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Biochemistry

The 28-kDa vitamin D-dependent calcium-binding protein has a six-domain structure

(cDNA cloning/nucleotide sequence)

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ABSTRACT **Vitamin D-dependent 28-kDa calcium-binding protein ($CaBP_{28}$) cDNA clones were isolated from a chicken intestinal library. The nucleotide sequence analysis of the $CaBP_{28}$ cDNA shows an open reading frame of 786 nucleotides, coding for a 262-amino acid 30.167-kDa protein. Interestingly, the protein contains six repeats of a domain with the feature of a calcium-binding site. In two of the six domains, oxygen-containing amino acids important for the positioning of calcium are absent, suggesting that these two sites have lost their calcium-binding capability and might have adopted a new function in evolution. In the chicken intestine, three different sized species of $CaBP_{28}$ mRNA (2.0, 2.8, and 3.1 kilobases) are detected. Primer extension and S1 nuclease mapping show that the three $CaBP_{28}$ mRNA species share a common 5′ end but differ in the length of their 3′ noncoding sequence. A similar triplet of $CaBP_{28}$ mRNAs is identified in the rat kidney by the chicken probe, showing an interspecies conservation of the $CaBP_{28}$. In the rat intestine, however, no $CaBP_{28}$ mRNA could be detected. Instead, a vitamin D-dependent 9-kDa CaBP ($CaBP_9$) is expressed, with an mRNA size of ≈0.7 kilobase that does not cross-hybridize with the $CaBP_{28}$ probe. This indicates that the $CaBP_{28}$ and $CaBP_9$ are the product of two independent genes.**

Vitamin D target tissues are characterized by the presence of an intracellular receptor for 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], the biologically active form of vitamin D. Most of the vitamin D target tissues also contain a vitamin D-dependent calcium-binding protein with a molecular mass of 28 kDa ($CaBP_{28}$) (for a review, see ref. 1). Exceptions include the mammalian intestine (2, 3) and skin (4), both containing a 9-kDa vitamin D-dependent calcium-binding protein ($CaBP_9$). These proteins are all three immunologically distinct from each other (4, 5) and bind calcium with high affinity ($1–10 \times 10^{-7}$ M) (6, 7). Kretsinger (8) has established the amino acid sequence required for the formation of an EF hand calcium-binding domain. All the structurally known intracellular CaBPs such as calmodulin, parvalbumin, and troponin C (9) comply with these rules.

The presence of two EF hand structures in the bovine intestinal $CaBP_9$, each binding 1 mol of Ca^{2+} per mol, was shown by calcium binding and by x-ray crystallography (10). Calcium-binding experiments with the $CaBP_{28}$ would by analogy suggest four EF hand domains.

$CaBP_{28}$ was originally discovered in the chicken intestine (11) and was initially thought to be directly involved in the $1,25(OH)_2D_3$-dependent transport of calcium across the intestinal epithelial cell. This hypothesis was dismissed after finding that the increase in calcium transport after administration of $1,25(OH)_2D_3$ to vitamin D-deficient animals occurred before an increase in the $CaBP_{28}$ protein or mRNA levels could be detected (12–14). Moreover, CaBP is present in a variety of tissues not involved in the transcellular transport of calcium, including brain, placenta, and testis (1). The physiological function apart from binding calcium remains unknown for both the $CaBP_{28}$ and the $CaBP_9$.

$1,25(OH)_2D_3$ has been shown to regulate the levels of the intestinal $CaBP_{28}$ from undetectable in vitamin D-deficient chickens to up to 1–3% of the cytoplasmic protein of the intestinal cell (15). The induction of the synthesis of $CaBP_{28}$ occurs via a receptor-mediated regulation of the $CaBP_{28}$ gene transcription (12–14, 16), although an additional translational regulation has not been ruled out. In other tissues, the levels of the $CaBP_{28}$ protein appear to be less stringently regulated by $1,25(OH)_2D_3$. For example, in the chicken kidney $1,25(OH)_2D_3$ only raises the $CaBP_{28}$ level to ≈6-fold above the basal level observed in the vitamin D-deficient control (15) and in the brain no dependence on the vitamin D status could be shown (15). Whereas the $CaBP_{28}$ is regulated by vitamin D in some tissues, it is not clear whether it is constitutively expressed in others. A possible explanation could be a long half-life of the protein in some tissues, resulting in continuously high $CaBP_{28}$ protein levels in the vitamin D-depleted state that could mask a possible increase upon $1,25(OH)_2D_3$ administration. To address the question of the regulation of gene expression and of the structure and function of the $CaBP_{28}$, we have cloned the $CaBP_{28}$ cDNA (16, 17). Since only part of the $CaBP_{28}$ nucleotide sequence was contained in these initial clones, a second cDNA library was established with improved procedures to obtain a full-length $CaBP_{28}$ cDNA.

