Immunoselection of cDNAs to Avian Intestinal Calcium Binding Protein 28K and a Novel Calmodulin-like Protein: Assessment of mRNA Regulation by the Vitamin D Hormone†

David J. Mangelsdorf,‡,⊥ Barry S. Komm,‡ Donald P. McDonnell,§ J. Wesley Pike,∥ and Mark R. Haussler*,‡

Department of Biochemistry, University of Arizona, Tucson, Arizona 85724, and Departments of Cell Biology and Pediatrics, Baylor College of Medicine, Houston, Texas 77030

Received April 28, 1987; Revised Manuscript Received July 31, 1987

ABSTRACT: Calcium's role in a variety of cellular processes has been well documented. The storage, distribution, and delivery of calcium are regulated by a family of binding proteins including troponin C, calmodulin, parvalbumin, and vitamin D dependent calcium binding protein (CaBP-28), all of which have evolved from a common ancestral gene. To evaluate vitamin D regulation of gene transcription, a CaBP-28 cDNA (767 base pairs) was isolated from a chicken intestine λgt11 library utilizing a polyvalent CaBP-28 antibody as a probe. Coincident with the identification of the CaBP-28 cDNA, a group of cDNAs also was isolated (with the anti-CaBP-28 antibody) that demonstrated 84% nucleotide homology and 99% deduced amino acid homology with chicken brain calmodulin (CaM). This new CaM-like cDNA was named neoCaM. There is little nucleotide homology between the CaBP-28 cDNA and neoCaM. The CaBP-28 cDNA hybridizes with three transcripts of 2000, 2900, and 3300 bases which are dramatically induced by 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], while the neoCaM cDNA recognizes three distinct (from CaBP-28) transcripts. Two of these mRNAs are 1400 and 1800 bases as described for brain CaM, but another larger 4000-base transcript is detected with neoCaM. Neither the CaM nor the neoCaM transcript reveals any modulation by 1,25$(OH)_2D_3$. Herein, we discuss the possible significance of not only the isolation of both cDNAs with a single antibody but also the relation of neoCaM to other well-characterized CaM cDNAs.

The biological role of calcium as a primary mediator of intracellular signaling is well-known. The study of these Ca^{2+}-mediated processes has generated a diversity of information which demonstrates the highly complex mechanism of action of calcium as a second messenger (Rasmussen, 1986). A key component to understanding part of this mechanism has been a group of calcium binding proteins which by virtue of their Ca^{2+} binding properties are capable of participating in calcium-dependent cellular events (Kilhoffer et al., 1983). These intracellular proteins bind Ca^{2+} with micromolar affinities within several discreet, evolutionarily related subdomains of each protein. Hence, it has been suggested that the family of proteins including troponin C, calmodulin, parvalbumin, and the vitamin D dependent calcium binding proteins have all evolved from a common, primordial ancestor gene coding for a polypeptide with one Ca^{2+} binding domain.

Our initial interest in this family of proteins has focused almost exclusively on the vitamin D dependent avian intestinal calcium binding protein (M_r 28 000) (CaBP-28)[1] and its use as a marker protein for studying the biochemical mechanism of action of the steroid hormone 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. CaBP-28 was first described by Wasserman and Taylor (1966) and has since been purified and sequenced (Fullmer & Wasserman, 1985). CaBP-28 is unique within the family of calcium binding proteins in that its transcription and synthesis in the intestine are hormonally regulated by 1,25$(OH)_2D_3$ (Theofan et al., 1986). This hormonal induction by 1,25$(OH)_2D_3$ is mediated through a high-affinity soluble receptor protein (Pike et al., 1987) in a manner analogous to other steroid hormone systems (Yamamoto, 1985). It is hypothesized that following steroid–hormone–receptor binding, this complex interacts with the promoter region of the CaBP-28 gene and regulates transcription of CaBP-28 mRNA. To completely elucidate the role 1,25$(OH)_2D_3$ plays in regulating the CaBP-28 gene, a reliable cDNA probe is required. Others (Hunziker et al., 1983) have synthesized and isolated cDNAs to the CaBP-28 transcript. More recently, Wilson et al. (1985) and Hunziker (1986) as well as our laboratories have characterized CaBP-28 cDNAs with sequences that correspond to the amino acid sequence of the protein (Fullmer & Wasserman, 1985).

Unlike the other groups who isolated CaBP cDNAs by differential hybridization techniques, we screened a λgt11 library with a polyvalent antibody raised against purified CaBP-28. Interestingly, not only did we identify CaBP-28 cDNA clones but also we identified a novel group of clones with a high degree of homology with calmodulin (CaM)—another member of the calcium binding protein family. In

† This work was supported in part by National Institutes of Health Grant AM-15781 and by National Institutes of Health grants to M.R.H., J.W.P., and B.S.K. This research constitutes a portion of a Ph.D. dissertation submitted to the Department of Biochemistry, University of Arizona, by D.J.M.

* Correspondence should be addressed to this author.

‡ Department of Biochemistry, University of Arizona.

§ Department of Cell Biology, Baylor College of Medicine.

∥ Department of Pediatrics, Baylor College of Medicine. J.W.P. is an Established Investigator of the American Heart Association.

⊥ Present address: Gene Expression Laboratory, The Salk Institute for Biological Studies, P.O. Box 85800, San Diego, CA 92138.

[1] Abbreviations: CaBP-28, vitamin D dependent intestinal calcium binding protein (M_r 28 000); CaM, calmodulin; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; kDa, kilodalton(s); bp, base pair(s); kb, kilobase(s); BSA, bovine serum albumin.

0006-2960/87/0426-8332$01.50/0

this paper, the CaBP-28 and CaM-like cDNAs are described, and the potential relationship between the CaM-like cDNA and authentic CaM is discussed.

