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Tissue-specific expression of a chicken calmodulin pseudogene lacking intervening sequences

(genomic cloning/DNA sequence/gene evolution)

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ABSTRACT **An eel calmodulin cDNA probe has been used to isolate a calmodulin gene from a chicken DNA library. Sequence analysis revealed this calmodulin gene (*cCM1*) to contain the nucleotides that code for 148 amino acids, a termination codon, and 486 residues of 3′-noncoding sequence before an A-A-T-A-A-A poly(A) addition signal. The amino acid sequence derived from these nucleotides is 87% homologous to that of bovine brain calmodulin. *cCM1* is one of two calmodulin genes in the chicken genome but is unique in that it does not contain intervening sequences to interrupt the structural segments of the protein. This suggests that *cCM1* originated as a processed gene copy derived from the other calmodulin gene, *cCL1*, a circumstance usually associated with pseudogenes. In contrast, *cCM1* appears to be a functional member of a multigene family whose expression is specific for muscle cells.**

Calmodulin is a Ca^{2+}-binding protein that has been found in virtually every eukaryotic cell type (1, 2). It exists as a monomer of molecular weight 17,000 and contains four Ca^{2+}-binding sites (3). Calmodulin plays multiple roles in the regulation of cell function by mediating intracellular Ca^{2+} interactions. It appears to be constitutively expressed in a number of hormonally regulated systems including the hen oviduct (1), but it is found in increased concentrations in virally transformed cells (4, 5) and is regulated during the cell cycle (6).

Amino acid sequences of calmodulin from human, rat, cow, rabbit, sea anemone, sea scallop, sea pansy, and a protozoan, *Tetrahymena pyriformis*, have been reported (see refs. 1 and 7 for reviews). The sequence conservation of these calmodulins is striking, with the most distantly related, *T. pyriformis* and cow, differing by only 12 amino acids. Furthermore, there are 4 calcium binding domains with significant regions of internal sequence homology, indicating that the present calmodulin evolved from a smaller ancestral precursor by successive gene duplication events (1, 8).

Here, we report the use of an eel calmodulin cDNA probe to isolate a calmodulin gene from a chicken DNA library. An analysis of the sequence organization of the calmodulin gene based on restriction endonuclease analysis, heteroduplex mapping, and DNA sequence determination establishes a lack of introns in this gene. This gene, *cCM1*, codes for a calmodulin, or calmodulin-like, protein that differs significantly from all known calmodulins. Furthermore, *cCM1* seems to be expressed in the chicken in a tissue-specific manner.

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MATERIALS AND METHODS

Screening of a Chicken Genomic Library. Recombinant Charon 4A phages carrying the chicken gene library were screened for the calmodulin gene by using the *in situ* plaque-hybridization technique of Benton and Davis (9). Hybridization with ^{32}P-labeled calmodulin cDNA from *Electrophorus electricus* (10) was performed in 6× NaCl/Cit [1× NaCl/Cit (standard saline citrate) is 0.15 M NaCl/0.015 M sodium citrate] containing 0.04% Ficoll, 0.04% polyvinylpyrrolidine, 0.04% bovine serum albumin, 0.5% $NaDodSO_4$, and 1 mM Na_2EDTA at 60°C for 16 hr. The filters were washed at the same salt concentration and temperature.

Restriction Endonuclease Digests and Gel Electrophoresis. Restriction endonucleases were purchased from Bethesda Research Laboratories and New England BioLabs. Reactions were carried out according to the recommended assay conditions. Analytical as well as preparative agarose gel electrophoresis was carried out in a Tris acetate buffer as described by Lai *et al.* (11).

Nick-Translation, DNA Transfer, and Filter Hybridization. The procedure used to label DNA fragments by nick-translation has been described (12). DNA in agarose slab gels was transferred onto nitrocellulose filters by the method of Southern (13). The filters were hybridized to ^{32}P-labeled DNA probes as described by Lai *et al.* (12).