METHODS

Animals. One-day-old White Leghorn chicks were raised from hatch on a vitamin D-deficient (−D) diet (18) or a vitamin D-deficient diet supplemented with vitamin D (+D). Similarly, rats (Fu-Albino) were raised from birth on a −D or a +D diet (19).

RNA Isolation, Cloning, and Screening. Intestinal and kidney RNA from rats and chickens were prepared by using guanidinium thiocyanate (20) as described (16). Poly(A)$^+$ RNA was obtained after one or two cycles of binding to oligo(dT)-cellulose. Double-stranded cDNA was synthesized by using avian myeloblastosis virus reverse transcriptase and RNase H/DNA polymerase I for the first and second strands, respectively (21). After (C)-tailing of the synthesized cDNA it was hybridized to the (G)-tailed *Pst* I site of pBR322 and used to transform *Escherichia coli* K-12 RRI as described (16). About 30,000 colonies were screened by the colony

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Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; CaBP, calcium-binding protein; $CaBP_{28}$, 28-kDa vitamin D-dependent CaBP; $CaBP_9$, 9-kDa vitamin D-dependent CaBP; nt, nucleotide(s); kb, kilobase(s); bp, base pair(s).

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hybridization method (22) using the *Dde* I/*Rsa* I fragment of the original CaBP cDNA clone (16) as a probe.

DNA Sequence Analysis. DNA sequencing was performed by the dideoxy-chain termination method (23) after subcloning restriction fragments into M13mp18 and M13mp19 bacteriophage (24). Terminal deletions of the fragments to be sequenced were made either with the nuclease BAL-31 (25), exonuclease III (26), or T4 DNA polymerase (27). To establish the 5′ end of the $CaBP_{28}$ mRNA that was not contained in the cDNA clones, the product of the primer-extension reaction was isolated from a 6% polyacrylamide/urea gel and subjected to a chemical cleavage sequencing method (28) on DEAE paper (Whatman DE81) (29).

5′ Primer Extension. First 10 pmol of a 33-mer 5′ ^{32}P-labeled oligonucleotide primer was hybridized to 100 μg of +D poly(A)$^+$ RNA under standard cDNA first-strand synthesis conditions at 42°C (21). After 5 min, 100 units of reverse transcriptase (Anglian Biotechnology, Colchester, England) was added and the reaction was continued for 60 min. The product was analyzed on a 6% polyacrylamide/urea gel.

RNA Blots. Poly(A)$^+$ RNA (10 μg) and DNA size standards were glyoxylated at 50°C for 15 min prior to separation on 1% agarose gels (30) followed by transfer to nitrocellulose (31) and hybridization with the appropriate ^{32}P-labeled probes.

3′ End S1 Nuclease Mapping. $CaBP_{28}$ cDNA clones 16 and 21 were joined at their *Hinc*II site (Fig. 1) and cloned into the *Pst* I site of M13mp19. The *Asp* 718 fragment [containing the 3′ half of $CaBP_{28}$ cDNA and 27 base pairs (bp) of vector] was ^{32}P-labeled by filling in the *Asp* 718 ends with Klenow DNA polymerase. The +D chicken intestinal poly(A)$^+$ RNA (10 μg) was hybridized with 8×10^5 cpm of the *Asp* 718 fragment in 80% formamide/40 mM Pipes, pH 6.4/1 mM EDTA/0.4 M NaCl (32), followed by digestion with 160 units of S1 nuclease (Pharmacia P-L Biochemicals) for 60 min. Pilot experiments showed that the optimal hybridization and S1 nuclease digestion temperatures were 45°C and 20°C, respectively. The S1 nuclease-protected fragments were analyzed on a 3.5% acrylamide/urea gel.