Materials and Methods

Reagents. All reagents were of ultrapure molecular biology and/or electrophoresis grade. Purified, bacterially synthesized chicken calmodulin, anti-calmodulin antibody, and calmodulin DNA probe were gifts from Dr. Anthony Means, Department of Cell Biology, Baylor College of Medicine.

Animals. Rhode Island red cockerels were obtained commercially on the day of hatch and raised on a vitamin D supplemented diet or a vitamin D deficient diet containing 3.0% calcium (McNutt & Haussler, 1973). In some cases, the rachitic chicks (3–4 weeks old) were injected intramuscularly with 10 nmol of $1,25(OH)_2D_3$ solubilized in sesame seed oil. Intestinal mucosa (primarily from the duodenum), kidney, brain, heart, oviduct, and liver were harvested immediately after sacrifice and washed thoroughly in ice-cold 0.25 M sucrose or phosphate-buffered saline (PBS). New Zealand white rabbits were used according to standard procedures for production of serum antibodies against CaBP-28 (see below).

Generation of CaBP Polyclonal Antisera. CaBP-28 was purified to homogeneity from vitamin D repleted chicken duodenal mucosa according to a slight modification of the technique described by Hitchman et al. (1973). This purification scheme involved three sequential chromatography steps as follows: (1) Sephadex G-100; (2) two DEAE-Sephadex anion-exchange columns with the first in the presence of EDTA and (3) the second in the presence of $CaCl_2$. CaBP-28 was monitored at each step by Chelex exchange (Wasserman et al., 1968). Protein purity was assessed electrophoretically on SDS–polyacrylamide gels stained with Coomassie blue.

Purified CaBP-28 was emulsified in Freund's adjuvant and injected into rabbits (200 μg of CaBP-28/animal). Antisera harvested from immunized animals were tested for CaBP-28 specificity by double immunodiffusion (Ouchterlony, 1962). Anti-CaBP-28 polyclonal antibodies were then purified by DEAE-Affigel Blue chromatography. The antibodies employed in screening λgt11-expressed epitopes were further purified over a Sepharose 4B resin covalently linked to a λgt11/Y1090 (*Escherichia coli*) lysate.

RNA Isolation. Total cellular RNA from fresh chicken tissues was isolated by the guanidinium/cesium chloride method (Glisin et al., 1974; Ullrich et al., 1977). Poly(A+) RNA was enriched by oligo(dT)–cellulose, type 3 (Collaborative Research, Inc.), affinity chromatography (Aviv & Leder, 1972).

In Vitro Translation. Poly(A+) RNA was translated in the presence of [^{35}S]methionine (>1100 Ci/mmol, New England Nuclear) utilizing components from a commercially available cell-free translation kit (Promega). The in vitro synthesized CaBP protein was immunoprecipitated as previously described with slight modification (Mangelsdorf et al., 1987). Briefly, in vitro translated lysates were first precleared of nonspecific, coprecipitating proteins by sequential incubations with nonspecific antisera and Pansorbin (Calbiochem). Immunoprecipitation was achieved by the addition of anti-CaBP polyclonal antisera (1 μL of a 10^4-fold dilution of antibody in 50 μL of lysate). Following incubation at 4 °C for 1 h, Pansorbin was added to adsorb antigen–antibody complexes which were subsequently isolated from the lysate by centrifugation. The pellets were washed extensively (Mangelsdorf et al., 1987), solubilized in a SDS-denaturing buffer, and electrophoresed on a 15% SDS–polyacrylamide gel as described by Laemmli (1970). The gel was fixed in 30% methanol/10% trichloroacetic acid/10% acetic acid, washed in distilled water, impregnated with 1 M sodium salicylate/0.5% glycerol, dried under vacuum, and fluorographed at −70 °C on Kodak Omat XAR film.

Construction and Immunoscreening of cDNA Libraries. Oligo(dT)-primed, double-stranded cDNA was synthesized according to the method of Gubler and Hoffman (1983) using intestinal poly(A+) RNA isolated from vitamin D replete chickens. The cDNA was ligated into λgt11, packaged, and expressed in *E. coli* strain Y1090 (McDonnell et al., 1987; Young & Davis, 1983). The bacteria were induced to express λgt11 β-galactosidase fusion proteins by overlaying nitrocellulose filters (Schleicher and Schuell) impregnated with 10 mM isopropyl β-thiogalactoside and incubating for 4–8 h at 37 °C. Colonies expressing immunoreactive epitopes were identified in the following manner. The filters were incubated for 1 h in Tris-buffered saline (TBS) containing 3% powdered nonfat milk "Blotto" (Carnation) and then overnight at 4 °C with a 10^4-fold dilution of purified anti-CaBP antisera in 1% Blotto and washed 5 times with 0.05% Tween-20 in TBS. The filters were incubated with $\sim 2 \times 10^5$ cpm/mL ^{125}I-labeled protein A (>30 μCi/μg, ICN) in 1% Blotto for 2 h, washed as above, dried, and autoradiographed. Plaque-purified clones expressing specific immunoreactive epitopes were verified by demonstrating that the antigen did not produce an autoradiographic signal when this procedure was performed in the absence of anti-CaBP antisera.