Subcloning of Calmodulin Gene Fragments in Plasmid pBR322. *Eco*RI-digested phage DNA (0.4 μg) was mixed with 0.1 μg of *Eco*RI-digested pBR322 and 75 units of T4 DNA ligase in 10 μl of ligase buffer (66 mM Tris·HCl, pH 7.5/6.6 mM $MgCl_2$/66 μm ATP/10 mM dithiothreitol). After incubation for 16 hr at 4°C, this ligated DNA was used to transform $CaCl_2$-treated *Eco*RI as described by Stein *et al.* (14). Plasmid DNA was isolated according to the procedure of Katz *et al.* (15).

DNA Sequence Determination. DNA fragments were treated with bacterial alkaline phosphatase and labeled at the 5′ ends with T4 polynucleotide kinase in the presence of γ-[^{32}P]ATP as described above. The labeled DNA was cut with an appropriate restriction endonuclease and the desired fragments were isolated from a 5% polyacrylamide slab gel. These fragments were degraded chemically according to the method of Maxam and Gilbert (16) and analyzed by electrophoresis on 0.3 mm urea/acrylamide gels according to the method of Sanger and Coulson (17).

Isolation of mRNA. White Leghorn hens were sacrificed by decapitation, and appropriate tissues were placed in liquid nitrogen immediately after dissection. Total nucleic acid was ex-

Abbreviations: NaCl/Cit, standard saline citrate; bp, base pair(s); kb, kilobase(s).

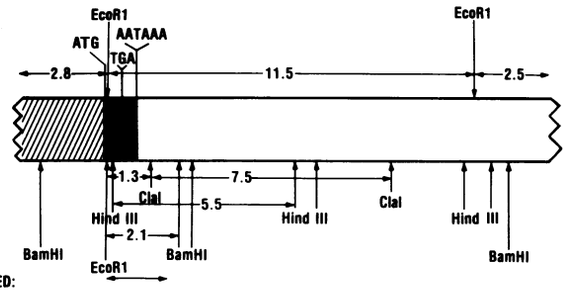

FIG. 1. Partial restriction map of a chicken calmodulin gene and its 5′ and 3′ flanking region. The sizes in kb of the three *Eco*RI fragments subcloned from the original phage isolate, CL10, are indicated above the figure. Also indicated above the figure are the positions in the sequence of the initiator codon ATG, the terminator codon TGA, and the polyadenylylation signal A-A-T-A-A-A. The extent of the sequenced region is indicated by the double-headed arrow. ■, Structural sequence; □, 3′ flanking sequence; ▨, 5′ flanking region.

tracted, and poly(A)$^+$ mRNA was isolated from the frozen tissue as reported (14).

RNA Blotting and Hybridization. mRNA (15–30 μg) was denatured in 50% formamide/6% formaldehyde, subjected to electrophoresis in a 1.5% agarose/6% formaldehyde gel (18), and transferred to nitrocellulose paper (19). The immobilized RNA was pretreated and hybridized to the appropriate [^{32}P]DNA probe at 42°C for 16 hr.

RESULTS

Structural Organization of a Calmodulin Gene Clone. pCM109 is a chimeric plasmid previously constructed in our laboratory containing a 350-base-pair (bp) calmodulin cDNA insert prepared from *E. electricus* mRNA (10). We screened 750,000 phage plaques from a chicken gene library for calmodulin gene clones by using ^{32}P-labeled pCM109 as the hybridization probe. One of the eight plaques that gave a positive hybridization signal, designated CL10, was selected for subcloning and sequence analysis. The genomic insert of phage CL10 was cut by *Eco*RI into fragments of 11.5, 2.8, and 2.5 kilobases (kb) organized as shown in Fig. 1. Only the 11.5-kb fragment hybridized to pCM109. Further restriction analysis resulted in the partial restriction map. The 0.25- and 5.5-kb *Hin*dIII fragments, the 2.1-kb *Eco*RI/*Bam*HI fragment, and the 1.3-kb *Cla* I/*Eco*RI fragment, but not the 0.5-kb *Bam*HI or 7.5-kb *Cla* I fragments, hybridized to a full-length eel calmodulin cDNA probe, pCM116 (20). Thus, the calmodulin sequences were located near the left-hand terminus of the 11.5-kb genomic fragment represented in Fig. 1.

