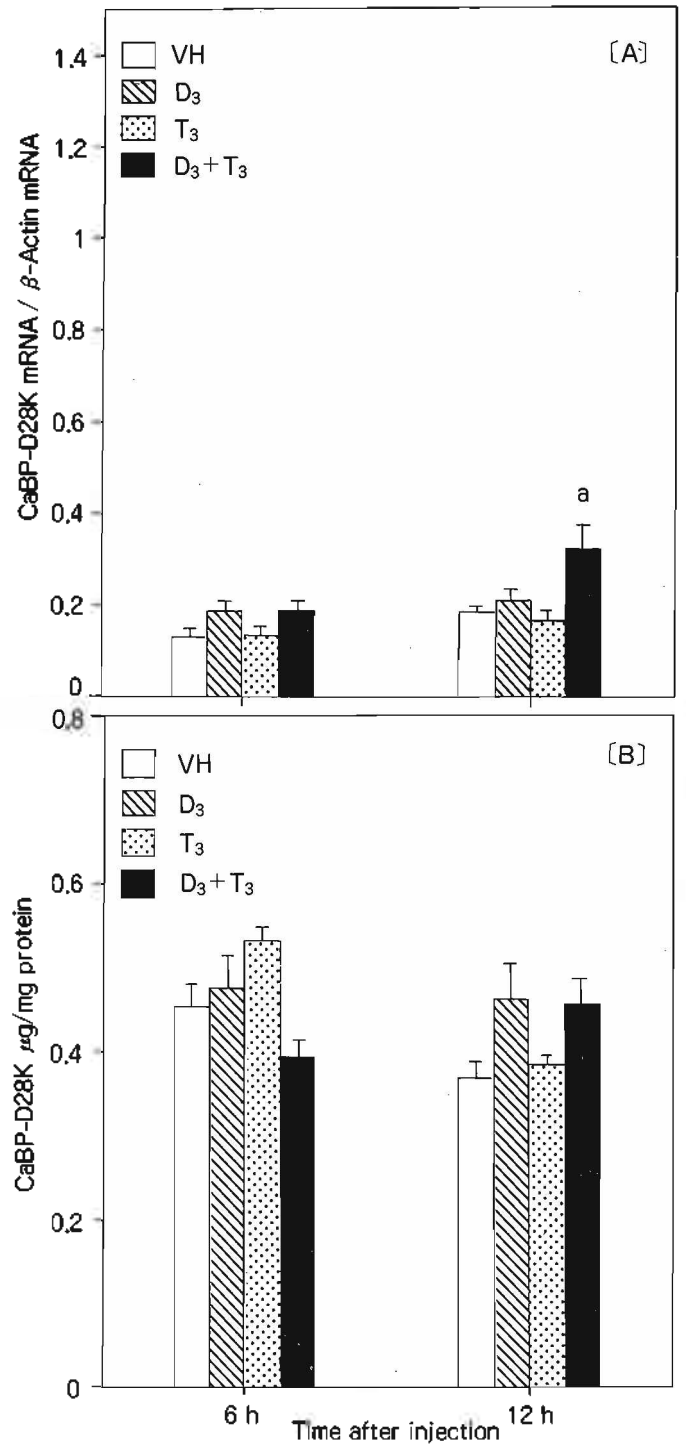
Figure 6. Levels of CaBP-D28K mRNA (A) and CaBP-D28K (B) in the kidney of one-day old chicks, 6 and 12 h after i.m. treatment with VH, D₃, T₃ and D₃ + T₃. Panel A: a − p < 0.05, when compared with other groups (mean ± SEM; n = 5).
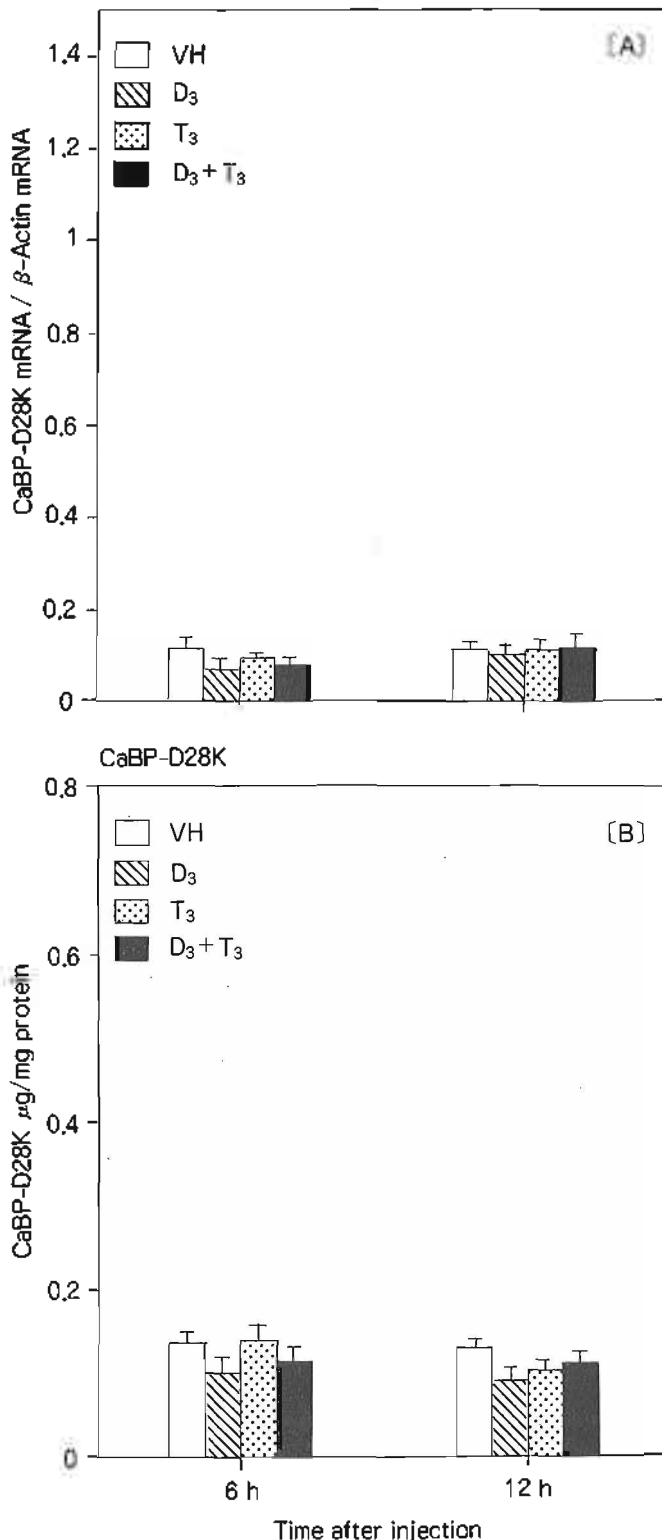
Figure 7. Levels of CaBP-D28K mRNA (A) and CaBP-D28K protein (B) in the cerebellum of one-day old chicks, 6 and 12 h after i.m. treatment with VH, D_3, T_3 and $D_3 + T_3$ (mean ± SEM; n = 5).