Oligonucleotide Synthesis. The 33-mer oligonucleotide primer with the sequence TCCTATTTTTCCATCAGTGGCCTTGCCATACTG complementary to nucleotides (nt) 295–327 of the $CaBP_{28}$ cDNA sequence (Fig. 2) and the primer used as a probe for the $CaBP_9$ with the sequence 5′ ATTGTCTAGAGTACTTGAAGCCTTCAGGAGGTT derived from the cDNA sequence of Desplan *et al.* (33) were synthesized simultaneously using phosphoramidite chemistry and controlled pore glass as solid support (34) by Willy Bannwarth.

RESULTS AND DISCUSSION

Isolation of $CaBP_{28}$ cDNA Clones. The initial chicken intestinal cDNA library only yielded short fragments of the $CaBP_{28}$ cDNAs (16). Therefore, a second library was generated using improved procedures (21).

About 30,000 clones of this library were screened yielding 30 positive clones with a cDNA insert size of 200–1800 bp. The 12 longest were selected for further analysis. All of them hybridized in an RNA blot to the same three mRNA species in +D intestinal poly(A) RNA that are absent in −D intestinal poly(A) RNA (data not shown), as did the CaBP cDNAs that had been confirmed by hybrid selected translation (16). Fig. 1 shows a restriction map with selected enzymes that allowed determination of the overlaps between the different $CaBP_{28}$ cDNA clones. Between them, a total of 2.7 kilobases (kb) of the $CaBP_{28}$ mRNA is represented (Figs. 1 and 2).

Nucleotide Sequence Analysis and 5′ End of the $CaBP_{28}$ mRNA. $CaBP_{28}$ cDNA clones 16 and 21 were subcloned in M13 vectors and sequenced in their entire length in both directions by the dideoxy method (23).

$CaBP_{28}$ 16 extends from nt 130 to 1874, whereas 21 starts at nt 1450 and extends to the poly(A)$^+$ tail beginning at nt 2791 (Fig. 2). To define the 5′ end of the $CaBP_{28}$ mRNA, a synthetic oligonucleotide primer (complementary to nt 295–327 of the sequence in Fig. 2) was 5′ ^{32}P-labeled and hybridized to +D chicken intestinal poly(A)$^+$ RNA followed by extension of the primer with reverse transcriptase. The major product of this extension was ≈330 nt long (Fig. 3) showing that the 5′ end of the $CaBP_{28}$ mRNA is homogenous and that ≈130 nt at the most 5′ end are not contained in the cDNA clones. The nucleotide sequence of this part was derived by sequencing the primer-extension product. Because of the heavy band of unreacted material, the first 3–5