Sequence Analysis of the Calmodulin Gene. For DNA sequence analysis all three of the genomic *Eco*RI fragments of phage CL10 were subcloned in pBR322. The sequence of a short region of genomic DNA within the fragments that hybridized to pCM116 was analyzed (Fig. 1). A 450-bp DNA sequence beginning near the end of the 2.8-kb *Eco*RI fragment and extending into the 11.5-kb *Eco*RI fragment is shown in Fig. 2. The sequence begins with the ATG initiation codon and extends in open reading frame through a termination codon at nucleotide 448. Not shown in this figure is the 3′-noncoding sequence that includes 486 nucleotides before an A-A-T-A-A-A polyadenylylation signal at nucleotide 937. The sequence of this gene, designated *cCM1*, is uninterrupted by intervening sequences.

The amino acid sequence translated from *cCM1* is also shown

```
    1
      ALA GLU ARG LEU SER GLU GLU GLN ILE ALA GLU PHE LYS GLU ALA PHE SER LEU PHE
ATG GCC GAG CGG CTG TCG GAG GAG CAG ATC GCT GAA TTC AAG GAG GCT TTT TCC CTT TTT
                                                                            60

20
  ASP ARG ASP GLY ASP GLY CYS ILE THR THR MET GLU LEU GLY THR VAL MET ARG SER LEU
  GAC CGC GAC GGC GAT GGC TGC ATC ACC ACG ATG GAG CTG GGC ACT GTC ATG CGC TCA CTG
                                                                           120

40
  GLY GLN ASN PRO THR GLU ALA GLU LEU GLN ASP MET VAL GLY GLU VAL ASP ALA ASP GLY
  GGG CAG AAC CCC ACC GAG GCG GAG CTG CAG GAC ATG GTG GGA GAG GTG GAC GCG GAC GGC
                                                                           180

60
  SER GLY THR ILE ASP PHE PRO GLU PHE LEU SER LEU MET ALA ARG LYS MET ARG ASP SER
  AGC GGC ACC ATT GAT TTC CCC GAG TTC CTC TCG CTG ATG GCG AGG AAG ATG AGG GAT TCA
                                                                           240

80
  ASP SER GLU GLU GLU ILE ARG GLU ALA PHE ARG VAL PHE ASP LYS ASP GLY ASN GLY TYR
  GAC AGC GAG GAG GAG ATC CGT GAA GCT TTC CGT GTC TTC GAC AAG GAC GGT AAT GGT TAC
                                                                           300

100
  ILE SER ALA ALA GLU LEU ARG HIS VAL MET THR ASN LEU GLY GLU LYS LEU THR ASP GLU
  ATC AGC GCG GCG GAG CTG CGG CAC GTC ATG ACC AAC CTG GGC GAG AAG CTG ACG GAC GAG
                                                                           360

120
  GLU VAL ASP GLU MET ILE LYS GLU ALA ASP CYS ASN ASN ASP GLY GLN VAL ASN TYR GLU
  GAG GTG GAT GAG ATG ATC AAG GAG GCA GAT TGC AAC AAT GAC GGG CAG GTC AAC TAC GAG
                                                                           420

140
  GLU PHE VAL ARG MET MET THR GLU LYS ***
  GAG TTT GTG AGG ATG ATG ACG GAG AAG TGA
                                       450
```

FIG. 2. Partial sequence of calmodulin gene *cCM1*. The sequence begins with the initiator codon, ATG, and ends with the terminator codon, TGA. The nucleotides are numbered below the line. The corresponding amino acid sequence is above the nucleotide sequence and is numbered above the line.

in Fig. 2. Encoded is a 148-amino acid protein that bears extensive sequence homology with the human, bovine, rat, and eel calmodulins. Of the 148 residues, 129 are identical to these other calmodulins (87% homology), including the invariant (trimethyl) lysine at position 115. However, this 13% sequence nonhomology represents a significant departure from the near identity of all other vertebrate calmodulins.