Discussion

The present study clearly demonstrated that 1, 25 $(OH)_2D_3$ increased the intestinal CaBP-D28K mRNA in the chicken. The intestinal response to 1, 25 $(OH)_2D_3$ was more prominent than that of the kidney. These results are consistent with the report of Clemens et al. (1988). However, we did not demonstrate significant increases in levels of CaBP-D28K protein 6 and 12 h after 1, 25 $(OH)_2D_3$ injection possibly because of limited period of the experiment. Theofan et al. (1986) and Norman et al. (1992) demonstrated a stimulatory effect of 1, 25 $(OH)_2D_3$ on both CaBP-D28K mRNA and CaBP-D28K protein production: CaBP-D28K mRNA begins to accumulate by 3-5 h but does not peak until 12 h following 1, 25 $(OH)_2D_3$ administration and the levels of CaBP-D28K protein start to increase by 5-8 h and do not peak until 48 h after the treatment.

In the present study, T_3 alone did not affect CaBP-D28K mRNA levels in the intestine and kidney, but T_3 augmented the effect of 1, 25 $(OH)_2D_3$. The similar synergistic effect of T_3 and 1, 25 $(OH)_2D_3$ on CaBP-D28K protein concentration was reported previously using the 19-day old chick embryo intestine *in vitro* by Corradino (1988). Although the T_3 receptor is present in the intestine and kidney (Forrest et al., 1990), T_3 may not be directly involved in gene expression of CaBP-D28K by itself, but it may require 1, 25 $(OH)_2D_3$. T_3 may increase the binding affinity of 1, 25 $(OH)_2D_3$ to its receptor or may stimulate 1, 25 $(OH)_2D_3$ receptor synthesis. Recent studies indicate that the thyroid hormone receptors, retinoic acid receptors and 1, 25 $(OH)_2D_3$ receptor are highly homologous in amino acid sequences (Minghetti and Norman, 1988; Beato, 1989). As suggested by Carlberg et al. (1993) and showed by Schrader et al. (1994), formation of the heterodimers of vitamin D_3 receptor and T_3 receptor may possibly explain for augmentation effect of T_3 on 1, 25 $(OH)_2D_3$-induced gene expression of CaBP-D28K in the chick intestine and kidney.

As reported previously (Clemens et al., 1988; Sechman et al., 1994), the cerebellar mRNA of CaBP-D28K was detected in this study. However, unlike the regulation of CaBP-D28K gene expression in the intestine and kidney, in the cerebellum 1, 25 $(OH)_2D_3$ may not be a key factor. Clemens et al. (1988) also showed that the cerebellar levels of CaBP-D28K mRNA were unchanged 24 h after 1, 25 $(OH)_2D_3$ injection in the vitamin D-deficient chicken. Although binding activity of the intestine and kidney to 1, 25 $(OH)_2D_3$ has been reported (Nakada and DeLuca, 1985; Theofan et al., 1986), no information is available concerning presence of 1, 25

(OH)$_2$D$_3$ receptor in the cerebellum. Recently, we have detected some message of vitamin D$_3$ receptor in the intestine and kidney, but not in the cerebellum of 1-day or 7-day old chicks (unpublished data).

In conclusion, this study using one-day-old chicks *in vivo* indicates that thyroid hormones may play a synergistic role with 1, 25 (OH)$_2$D$_3$ for CaBP-D28K gene expression in the intestine and kidney.

Acknowledgements

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