Southern Analysis. Purified cDNA inserts and DNA molecular weight markers were electrophoresed in a 1% agarose gel using a Tris–borate running buffer (Maniatis et al., 1982). Prior to the transfer, the gel was stained with ethidium bromide (1 μg/mL) and photographed. The Southern transfer was performed as outlined (Southern, 1975) with the following modifications. Before DNA transfer, the gel was briefly (7–15 min) depurinated with 0.25 M HCl, denatured with 0.5 M NaOH, and neutralized with 1 M Tris-HCl, pH 7.4. The DNA was electrotransferred to a nylon membrane (Nytran, Schleicher & Schuell) at 4 °C in a Tris–acetate transfer buffer (Maniatis et al., 1982) and fixed by baking at 80 °C in vacuo. After being prehybridized in 50% formamide, 5× SSC, 1X Denhardt's solution, 50 mM sodium phosphate, pH 7.0, 0.5% SDS, and 0.1 mg/mL single-stranded salmon sperm DNA for 4 h at 42 °C, the Nytran-bound DNA was hybridized with 10^6 cpm/mL nick-translated cDNA for 16 h at 42 °C, washed (Thomas, 1980), and exposed to X-ray film at −70 °C.

Northern Analysis. Northern hybridization analysis was performed as described previously (McDonnell et al., 1987). Briefly, poly(A+) RNA extracted from chicken tissue was denatured at 55 °C in 50% formamide and 6.5% formaldehyde, electrophoresed in a 1% agarose/formaldehyde gel, and electrotransferred to a Nytran membrane in a MOPS/sodium acetate/EDTA buffer (Maniatis et al., 1982). The membrane was dried at 80 °C in vacuo, prehybridized, hybridized, washed, and autoradiographed as described above.

Western Analysis. Purified CaBP and bacterially synthesized calmodulin were electrophoresed on 12.5% polyacrylamide–SDS gels (Laemmli, 1970) and electrotransferred to nitrocellulose (Schleicher & Schuell) in a buffer containing 25 mM Tris-HCl, pH 7.4, 192 mM glycine, 100 mM NaCl, and 20% methanol (Towbin et al., 1979). Immunodetection of CaBP-specific protein was performed as described above, and the immunoreactive protein was identified by autoradiography.

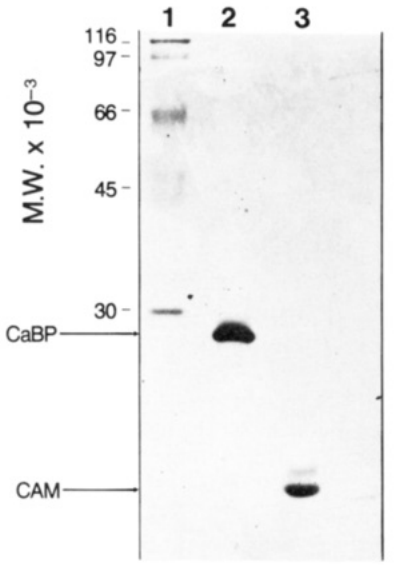

FIGURE 1: Electrophoretic analysis of purified CaBP-28 and CaM on a SDS–polyacrylamide gel. Proteins were electrophoresed on a 12.5% polyacrylamide gel and visualized by Coomassie blue staining. Lane 1, molecular weight standards [β-galactosidase (116K), phosphorylase B (97K), bovine serum albumin (66K), ovalbumin (45K), and carbonic anhydrase (30K)]; lane 2, 5 μg of purified CaBP-28; lane 3, 5 μg of bacterially synthesized purified CaM (the one fainter band in the doublet is slower migrating CaM complexed with Ca^{2+}. Arrows indicate the positions of CaBP-28 (28 kDa) and CaM (17 kDa).

Molecular Biology Techniques. cDNA inserts isolated from λgt11 were subcloned into the *EcoRI* site of pGEM-3 or -4 (Promega) and phage m13 for sequencing by the dideoxy technique (Sanger et al., 1977). The preparation of phage and plasmid DNA, the isolation of cDNA inserts, and nick translations were performed as detailed elsewhere (Maniatis et al., 1982).

RESULTS

Specificity of Anti-CaBP Antibodies. Vitamin D dependent CaBP-28 was purified from avian intestinal mucosa and used to generate polyclonal anti-CaBP-28 antibodies. When these antibodies were used to immunoscreen an avian intestinal λgt11 cDNA expression library, they reacted strongly with two unique populations of cDNA clones. We have identified one of these clones as CaBP-28 and the other as a novel chicken calmodulin-like protein (see below). Because of the apparent cross-reactivity of the polyclonal antisera during immunoscreening, we rigorously tested both the specificity of the antisera and the purity of the CaBP-28 that was used for immunization. The data in Figure 1 demonstrate the purity of this CaBP-28. When 5 μg of purified CaBP-28 was analyzed by SDS–polyacrylamide gel electrophoresis, one Coomassie blue stained band was identified at 28 kDa (lane 2) which is the estimated molecular mass of avian CaBP-28 (Wasserman et al., 1968). No other contaminating proteins appear in the CaBP-28 preparation, including CaM which migrates at 17 kDa (lane 3).

In order to demonstrate the specificity of the polyclonal antisera and eliminate the possibility that it contains antibodies generated against a contaminant protein not detected by Coomassie staining, we performed direct immunoblot analysis under both denaturing and nondenaturing conditions. It was clear from the Western blot in Figure 2 (lane 1) that the anti-CaBP-28 antisera react with only the 28-kDa CaBP and that this purified preparation was free from other contaminating, immunoreactive species of different molecular weights.

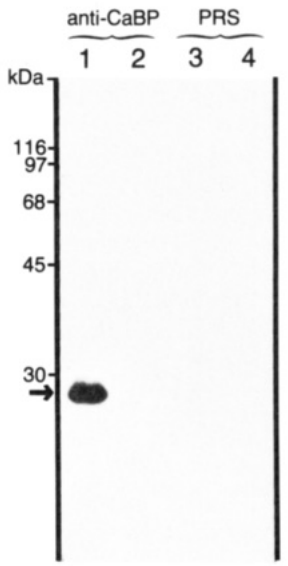

FIGURE 2: Western blot analysis of denatured, purified CaBP-28 and CaM. Proteins were electrophoresed as in Figure 1, electrotransferred to nitrocellulose, immunodetected with antiCaBP-28 antibody (anti-CaBP) or preimmune rabbit sera (PRS) as described under Materials and Methods, and autoradiographed. Lanes 1 and 3, 5 μg of purified CaBP-28; lanes 2 and 4, 5 μg of bacterially synthesized, purified CaM. The arrow indicates the position and the only immunoreactive band (lane 1) at 28 kDa.