Hybridization of *cCM1* with Chicken DNA. Using the cloned 1.3-kb *Eco*RI/*Cla* I fragment of *cCM1* (CM1.3), we investigated its corresponding genetic complexity. High molecular weight DNA was prepared from hen liver and digested with *Bam*HI, *Eco*RI, and *Hin*dIII. Only *Hin*dIII cut within the sequences represented by the cloned probe. We subjected 15 μg of each digest to electrophoresis, transferred the DNA to nitrocellulose paper (13), and hybridized it with ^{32}P-labeled CM1.3 (Fig. 3). In all cases only one band was observed, and all had an intensity corresponding to that expected for a gene present in one copy per haploid genome. Fig. 1 would have predicted the presence of a second *Hin*dIII fragment 0.25 kb long. Presumably, its absence reflects the use of a 1% agarose gel to resolve the fragments.

Quite recently, however, as a result of screening a brain cDNA library, a second chicken calmodulin gene has been isolated (21). This gene, designated *cCL1*, codes for a calmodulin that is identical to the bovine, rat, and human calmodulins, and thus is 87% homologous to *cCM1* (see *Discussion*). It does, however, contain multiple intervening sequences. Southern blot analysis of *cCL1* under stringent hybridization conditions yields a unique set of hybridizing bands, again corresponding to one copy per haploid genome. Only under conditions of decreased stringency do *cCL1* and *cCM1* cross-hybridize. It thus appears that the chicken genome contains two different calmodulin genes in which coding sequences are related but sequence organization is considerably different.

Expression of Calmodulin mRNAs in Chicken Tissue. In light of the fact that two potential calmodulin genes exist in the chicken genome, it was of interest to examine their expression in different tissues. A cDNA probe for the *cCL1* gene and the cloned

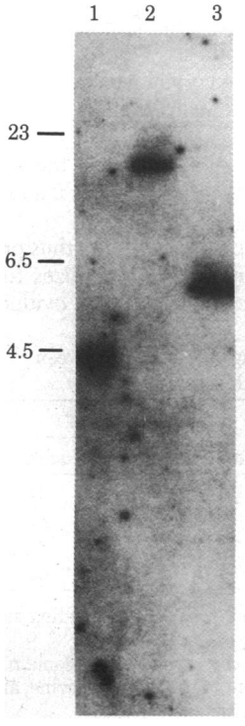

FIG. 3. Hybridization of calmodulin gene *cCM1* to hen liver DNA. *Bam*HI- (lane 1), *Eco*RI- (lane 2), or *Hin*dIII-digested (lane 3) DNA was separated on a 1% agarose gel, transferred to filter paper, and hybridized to CM1.3. Approximate size in kb is indicated.

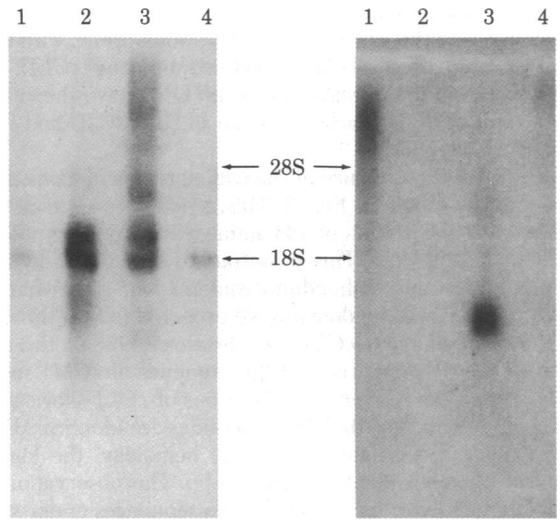

FIG. 4. Identification of calmodulin mRNA species in different chicken tissues. Poly(A)$^+$ mRNA (20 μg) prepared from chicken oviduct (lanes 1), brain (lanes 2), breast muscle (lanes 3), and liver (lanes 4) was run on a 1.5% agarose/6% formaldehyde gel. After transfer to nitrocellulose paper, the RNA was hybridized to a cDNA probe for gene *cCL1* (*Left*) or the 1.3-kb genomic *cCM1* fragment (*Right*). The positions of 28S and 18S rRNAs are indicated.