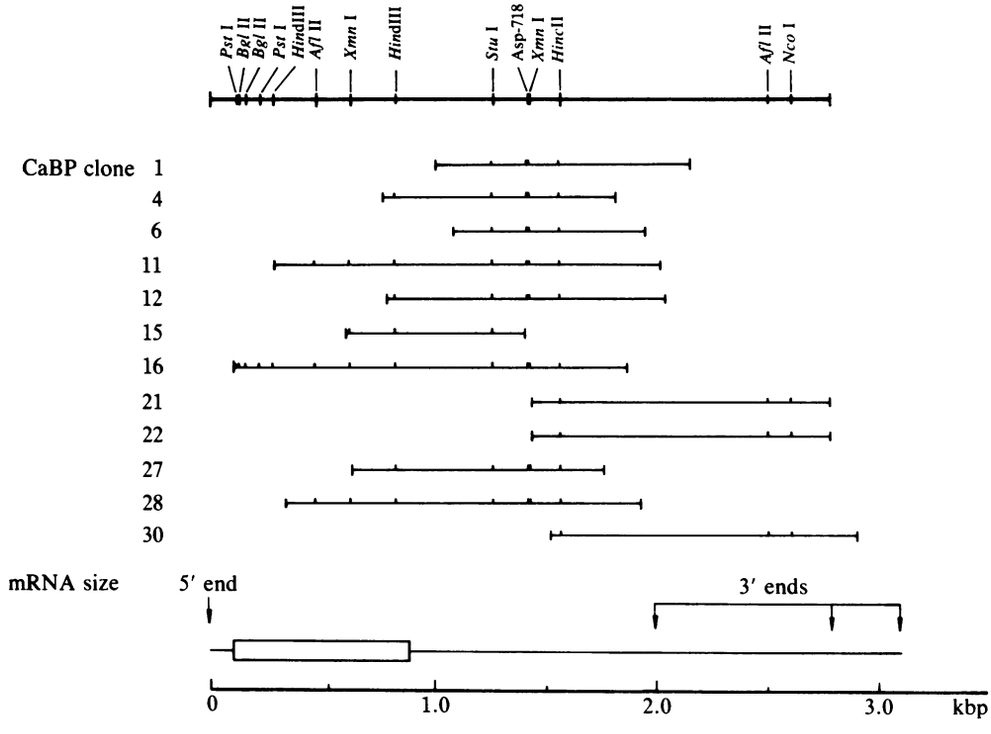

FIG. 1. Restriction map of the chicken $CaBP_{28}$ clones. Plasmid DNA of the 30 positive clones was digested with *Pst* I to estimate the size of the cDNA inserts. The clones containing the 12 largest inserts were subjected to a more detailed analysis with selected restriction enzymes to establish the overlaps. The 786-bp open reading frame is boxed, whereas the 5′ and 3′ noncoding sequences are indicated by lines. The 5′ end and the 3′ end of the three mRNA species are indicated by arrows.

FIG. 2. Complete nucleotide sequence of the 2.8-kbp $CaBP_{28}$ mRNA and deduced amino acid sequence. The open reading frame starts at nt 103 and extends to nt 888.

nt of the 5′ end of the mRNA could not be determined, so that the first nt of the sequence in Fig. 2 corresponds to nt 4–6 of the mRNA. The first ATG at nt 103 is followed by a long open reading frame that ends with a TAA stop codon at nt 889. This open reading frame is in agreement with the sequence recently published by Wilson *et al.* (35) and encodes a protein of 262 amino acids.

The calculated size (30,167 Da) is slightly larger than the 28 kDa observed for $CaBP_{28}$ on $NaDodSO_4$ gel electrophoresis. The discrepancy in molecular mass is most likely due to aberrant migration of the protein in gel electrophoresis. A posttranslational cleavage of $CaBP_{28}$ as Wilson *et al.* (35) suggested is unlikely since the NH_2 and COOH termini of the predicted protein are consistent with the amino acid composition of NH_2- and COOH-terminal peptides (36).

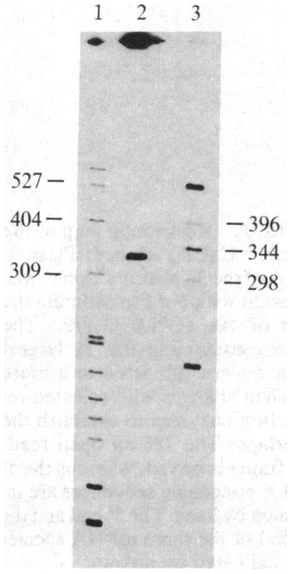

FIG. 3. Primer extension on the CaBP mRNA. A 33-mer synthetic oligonucleotide (corresponding to nt 295–327 in Fig. 2) was 5′ ^{32}P-labeled and hybridized to chicken intestinal poly(A)$^+$ RNA followed by extension with reverse transcriptase under standard conditions. The product was separated on a 6% polyacrylamide/urea gel (lane 2). Standards: lane 1, pBRxHpaII; lane 3, pBRxHinfI.

Interestingly, our analysis of the sequence shows that the predicted protein contains six repeats (Figs. 2 and 4) of about 40 amino acids, whereas Wilson *et al.* (35) only described five. Each repeat has the general feature of a calcium-binding EF hand domain according to Kretsinger's rules (8), even though the protein is known to bind only four Ca^{2+} per mol of protein (6). There is a stronger amino acid homology between domains I, II, and IV on one hand (type A) and domains Ia, III, and IVa on the other (type B).