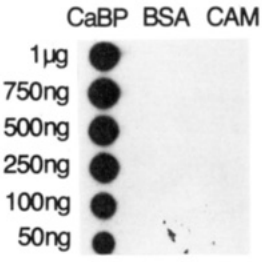

FIGURE 3: Dot blot analysis of nondenatured purified CaBP-28 and CaM. Serial dilutions of nondenatured purified CaBP-28, bovine serum albumin (BSA), and CaM were spotted directly onto a nitrocellulose filter, screened with anti-CaBP-28 antibody as described in Figure 2, and visualized by autoradiography.

Furthermore, these antibodies do not cross-react with CaM (lane 2). In a similar experiment, preimmune rabbit serum (lanes 3 and 4) also shows no interaction with either CaBP-28 or CaM, further demonstrating the specificity of the primary antibody. The data in Figure 3 illustrate that when CaBP-28 and CaM were spotted directly onto a nitrocellulose membrane and incubated with anti-CaBP-28 antisera, there was no apparent antibody cross-reactivity with nondenatured CaM. Taken together, these data strongly support the specificity of the anti-CaBP-28 polyclonal antibodies for CaBP-28.

In Vitro Translation and Immunoprecipitation of CaBP-28. To test the integrity of the poly(A+) RNA utilized to generate a cDNA library, and as a further evaluation of the CaBP-28 antibody, we have coupled in vitro translation and immunprecipitation to identify CaBP-28 transcripts. CaBP-28-specific immunoprecipitation of protein translated in vitro from chicken intestinal mRNA results in one major fluorographic band at 28 kDa (Figure 4, lane 2). The electrophoretic mobility of this protein is identical with purified CaBP-28 (Figures 1 and 2). When intestinal mRNA from vitamin D deficient, rachitic chickens is used to direct in vitro translation, no CaBP-28 was detected by immunoprecipitation (Figure 4, lane 3). However, upon repleting the rachitic chickens with injections of $1,25(OH)_2D_3$, there was a dramatic increase in the amount of immunoprecipitable CaBP-28 (lane 4). This vi-

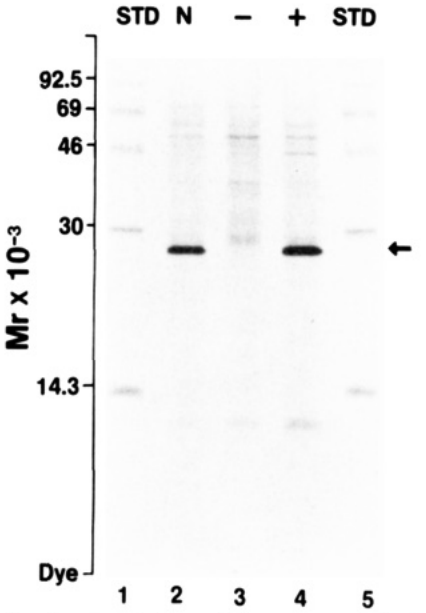

FIGURE 4: In vitro translation and immunoprecipitation of avian intestinal poly(A+) RNA. Poly(A+) RNA (0.5 μg) was translated in the presence of [^{35}S]methionine, immunoprecipitated with anti-CaBP-28 antibody, electrophoresed, and fluorographed as described under Materials and Methods. Lanes 1 and 5, ^{14}C-labeled molecular weight standards; lane 2, intestinal mRNA from chickens fed a normal calcium/vitamin D diet (N); lane 3, intestinal mRNA from rachitic chickens (–); lane 4, intestinal mRNA from rachitic chickens supplemented with 1,25(OH)$_2$D$_3$ 48 and 24 h before sacrifice (+). The arrow indicates the only major immunoprecipitable band at 28 kDa.

Table I: Comparison of Calcium Binding Protein cDNA Clones

cDNA clone	size (bp/aa)[a]	CaBP homology (%) nucleotide	CaBP homology (%) amino acid	CaM homology (%) nucleotide	CaM homology (%) amino acid
CaBP (9-1B)	676/241	100	100		
neoCaM (C-2)	393/131			84	99

[a] The first number represents base pairs of DNA sequence; the second number represents amino acid residues of protein sequence.

tamin D dependent effect is consistent with the known regulatory role of 1,25(OH)$_2$D$_3$-induced CaBP-28 synthesis (Theofan et al., 1986). The CaBP-28 antibody does not immunoprecipitate any other major protein synthesized from normal or rachitic chicken mRNA (lanes 2–4).

Identification of cDNA Clones to Calcium Binding Proteins. A λgt11 cDNA library (1.2×10^7 recombinants) was constructed from avian intestinal poly(A+) RNA shown to be CaBP-28 positive by in vitro translation (Figure 4). Immunoscreening of 4×10^5 recombinants from this library with anti-CaBP-28 antibodies yielded 24 immunoreactive clones. Several of the cDNA inserts from these clones were isolated and examined by cross-hybridization Southern analysis (Figure 5). Approximately 80% of the cDNA inserts contained sequences that were hybridized with a 767 bp clone designated 9-1B (Figure 5, left panel). Sequence analysis of this clone revealed it to be 100% homologous with the published nucleotide sequence of CaBP-28 (Table I; Wilson et al., 1985). The CaBP-28 clone 9-1B begins at nucleotide position 81 that corresponds to amino acid 27 of CaBP-28 and extends ~60 nucleotides into the 3′ untranslated region of the mRNA.