CM1.3 probe for *cCM1* were both labeled by nick-translation and hybridized against cytosolic poly(A)$^+$ mRNA prepared and separated on 1.5% agarose/formaldehyde gels. The results with mRNA from chicken oviduct, brain, breast muscle, and liver are shown in Fig. 4. Whereas the *cCL1* gene probe hybridizes to one (or more) mRNA species in all tissues examined, the *cCM1* probe hybridizes to a mRNA species in muscle only. The heterogeneous (>28S) oviduct RNA that hybridizes is not usually observed and presumably represents a hybridization artifact. The lone mRNA species to which *cCM1* hybridizes is smaller than any of the calmodulin 2 mRNAs. These data suggest that *cCL1* is the primary gene responsible for the production of calmodulin and that *cCM1* is a muscle-specific calmodulin (or calmodulin-like) gene.

DISCUSSION

Here, we report the sequence of a calmodulin gene. Because of the extensive sequence homology of calmodulins from diverse species, a short eel calmodulin cDNA was used to screen the chicken DNA library. This cDNA probe contained only 168 bp of coding sequences, which code for amino acids 93–148 of eel calmodulin (10); 182 bp of the probe represent 3′-noncoding sequences of the eel mRNA and show very little sequence homology to the 3′-noncoding region of the chicken calmodulin gene (*cCM1*) that we isolated. However, under the hybridization conditions of decreased stringency that were ultimately used (60°C and 6× NaCl/Cit), a number of positive hybridization signals were observed. All of these clones proved to contain identical or overlapping fragments of the same chicken gene. This fact together with the Southern hybridization data in Fig. 3 argue that the *cCM1* sequence represents a unique gene. However, our preliminary data suggested that a mRNA complementary to this gene is not present in every chicken tissue. These observations, together with the fact that calmodulin exists in all chicken tissues, raised the possibility that multiple calmodulin genes exist in the chicken genome. To test this hypothesis, a cDNA library prepared from chicken brain mRNA was screened by using a full-length eel calmodulin cDNA (20). Indeed, a brain cDNA representing a calmodulin mRNA not

identical to the chicken gene *cCM1* was found, isolated, and recloned. Using this cDNA as a hybridization probe, Putkey *et al.* (21) isolated a second chicken calmodulin gene (*cCL1*). Hybridization experiments against chicken DNA have shown that it is also unique in the chicken genome and that *cCM1* and *cCL1* cross-hybridize poorly.

The nucleotide sequence of the coding region of calmodulin gene *cCM1* is shown in Fig. 2. This gene sequence codes for a calmodulin-like protein of 148 amino acids uninterrupted by intervening sequences. This is contrary to our original hypothesis (22–24); because calmodulin contains four somewhat homologous Ca^{2+}-binding domains we expected to find three introns. Calmodulin gene *cCL1* does, however, contain three (or more) introns. A comparison of the sequence of *cCM1* shown in Fig. 2 with the sequence of the exons of *cCL1* shows a homology of 74%; 333 of the 450 nucleotides are identical. Even though this is a substantial sequence homology, the longest stretch of sequence identity is only 14 bp. This observation explains the poor hybridization of the two sequences under stringent hybridization conditions. Furthermore, the overall sequence homology demonstrates that the two genes are related and suggests that gene *cCM1* might have originated as a processed gene copy derived from *cCL1*.

Processed genes normally contain a poly(A) sequence at their 3′ end. So far a sequence of 750 nucleotides 3′ to the TGA termination codon in *cCM1* has been determined, and no poly(A) sequence has been found. However, *cCL1* contains at least two polyadenylylation signals, one located at 857 and the other located at 1,157 nucleotides past the TGA codon (21). One of these termination sites would have been used in creating the mRNA from which gene *cCM1* was presumably processed, and so the poly(A) sequence has not yet been reached by our sequence analyses. This conclusion suggests that the A-A-T-A-A-A polyadenylylation signal of *cCM1* indicated in Fig. 1 that would produce a mRNA of the size observed in Fig. 4 was created after reinsertion of gene *cCM1* into the genome.