This suggests that in evolution there was a gene duplication resulting in the type A and B domains. Later in evolution, this doublet was further replicated twice to yield the six domains. Comparing the amino acid sequence of these domains to the sequence requirements for a perfect EF hand calcium binding site (8, 10), there is a varying degree of divergence. The central glycine is conserved in all the predicted sites. The five oxygen-containing residues important for the coordinating of Ca^{2+} (8, 10) are present in sites I, II, III, and IV, whereas in site Ia only the third and fifth and in site IVa the second, fourth, and fifth oxygen-containing residue are conserved (Fig. 4). By analogy to the bovine defective troponin C site I where the first two oxygen-containing residues are replaced (37), sites Ia and IVa of the $CaBP_{28}$ probably do not bind Ca^{2+} anymore. The Ca-binding sites would then be constituted by three type A domains (I, II, and IV) and one type B domain (III). Interestingly, the helix and linker sequence that follows the loop shows extensive sequence homology among the type A sites (Ia, III, and IVa), even though sites Ia and IVa most likely do not bind Ca^{2+}. These sequences could, however, have adopted a new function and therefore be under a different conservation pressure.

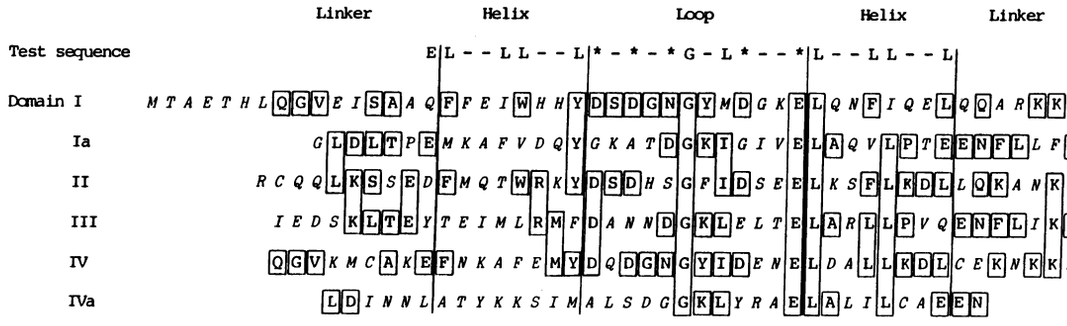

FIG. 4. Calcium-binding domains of $CaBP_{28}$. The protein sequence (Fig. 2) was analyzed for homologies to the test sequence for an EF hand Ca^{2+} binding site (8). Each line shows one domain, whereas the linker sequences are divided arbitrarily between the lines. The amino acid sequence is shown in the one-letter code. Homologous amino acids between two domains are boxed.

mRNA Size of the $CaBP_9$ and the $CaBP_{28}$ and Conservation Between Species. An RNA blot of poly(A)$^+$ RNA isolated from vitamin D-deficient (−D) and repleted (+D) chicken intestine as well as −D and +D rat intestine and kidney was hybridized with a $CaBP_{28}$ probe (*Pst* I/*Asp* 718 fragment of CaBP clone 16 in Fig. 1). As previously reported (16), the vitamin D-replete chicken intestine contains three mRNA species (Fig. 5*a*), a major species of 2 kb and two minor species with 2.8 and 3.1 kb, respectively. None of these mRNAs is present in the −D mRNA. In the rat intestinal RNA, there is no hybridization to the $CaBP_{28}$ probe regardless of the vitamin D status. The rat kidney mRNA, however, shows three hybridizing species similar to those observed in chicken intestine. The three rat mRNAs are ≈250 nt shorter than the corresponding ones in the chicken and are present at lower levels. The presence of these three species, albeit at reduced levels in the −D rat kidney, is most likely explained by the rats not being totally vitamin D-deficient.

The same blot was then additionally (without dehybridization) hybridized with a synthetic oligonucleotide probe for the $CaBP_9$. The rat intestine shows a strongly hybridizing single mRNA species ≈700 nt long, which again is also present at a reduced level in the −D RNA (Fig. 5*b*). The +D rat kidney also expresses this $CaBP_9$ mRNA, although at a low level.