The second set of cDNAs represented 20% of the original immunopositive clones and did not cross-react with the authentic CaBP-28 cDNA (via Southern analysis) (Figure 5, right panel, lane 1). The largest of these cDNAs was a 393 bp insert designated C-2. Somewhat surprisingly, clone C-2

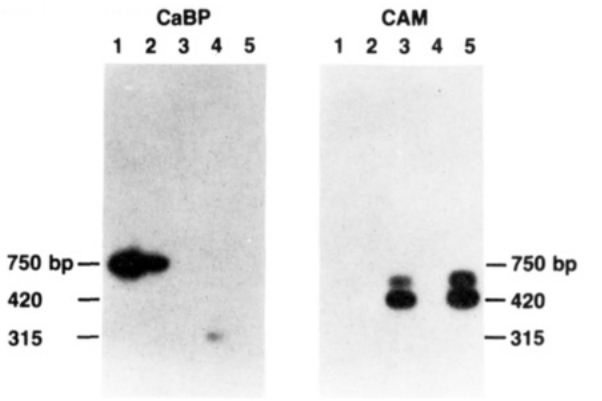

FIGURE 5: Southern analysis of immunoreactive calcium binding protein cDNA clones. A λgt11 cDNA library constructed from avian intestinal poly(A+) RNA was screened with polyclonal antisera to CaBP-28. Several immunopositive clones were identified; their cDNA inserts were isolated and then compared to each other by cross-hybridization Southern analysis. Lanes 1–5 represent cDNA clones designated 9-1B (lane 1), 9-2A (lane 2), 9-3A (lane 3), 10-1A (lane 4), and C-2 (lane 5). The left panel (CaBP) was probed with nick-translated 9-1B cDNA which codes for the translated domain of CaBP-28 (see Table I). The right panel (CaM) was probed with nick-translated C-2 cDNA which codes for a CaM-like protein (see Table I and Figure 6).

shared no homology with CaBP in either its nucleotide or its peptide sequence. However, clone C-2 contained one open-ended reading frame of 131 amino acids. When this 393 bp sequence was compared to several known cDNAs, it demonstrated a striking resemblance to that of chicken CaM described by Simmen et al. (1985). The chicken CaM gene codes for a mature mRNA of 1404 nucleotides which translates into a protein of 148 amino acid residues (Simmen et al., 1985). Clone C-2 displayed 99% conservation of peptide sequence corresponding to residues 11–141 in the authentic chicken CaM protein (Table I; Figure 6). This portion of the sequence included all four of the Ca^{2+} binding domains found in the structural CaM protein (Figure 6). There was one amino acid difference between the two proteins. The glycine at position 96 in CaM was a serine in clone C-2, a mutation which involved a single nucleotide substitution and occurred in Ca^{2+} binding domain III (Figure 6). Whereas the amino acid sequence was nearly identical, the nucleotide sequence homology between the two proteins was significantly less, only 84% (Table I). The lack of identity between the two nucleotide sequences is interesting considering both proteins are from the same species and suggests that, in fact, they are two distinct proteins derived from two different genes. In light of this information, we have tentatively named the new CaM-like protein neoCaM.

Northern Analysis of Cloned Calcium Binding Proteins. To more thoroughly evaluate the two cloned calcium binding proteins, we have examined the transcripts by Northern blot analysis. Nick-translated DNAs to authentic chicken CaM (CaM), clone C-2 (neoCaM), and clone 9-1B (CaBP) were used to probe intestinal mRNA prepared from both rachitic and vitamin D replete chickens (Figure 7). CaM [^{32}P]DNA hybridizes to two sizes of transcripts of 1400 and 1800 bases (lanes 1 and 2). The neoCaM probe appears to recognize two similar sized transcripts but, in addition, identifies a unique, substantially larger mRNA of ~4000 bases (lanes 3 and 4). Neither the CaM nor the neoCaM transcripts demonstrate hormonal regulation by vitamin D. However, when the CaBP-28 cDNA is utilized for hybridization, there is a vitamin D dependent induction of three different CaBP-28 transcripts of 2000, 2900, and 3300 bases in agreement with previously