The amino acid sequence coded for by *cCM1* is also shown in Fig. 2. The sequence of 148 amino acids after the initiator methionine is typical of all calmodulins isolated to date. Eighty-seven percent of the amino acid residues are identical to human (25), rat (26), and bovine (27) calmodulins and to the protein sequence translated from *cCL1* (21). The 19 amino acid differences between calmodulin 1 and these other calmodulins are listed in Table 1. Interestingly, 11 of these differences are nonconservative substitutions. This represents a significant variation for calmodulins, because the largest difference among vertebrate calmodulins previously analyzed is one amino acid. Even *T. pyriformis* contains a calmodulin that differs from human calmodulin at only 12 positions, and only 5 of those substitutions are nonconservative.

Examination of the amino acid substitutions in the Ca^{2+}-binding subdomains of *cCM1* suggests the possibility of functional changes in the calmodulin coded for by this gene. Based on sequence analogies to metal-binding sites proposed by Kretsinger and Barry (28) for parvalbumin and troponin C, Dedman *et al.* (26) determined the probable metal-binding sites of calmodulin proteins. These four Ca^{2+}-binding subdomains of *cCM1* are listed in Table 2. Subdomain 3 is identical to previously sequenced calmodulins, and subdomain 2 is 91% homologous (only one amino acid difference). However, subdomains 1 and 4 are only 75% homologous to the corresponding Ca^{2+}-binding subdomains in the other vertebrate calmodulins. Significantly, the single change in subdomain 2 is an asparagine, which is one of the six coordination residues for Ca^{2+}, to a serine, which still could participate in the coordination of a Ca^{2+}. Thus, subdomains 2 and 3 could have fully functional Ca^{2+}-binding sites.

Table 1. Differences in amino acid sequences derived from chicken calmodulin genes *cCM1* and *cCL1*

Position	*cCM1*	*cCL1*
2	Glu	Asp
3*	Arg	Gln
5	Ser	Thr
21	Arg	Lys
26*	Cys	Thr
30*	Met	Lys
52*	Val	Ile
53*	Gly	Asn
60*	Ser	Asn
70	Ser	Thr
71*	Leu	Met
77	Arg	Lys
79	Ser	Thr
126	Lys	Arg
130*	Cys	Ile
131	Asn	Asp
132*	Asn	Gly
143*	Arg	Gln
147*	Glu	Ala

* Nonconservative substitutions.

The changes in subdomains 1 and 4 are more extensive, however. Two of the three differences in subdomain 4 are in critical residues implicated in Ca^{2+}-binding (asparagine-131 and glycine-132), and the third change is the substitution of a bulky cysteine residue for the adjacent isoleucine-130. In subdomain 1, three residues are replaced by bulkier groups, including the substitution of a cysteine for threonine-26, which is located in the center of the Ca^{2+}-binding subdomain. These changes themselves would probably be sufficient to severely impede the binding of Ca^{2+} to these two subdomains. In addition, the presence of a cysteine residue in both subdomains suggests that a disulfide bond might even be formed that would bridge the central portions of the first and fourth Ca^{2+}-binding sites. It thus seems likely that, for whatever reason, this variant calmodulin has evolved to the point where it functions as a two-domain Ca^{2+}-binding protein.

The nucleotide homologies of the Ca^{2+}-binding subdomains listed in Table 2 also support this hypothesis. Whereas the second and third subdomains of *cCM1* are very homologous to the corresponding regions in *cCL1*, subdomains 1 and 4 are considerably less so (64% and 61%, respectively). If these two subdomains no longer functioned as Ca^{2+}-binding sites, then the sequence would be less constrained than subdomains 2 and 3, and evolutionary "drift" would be more rapid.

cCL1 is expressed in all tissues (Fig. 4; ref. 21) and thus provides the bulk of chicken calmodulin. *cCM1* hybridizes to a smaller mRNA found in breast muscle but not in brain, oviduct,

Table 2. Conservation of Ca^{2+}-binding subdomains between chicken calmodulin 1 and other calmodulins

		% Homology†	
Subdomain	Amino acids*	Amino acid	Nucleotide
1	20–31	75	64
2	56–67	91	81
3	93–104	100	83
4	129–140	75	61

* Position of amino acid residues involved in each Ca^{2+}-binding subdomain.