These experiments show that the $CaBP_{28}$ and the $CaBP_9$ mRNA are unrelated, since they do not cross-hybridize and therefore must represent two different genes. The rat $CaBP_{28}$ mRNA, however, cross-hybridizes with the chicken probe, showing a strong conservation of the $CaBP_{28}$ gene between the two species. Recently, we also established the nucleotide sequence of the rat $CaBP_{28}$ cDNA. The predicted protein is one amino acid shorter and shows an overall 80% homology to the chicken protein; allowing for conservative changes, the homology increases to 93%.

This high homology extends to the two non-calcium-binding domains Ia and IVa, which further supports the hypothesis that they have an important physiological function. The conservation of the $CaBP_{28}$ protein between species is further shown by the fact that antibodies to the chicken protein cross-react with rat, bovine, and human $CaBP_{28}$ (5). If the sole function of $CaBP_{28}$ was to keep the ionized Ca^{2+} below toxic levels, there would not be enough selection pressure to explain the observed conservation of the $CaBP_{28}$ during the course of evolution. The amino acid requirement for an EF hand calcium-binding site allows for a variation as shown in the test sequence in Fig. 4 (8, 9). Divergence of calcium-binding sites is observed for calmodulin, parvalbumin, troponin C, $CaBP_{28}$, and $CaBP_9$, which all bind calcium by means of an EF hand structure, but show no cross-reactivity with antibodies nor cross-hybridization of cDNA probes. More sequence data from $CaBP_{28}$ of other species are needed to determine the exact degree of conservation. Conserved domains could yield clues to the function of the protein.

What Is the Difference Between the Three mRNA Species? The size heterogeneity of the $CaBP_{28}$ mRNA could be due to a heterogeneous start of transcription resulting in a heterogeneity in the 5′ end of the mRNA. However, this possibility has been ruled out since the primer extension experiment only yielded one band with a size of 330 nt (Fig. 2).

Alternatively, a differential splicing of an RNA precursor or a different length of the 3′ end could be responsible for the size heterogeneity. To distinguish between these two possibilities, a fragment starting from the *Asp* 718 site and containing the entire cloned 3′ region (Fig. 1) and an additional 27 bp of the vector was 3′-end-labeled and used for S1 nuclease mapping. At the optimal hybridization (45°C) and S1 nuclease digestion temperatures (20°C), three bands of ≈650, ≈1350, and ≈1400 bp can be detected under denaturing conditions (Fig. 6). This shows that the probe was colinear

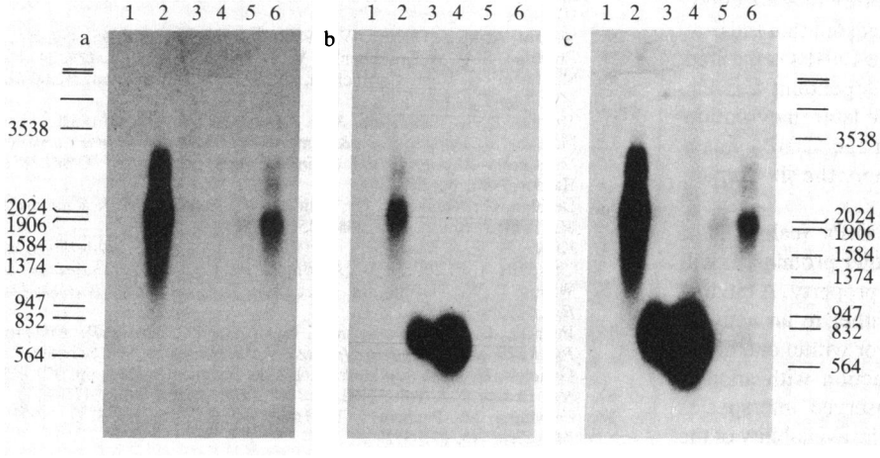

FIG. 5. RNA blot. Poly(A)$^+$ RNA (10 μg) isolated from vitamin D-deficient (−D) and replete (+D) chicken as well as −D and +D rat intestine and kidney were electrophoresed on a 1% agarose gel, followed by transfer to nitrocellulose. Chicken intestine: lanes 1, −D; lanes 2, +D. Rat intestine: lanes 3, −D; lanes 4, +D. Rat kidney: lanes 5, −D; lanes 6, +D. (*a*) The blot was first hybridized with the $CaBP_{28}$ probe (*Pst* I/*Asp* 718 fragment of $CaBP_{28}$ clone 16 in Fig. 1); (*b*) the same blot was hybridized with a synthetic $CaBP_9$ probe; (*c*) overexposure of *b* to show the longer $CaBP_{28}$ bands as well as the $CaBP_9$ band present in the kidney.