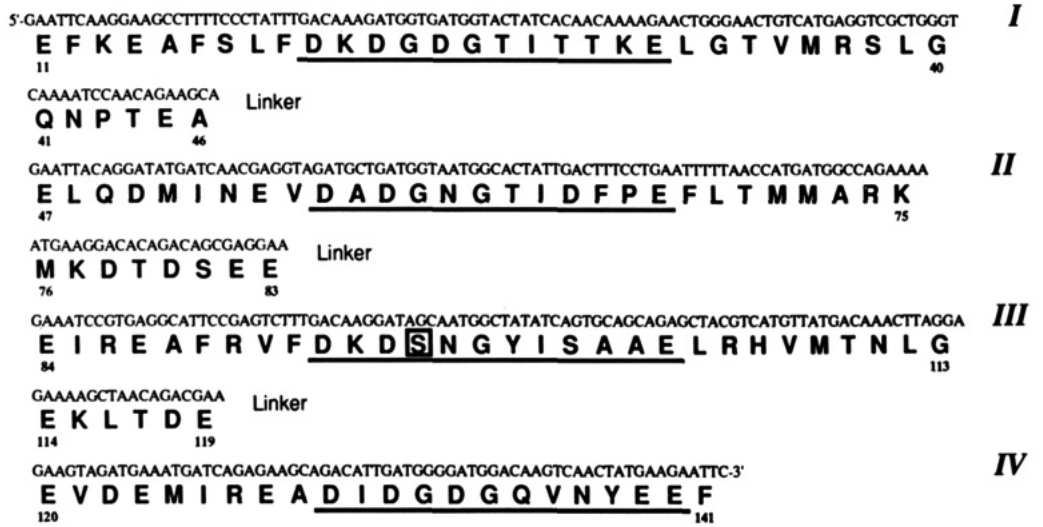

FIGURE 6: Nucleotide and deduced amino acid sequences of clone C-2 (neoCaM). The amino acid sequence (depicted by the one-letter code) is located below the clone C-2 nucleotide sequence from which it is deduced. I, II, III, and IV represent the four Ca^{2+} binding domains of chicken CaM (Simmen et al., 1985), each of which is separated by an intervening "linker" region. The underlined amino acids are those residues which are directly involved in binding a single calcium ion within each domain. The numbers under certain amino acids in neoCaM refer to the corresponding position of amino acids in chicken CaM (Simmen et al., 1985). The boxed amino acid in neoCaM shows the position of the amino acid substitution in Ca^{2+} binding domain III of chicken CaM.

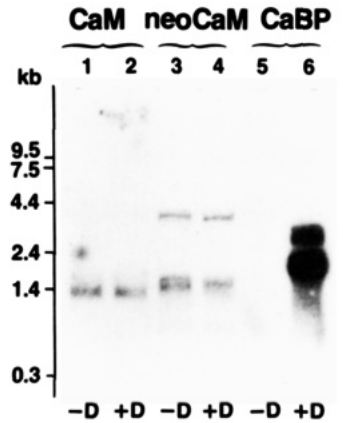

FIGURE 7: Northern analysis of avian intestinal poly(A+) RNA using various calcium binding protein cDNAs. Two micrograms of intestinal poly(A+) RNA from vitamin D depleted chickens (lanes 1, 3, and 5) or vitamin D repleted chickens (lanes 2, 4, and 6) was subjected to Northern hybridization analysis using nick-translated cDNAs (10^6 cpm/mL, $>10^8$ dpm/μg of cDNA) from authentic calmodulin (CaM, lanes 1 and 2), clone C-2 (neoCaM, lanes 3 and 4), or clone 9-1B (CaBP, lanes 5 and 6). Autoradiography was performed for 24 h.

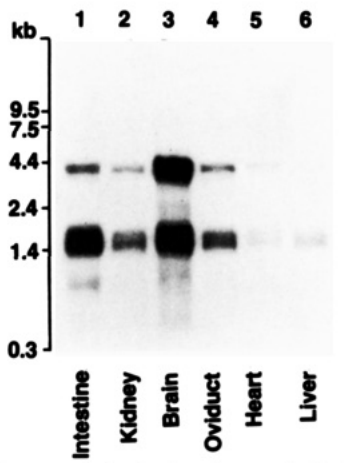

FIGURE 8: Northern analysis of avian tissue poly(A+) mRNA using clone C-2 (neoCaM). Chicken intestine, kidney, brain, oviduct, heart, and liver poly(A+) RNAs (2.5 μg of each) were isolated as described under Materials and Methods and subjected to Northern hybridization analysis using nick-translated clone C-2 (neoCaM) under conditions identical with Figure 7. Autoradiography was performed for 39 h.

published reports (lanes 5 and 6; King & Norman, 1986; Hunziker, 1986). These results verify the authenticity of the CaBP-28 cDNA clones and provide strong evidence for the existence of a yet undiscovered novel calcium binding protein, neoCaM.

Because CaM is expressed in many, if not all, eukaryotic cells, it was of interest to know whether neoCaM also exhibited a similar distribution. We have used the neoCaM probe to hybridize to mRNA isolated from various chicken tissues in a Northern analysis. As shown in Figure 8, the neoCaM probe hybridizes to three major transcripts (two CaM-like species and a unique 4-kb species) in all chicken tissues examined thus far. Although all three mRNA species are represented, the relative concentration of each mRNA appears to be tissue-specific. For example, brain has the highest concentration of both the CaM-like (1.4 and 1.8 kb) and strictly neoCaM (4 kb) transcripts (Figure 8, lane 3). The other tissues, however, express much reduced amounts of the 4-kb neoCaM mRNA but varying amounts of the CaM-like mRNAs. These results are consistent with the hypothesis that neoCaM is a ubiquitous protein and that is mRNA is transcribed from a distinct gene.

Discussion

Employing polyclonal antisera directed against vitamin D dependent CaBP-28, we have immunoscreened a chick intestinal λgt11 cDNA library and identified the translated regions of two distinct calcium binding protein cDNAs. Isolation of these cDNAs has allowed us to characterize one of the proteins as authentic CaBP-28 and the other as a novel CaM-like protein (neoCaM). Although both of these proteins are similar in that they contain conserved Ca^{2+} binding domains which display EF hand structures (Kretsinger, 1976), they remain immunologically distinct and share no cross-hybridizing sequence homology. These data are in agreement with the observation that the superfamily of calcium binding proteins including CaM, parvalbumin, troponin C, and CaBP-28 is evolutionarily related (Kilhoffer et al., 1983) but has diverged sufficiently to show no immunogenic similarity or stringent cross-hybridization. The fact that both proteins were identified immunologically by expression of their cDNAs

is therefore somewhat surprising. Goodman et al. (1979) and Kilhoffer et al. (1983) have postulated that this family of calcium binding proteins evolved from the duplication and subsequent divergence of a primordial Ca^{2+} binding domain. This suggests that although both CaBP-28 and neoCaM are not normally cross-immunogenic (Figures 2 and 3), similar immunoreactive epitopes may still exist and have been exposed when expressed artificially as a β-galactosidase fusion protein in the λgt11/*E. coli* system. Another plausible explanation is that the complete, intact neoCaM mRNA is a nontranslatable transcript and, therefore, its translation product would not be immunodetected by Western blots (Figures 2 and 3) or in vitro translation (Figure 4) but would be immunodetected when expressed as a λgt11 fusion protein. The possibility that neoCaM was a minor contaminant in the preparation of CaBP-28 antisera cannot be absolutely eliminated, although the data in Figures 1–4 convincingly argue that such a contaminant was not present. Furthermore, it is not likely that minor contamination of a conserved protein like CaM would be so highly immunogenic.