† % homology of each subdomain with the corresponding subdomain in chicken calmodulin 1, which is equivalent to the human, bovine, and rat sequence.

or liver. Preliminary experiments further indicate that *cCM1* is expressed in skeletal and cardiac muscle but not in smooth muscle. *cCM1* clearly does not code for a troponin C, because only 54% of the residues are identical to bovine troponin C, which is roughly 50% homologous to calmodulin. The sequence homologies and tissue specificity of expression indicate that *cCM1* codes for a calmodulin-like protein that may have a Ca^{2+}-binding function somehow concerned with striated muscle physiology.

The lack of intervening sequences in this calmodulin gene was unexpected. The extraordinarily high sequence homology of the four domains of calmodulin is a cogent argument that this protein has evolved via two successive duplications of a primordial single-domain Ca^{2+} binding protein (8). In light of other evidence concerning the sequence organization of genes that have evolved by successive duplications, such as ovomucoid (24), we believe that the evolution of this calmodulin gene would have resulted in the presence of three intervening sequences located between domains 1 and 2, 2 and 3, and 3 and 4. It is likely, therefore, that three (or more) introns were present in a primordial calmodulin gene but were subsequently lost by some mechanism of gene conversion. Several examples of such processed genes have recently been published. A mouse pseudo α-globin gene has moved from the α-globin cluster on chromosome 11 to a position on chromosome 15 and is flanked by retrovirus-like elements (29). Other processed genes that are flanked by short direct repeats have been described, including human small nuclear RNA genes (30), a rat α-tubulin gene (31), and human immunoglobulin (32) and β-tubulin genes (33). All of these processed genes are pseudogenes; they are nonfunctional because of numerous point mutations and deletions. A different type of processed pseudogene has also been discovered—the human metallothionein II_B gene (34). Even though this gene has an intact coding sequence, it is apparently not expressed because it lacks functional promoter sequences near its 5′ end. These discoveries imply that RNA transcripts of functional genes, or their DNA cognates, can be reinserted at a distant chromosomal site to generate processed gene copies.

The potential applicability of this type of mechanism to the origin of *cCM1* was enhanced by the discovery of a second chicken calmodulin gene, *cCL1*, that apparently contains the predicted three introns. We hypothesize that gene *cCM1* was originally derived as a processed copy of the primordial calmodulin gene *cCL1*. This processed copy, lacking the intervening sequences, was inserted back into the genome and has since evolved into a functional calmodulin-like gene, *cCM1*, that codes for a Ca^{2+}-binding protein that binds only two molar equivalents of Ca^{2+}. This gene has an intact coding sequence, and preliminary evidence that it produces a mRNA in certain muscle tissues has been obtained. This hypothesis predicts that some form of repeat sequence flanks both ends of the gene, a poly(A) tract exists at the 3′ end of the gene, and regulatory sequences that direct tissue-specific expression of this gene exist in the 5′ flanking region. Indeed, preliminary sequence analysis has revealed the presence of a 9-bp direct repeat flanking both ends of *cCM1*.