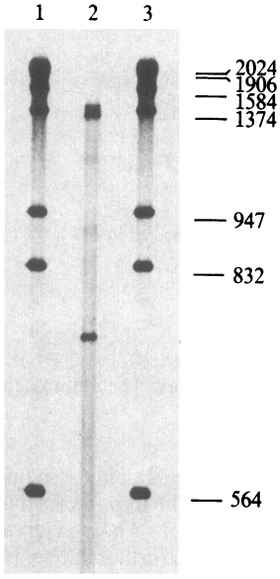

FIG. 6. S1 nuclease mapping of the 3′ end of the CaBP mRNA. A fragment containing the entire cloned 3′ end sequence starting from the Asp 718 site and an additional 27 bp of the vector was 3′ end-labeled (by filling in with Klenow fragment of DNA polymerase I) and used for the S1 nuclease mapping. Ten micrograms of chicken intestinal poly(A)$^+$ RNA was hybridized with the labeled probe (8×10^5 cpm) in 80% formamide for 3 hr followed by digestion with S1 nuclease (160 units), followed by analysis of a 3.5% polyacrylamide/urea gel (lane 2). Lanes 1 and 3, λ DNA digested with *Eco*RI and *Hin*dIII as size standards.

with all three mRNA species, ruling out a differential splicing. The shortest band of ≈650 nt shows that the end of the shortest mRNA species extends to ≈650 nt downstream of the *Asp* 718 site (nt 1438). Indeed, at nt 2002 and nt 2020 there are two AATAAA polyadenylylation signals (Fig. 2). Since the 2-kbp band is the predominant mRNA species (Fig. 5), most of the transcribed RNA apparently is polyadenylylated at one of these two positions. A second part is polyadenylylated at nt 2791, as deduced from the second band seen on the S1 nuclease map and confirmed by sequence analysis, and the rest of the mRNA extends even further and is not contained in our clones. The polyadenylylation at nt 2791 is not preceded by a typical polyadenylylation signal. The sequence CACAAGA (nt 2026–2032) just following the second polyadenylylation signal (nt 2020–2025) is complementary to the sequence TCTTGTG (nt 2783–2789) preceding the poly(A)$^+$ tail (nt 2791). One could therefore speculate that the RNA folds back, which brings position 2791 in the vicinity of the two AATAAA polyadenylylation signals and thereby decreases their efficiency.

Evolutionary Implications. Analyzing the amino acid seqences of 25 CaBPs by the maximum parsimony method, Goodman *et al.* (38) concluded that all CaBPs evolved from a four-domain structure. Their data suggest that there were two duplication events so that domain I is genetically closer to III and II is closer to IV. The calcium-binding domain itself probably also arose by a duplication of a precursor gene to yield a helix–loop–helix Ca^{2+} binding structure of ≈40 amino acids long. Goodman *et al.* (38) further suggested that the first branch point in the evolutionary tree of the CaBPs separated all the other CaBPs from the vitamin D-dependent CaBPs, some of which also lost domains III and IV later in evolution (e.g., $CaBP_9$). In view of our finding that the $CaBP_{28}$ has a six-domain structure, an additional branch for the six-domain proteins has to be postulated.

Another feature common to some CaBPs (calmodulin, troponin C) is the ability to interact with other proteins. It will be interesting to see if $CaBP_{28}$ retained this property. A binding site for other proteins could be located either in an actively calcium-binding domain (as in calmodulin) or within one of the degenerated domains. A functional interaction with another protein(s) may be the reason for the observed interspecies conservation of $CaBP_{28}$ discussed above. The availability of the full-length cDNA probe will facilitate elucidation of the physiological function of the $CaBP_{28}$ protein.

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