The identity of the CaBP-28 cDNA clone has been demonstrated by its 100% sequence homology with other known CaBP-28 clones (Table I; Wilson et al., 1985; Hunziker, 1986) and by its ability on Northern blots to hybridize with three characteristic mRNA transcripts which are regulated by $1,25(OH)_2D_3$ (Figure 7; Hunziker, 1986). We are currently utilizing this cDNA as a probe to isolate the promoter regions of the CaBP-28 gene which, in concert with cDNAs to the $1,25(OH)_2D_3$ receptor (McDonnell et al., 1987), will provide an invaluable tool for dissecting the molecular events of vitamin D induced regulation.

In contrast to CaBP-28, the identity of the neoCaM protein is unknown and at present remains open to speculation. We do know that unlike CaBP-28, neoCaM poly(A+) RNA (like authentic CaM) is not regulated by $1,25(OH)_2D_3$ (Figure 7) and that if it is translated it probably functions as a constitutively expressed protein much like authentic CaM. Furthermore, we have established by DNA sequencing (Table I; Figure 6), Northern analysis (Figures 7 and 8), and genomic Southern analysis (data not shown) that while neoCaM shows considerable homology with authentic CaM, the two proteins appear to be encoded by two different genes. Although its full sequence has not yet been isolated, neoCaM contains all four of the CaM domains for Ca^{2+} binding, and the nucleotide sequence homology between the two proteins remains high (84%). Due to the redundancy of the genetic code, the 16% divergence between the two nucleotide sequences has resulted in only one amino acid substitution: glycine-96 in CaM to a serine in neoCaM occurring in Ca^{2+} binding domain III (Figure 6). Phylogenetically, calmodulin exhibits one of the most conserved protein sequences known (Means et al., 1982; Nojima & Sokabe, 1986). Mammalian and avian calmodulins are nearly identical, the most evolutionarily divergent being in the protozoan, which differs in only 12 out of 148 amino acids. The high degree of homology (99%) between neoCaM and authentic CaM is therefore not surprising, and we would predict that the neoCaM gene codes for a functional, albeit different, protein than authentic CaM. Recently, Nojima and Sokabe (1986) reported the isolation of two processed CaM genes in addition to the authentic CaM gene in the rat. One of these processed genes (λSC9) was identified as a CaM pseudogene and the other (λSC8) as an intronless CaM-like gene whose mRNA could be detected in brain and muscle, but not liver. Interestingly, λSC8 differs from the authentic rat CaM at 19 of 148 amino acids (87% homology) and includes 2 amino acid deletions—relatively high divergence for two calmodulins from the same species. Although the amino acid substitutions in λSC8 are probably not significant because they occur in nonconserved residues, the two deletions—which occur in Ca^{2+} binding domain III—probably are significant. One of these deletions is at one of the invariant glycines which are thought to be critical in allowing the polypeptide to bend into a high-affinity metal binding domain (Dedman et al., 1978). Nojima et al. (1986) have suggested that this deletion may be responsible for hypertension in SHR rats. By analogy, therefore, it is possible that the amino acid substitution in neoCaM represents a significant structural/functional change in the CaM protein, since this mutation also occurs at an invariant glycine in the third Ca^{2+} binding domain (Figure 6).

The significance of the change from a glycine to a serine in neoCaM is unknown, although serines are target residues for phosphorylation. Plancke and Lazarides (1983) were able to demonstrate that a phosphorylated form of CaM exists in chick brain and that this phosphorylation occurred exclusively at serine residues. We are currently investigating the data from Northern analysis that show neoCaM transcripts are found predominantly in the brain, but expressed at low levels in all other tested tissues (Figure 8), suggesting that the phosphoCaM reported by Plancke and Lazarides may be neoCaM. Regardless of the identity of neoCaM, it is clear from the sequence data comparison of the two CaMs and the fact that neoCaM is derived from a distinct gene (and has a larger transcript) that this protein is not CaM but a different perhaps even larger protein which contains a CaM-like domain. We are currently in the process of determining the full size of the neoCaM protein as a means of furthering its identification.

In conclusion, we have used polyclonal antisera to isolate the translated domains of two different calcium binding proteins. The cDNA to one of these proteins, CaBP-28 should provide an excellent tool for characterizing the mechanism of $1,25(OH)_2D_3$-dependent regulation. The cDNA to the other protein, neoCaM, has tentatively been identified to code for a variant CaM-like protein, the full characterization of which is now under study.

ACKNOWLEDGMENTS

We thank Samuel L. Marion for his expert technical assistance in purifying CaBP-28 and preparing CaBP-28 antisera and Paula Leece for typing the manuscript.

Registry No. $1,25(OH)_2D_3$, 32222-06-3.

REFERENCES

Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408–1412.

Dedman, J. R., Jackson, R. L., Schreiber, W. E., & Means, A. R. (1978) *J. Biol. Chem. 253*, 343–346.

Fullmer, C. S., & Wasserman, R. H. (1985) in *Vitamin D: Chemical, Biochemical and Clinical Update* (Norman, A. W., Schaefer, K., Grigoleit, H.-G., & Herrath, D. V., Eds.) pp 323–332, de Gruyter, Berlin.

Glisin, V., Crkvenjakov, R., & Byus, C. (1974) *Biochemistry 13*, 2633–2637.

Goodman, M., Pechere, J. F., Haiech, J., & Demaille, J. G. (1979) *J. Mol. Evol. 13*, 331–352.