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1. Means, A. R., Tash, J. S. & Chafouleas, J. G. (1982) *Physiol. Rev.* **62**, 1–39.
2. Chafouleas, J. G., Dedman, J. R., Munjaal, R. P. & Means, A. R. (1979) *J. Biol. Chem.* **254**, 10262–10267.
3. Watterson, D. M., Sharief, F. & Vanaman, T. C. (1980) *J. Biol. Chem.* **255**, 962–975.
4. Watterson, D. M., Van Eldik, L. J., Smith, R. E. & Vanaman, T. C. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2711–2715.
5. Chafouleas, J. G., Pardue, R. L., Brinkley, B. R., Dedman, J. R. & Means, A. R. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 996–1000.
6. Chafouleas, J. G., Bolton, W. E., Hidaka, H., Boyd, A. E. & Means, A. R. (1982) *Cell* **28**, 41–50.
7. Klee, C. B. & Vanaman, T. C. (1982) *Adv. Protein Chem.* **35**, 213–321.
8. Goodman, M., Pechere, J. R., Haiech, J. & Demaille, G. (1979) *J. Mol. Evol.* **13**, 331–352.
9. Benton, W. D. & Davis, R. W. (1977) *Science* **196**, 180–182.
10. Munjaal, R. P., Chandra, T., Woo, S. L. C., Dedman, J. R. & Means, A. R. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2330–2334.
11. Lai, E. C., Woo, S. L. C., Dugaiczyk, A. & O'Malley, B. W. (1979) *Cell* **16**, 201–211.
12. Lai, E. C., Stein, J. P., Catterall, J. F., Woo, S. L. C., Mace, M. L., Means, A. R. & O'Malley, B. W. (1979) *Cell* **18**, 829–842.
13. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
14. Stein, J. P., Catterall, J. F., Woo, S. L. C., Means, A. R. & O'Malley, B. W. (1978) *Biochemistry* **17**, 5763–5772.
15. Katz, L., Kingsbury, D. R. & Helinski, D. R. (1973) *J. Bacteriol.* **114**, 577–591.
16. Maxam, A. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 560–564.
17. Sanger, F. & Coulson, A. R. (1977) *FEBS Lett.* **87**, 107–110.
18. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5201–5205.
19. Rawe, N., Crkvensjakov, R. & Boedtker, H. (1979) *Nucleic Acids Res.* **6**, 3559–3567.
20. Lagace, L., Chandra, T., Woo, S. L. C. & Means, A. R. (1983) *J. Biol. Chem.* **258**, 1684–1688.
21. Putkey, J., Tsui, K.-F., Tanaka, T., Lagace, L., Stein, J. P., Lai, E. C. & Means, A. R. (1983) *J. Biol. Chem.* **258**, in press.
22. Gilbert, W. (1978) *Nature (London)* **271**, 501.
23. O'Malley, B. W., Roop, D. R., Lai, E. C., Nordstrom, J. L., Catterall, J. F., Swaneck, G. E., Colbert, D. A., Tsai, M.-J., Dugaiczyk, A. & Woo, S. L. C. (1979) *Recent Prog. Horm. Res.* **35**, 1–46.
24. Stein, J. P., Catterall, J. F., Kristo, P., Means, A. R. & O'Malley, B. W. (1980) *Cell* **21**, 681–687.
25. Sasagawa, T., Ericsson, L. H., Walsh, K. A., Schreiber, W. E., Fischer, E. H. & Titani, K. (1982) *Biochemistry* **21**, 2565–2569.
26. Dedman, J. R., Jackson, R. L., Scheiber, W. E. & Means, A. R. (1978) *J. Biol. Chem.* **253**, 343–346.
27. Kasai, H., Kato, Y., Isobe, T., Kawasaki, H. & Okuyama, T. (1980) *Biomed. Res.* **1**, 248–264.
28. Kretsinger, R. H. & Barry, C. D. (1975) *Biochim. Biophys. Acta* **405**, 40–52.
29. Leuders, K., Leder, A., Leder, P. & Kuff, E. (1982) *Nature (London)* **295**, 426–428.
30. Van Arsdell, S. W., Denison, R. A., Bernstein, L. B., Weiner, A. M., Manser, T. & Gesteland, R. F. (1981) *Cell* **26**, 11–17.
31. Lemischka, I. & Sharp, P. A. (1982) *Nature (London)* **300**, 330–335.
32. Hollis, G. F., Hieter, P. A., McBride, O. W., Swan, D. & Leder, P. (1982) *Nature (London)* **296**, 321–325.
33. Wilde, C. D., Crowther, C. E., Cripe, T. P., Lee, M. G.-S. & Cowan, N. J. (1982) *Nature (London)* **297**, 83–84.
34. Karin, M. & Richards, R. (1982) *Nature (London)* **299**, 797–802.