Gubler, U., & Hoffman, B. J. (1983) *Gene 25*, 263–269.

Hitchman, A. J. W., Kerr, M.-K., & Harrison, J. E. (1973) *Arch. Biochem. Biophys. 155*, 221–222.

Hunziker, W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7578–7582.

Hunziker, W., Siebert, P. D., King, M. W., Stucki, P., Dugaicyk, A., & Norman, A. W. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4228-4232.
Kilhoffer, M. C., Haiech, J., & Demaille, J. G. (1983) *Mol. Cell. Biochem. 51*, 33-54.
King, M. W., & Norman, A. W. (1986) *Arch. Biochem. Biophys. 248*, 612-619.
Kretsinger, R. H. (1976) *Annu. Rev. Biochem. 45*, 239-266.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Mangelsdorf, D. J., Pike, J. W., & Haussler, M. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 354-358.
Maniatis, T., Fritsch, E., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
McDonnell, D. P., Mangelsdorf, D. J., Pike, J. W., Haussler, M. R., & O'Malley, B. W. (1987) *Science (Washington, D.C.) 235*, 1214-1217.
McNutt, K. W., & Haussler, M. R. (1973) *J. Nutr. 103*, 681-689.
Means, A. R., Tash, J. S., & Chafouleas, J. G. (1982) *Physiol. Rev. 62*, 1-38.
Nojima, H., & Sokabe, H. (1986) *J. Mol. Biol. 190*, 391-400.
Nojima, H., Kishi, K., & Sokabe, H. (1986) *J. Hypertens. 4 (Suppl. 3)*, S275-S277.
Ouchterlony, O. (1962) *Prog. Allergy 6*, 30-75.
Pike, J. W., Sleator, N. M., & Haussler, M. R. (1987) *J. Biol. Chem. 262*, 1305-1311.
Plancke, Y. D., & Lazarides, E. (1983) *Mol. Cell. Biol. 3*, 1412-1420.
Rasmussen, H. (1986) *N. Engl. J. Med. 314*, 1094-1101.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Simmen, R. C. M., Tanaka, T., Ts'ui, K. F., Putkey, J. A., Scott, M. J., Lai, E. C., & Means, A. R. (1985) *J. Biol. Chem. 260*, 907-912.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503-517.
Theofan, G., Nguyen, A. P., & Norman, A. W. (1986) *J. Biol. Chem. 261*, 16943-16947.
Thomas, P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5201-5205.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350-4354.
Ullrich, A., Shine, J., Chirgwin, J., Pictet, R., Tischer, E., Rutter, W. J., & Goodman, H. M. (1977) *Science (Washington, D.C.) 196*, 1313-1319.
Wasserman, R. H., & Taylor, A. N. (1966) *Science (Washington, D.C.) 152*, 791-793.
Wasserman, R. H., Corradino, R. A., & Taylor, A. N. (1968) *J. Biol. Chem. 243*, 3978-3986.
Wilson, P. W., Harding, M., & Lawson, D. E. M. (1985) *Nucleic Acids Res. 13*, 8867-8881.
Yamamoto, K. R. (1985) *Annu. Rev. Genet. 19*, 209-252.
Young, R. A., & Davis, R. W. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1194-1198.

Phase-Resolved Spectral Measurements with Several Two Tryptophan Containing Proteins†

Maurice R. Eftink,*,‡ Zygmunt Wasylewski,§ and Camillo A. Ghiron‖

Department of Chemistry, University of Mississippi, University, Mississippi 38677, Department of Biochemistry, Jagellonian University, Krakow, Poland, and Department of Biochemistry, University of Missouri, Columbia, Missouri 65201

Received June 12, 1987; Revised Manuscript Received August 12, 1987

ABSTRACT: We have used frequency domain fluorescence techniques to resolve the component emission spectra for several two tryptophan containing proteins (e.g., horse liver alcohol dehydrogenase, sperm whale apomyoglobin, yeast 3-phosphoglycerate kinase, apoazurin from *Alcaligenes denitricans*). We have first performed multifrequency phase/modulation measurements and have found the fluorescence of each of these proteins to be described by a double exponential. Then, using phase-sensitive detection and the algorithm of Gratton and Jameson [Gratton, E., & Jameson, D. M. (1985) *Anal. Chem. 57*, 1694-1697], we have determined the emission spectrum associated with each decay time for these proteins. We have compared these phase-resolved spectra with the fractional contributions of the component fluorophores determined by selective solute quenching experiments. Reasonably good agreement is seen in most cases, which argues that the individual Trp residues emit independently. In the case of apoazurin, however, a negative amplitude is seen for the phase-resolved spectrum of the short-lifetime component. This pattern is consistent with the occurrence of energy transfer from the internal Trp residue to the surface Trp of this protein. We also present multifrequency lifetime measurements, phase-resolved spectra, and solute quenching data for a few protein–ligand complexes, to illustrate the utility of this approach for the study of changes in the fluorescence of proteins.

The fluorescence emission of most proteins and other biochemical assemblies is heterogeneous, due to ground-state hetereogeneity or excited-state reactions (Beechem & Brand, 1985; Longworth, 1983). For example, most proteins possess more than one Trp residue, and since the fluorescence properties of the indole nucleus are environment dependent, the experimentally measured fluorescence spectra, quantum yields, lifetimes, or anisotropies will be a composite or average over the individual components. Such heterogeneity makes the interpretation of fluorescence data much more difficult. It is often desirable to be able to resolve the fluorescence of a system into its components. For example, if one can resolve and assign the fluorescence signals of individual Trp residues

† This research was supported by NSF Grant DMB 85-11569 to M.R.E.
‡ University of Mississippi.
§ Jagellonian University.
‖ University of Missouri.

0006-2960/87/0426-8338$01.50/0