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COMMON STRUCTURAL AND EXPRESSIONAL PROPERTIES OF VERTEBRATE CALDESMON GENES+

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SUMMARY: We have determined the genomic structure of chicken caldesmon (CaD) gene. The gene, 100-150 kilobases long, is composed of 17 exons. Exons 1a-1, 1a-2, and 1a-3 encode the 5'-terminal sequence specific to the mRNAs for CaDs expressed in gizzard. Exon 1b encodes the 5'-terminal sequence of the brain *l*-CaD and locates downstream of exons 1a-1, 1a-2, and 1a-3. The genomic construction of the chicken CaD resembles with that of the human CaD. Exon 3 of chicken CaD gene posseses the unique structure similar to that of human CaD gene; the common domain in both *h*- and *l*-CaDs (amino acid residues 74-199 for *h*-CaD and residues 66-191 for *l*-CaD) and the central repeating domain specific to *h*-CaD (amino acid residues 200-419) are encoded in exons 3a and 3b, respectively. Of particular interest is that the two consensus 5'-splice sites are found in the borders between exons 3a and 3b, and exon 3b and intron. Therefore, the expressional regulation between *h*- and *l*-CaDs can be explained by selection of these 5'-splice sites. Alternative 3'-splice sites also exist at intron/exon junction of exon 14 and the difference in selection of the sites would induce the specific Ala-508 insertion in the brain *l*-CaD.

Caldesmon (CaD), a calmodulin- and actin-binding protein originally purified from gizzard smooth muscle, plays a vital role in the regulation of smooth muscle and nonmuscle actin-myosin interaction (1,2). It is widely expressed in smooth muscle and nonmuscle cells but not in skeletal and heart muscles. The two major isoforms of CaD have been identified; *h*-CaD (high *Mr*, 120,000-150,000) and *l*-CaD (low *Mr*, 70,000-80,000) as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sequencing analyses have revealed that the deduced *Mrs* of *h*- and *l*-CaDs are 89,000 and 59,000, respectively (3-5). *h*-CaD is predominantly expressed in smooth muscles, while *l*-CaD widely distributes in nonmuscle tissues and cells (6-8). Notably, the change in expression of the CaD isoforms is tightly associated with phenotypic modulation of smooth muscle cells, in which the CaD expression converts from *l*- to *h*-form during differentiation and *vice versa* (9-12). Chicken and human CaD isoforms are well characterized by cDNA cloning (3-5 and 13-16). The calmodulin-,

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Abbreviations: CaD, caldesmon; kb, kilobases; PCR, polymerase chain reaction.

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actin-, and tropomyosin-binding sites of chicken CaD isoforms have been identified in the common COOH-terminal domain involving the regulation of actin-myosin interaction (4, 5). The major parts of the NH_2-terminal domain are highly conserved in CaD isoforms. The short NH_2-termini of chicken gizzard *h*-CaD and brain *l*-CaD are individually unique sequences, and the central repeating domain of *h*-CaD is deleted in the *l*-CaD molecule. In the brain *l*-CaD, alanine is inserted at amino acid residue 508 (5). The identical sequence of the short NH_2-terminus of the gizzard *h*-CaD has been also found in the chicken *l*-CaD isoform (13). Human CaD subtypes are categorized by their short NH_2-terminal sequence, HeLa- and fibroblast (WI-38)-types (14-16). Genomic analysis has revealed that human CaD gene is composed of, at least, 14 exons and is mapped to a single locus, 7q33-q34. Exons 1 and 1' encode the short NH_2-termini specific to HeLa and fibroblast CaDs, respectively. Exon 3 shows the unique structure; the common domain of *h*- and *l*-CaDs is encoded in exon 3a and the central repeating domain of *h*-CaD is in exon 3b. The consensus sequences for the 5'-splice site are found in the borders between exons 3a and 3b, and exon 3b and intron (16). Therefore, alternative selections of exon 3a or 3ab arise isoform diversity of human CaDs. Here, we demonstrate the genomic structure of chicken CaD gene and discuss regarding the common expressional mechanism of vertebrate CaD genes.

MATERIALS AND METHODS

Genomic library screening and sequencing. A chicken genomic library was constructed with λDASH II. First, we introduced the cassette primer-linked PCR (polymerase chain reaction) method (17, 18) using specific antisence primer corresponding to the sequences of NH_2-terminal and/or 5'-noncoding regions of the *h*- and *l*-CaD cDNAs, and then used amplified DNA fragments as probes to isolate genomic clone carrying 5'-noncoding region of *h*- or *l*-CaD mRNA. We have obtained three nonoverlapping clones (λG102, λG211, and λB11) by this method. We have further cloned three overlapping clones (λ1, λ12, and λ9) using chicken *h*-CaD cDNA (4) as a probe. Each genomic clone was characterized by Southern blotting to identify the DNA fragments containing exons, and subcloned the fragments and sequenced by dideoxy-chain termination methods (19).

Southern blot analysis. High molecular weight DNA was purified from chicken embryonic tissues as described elsewhere (20) and was digested with indicated restriction enzyme. The DNA fragments were separated on 0.7% agarose gel electrophoresis, and transferred onto a nylon membrane (Hybond-N+, Amersham) according to the method of Southern (21). ^{32}P-labeled probes were prepared by *Bca Best* DNA polymerase (Takara Shuzo, Japan) using random primers or specific primer or by T_4 polynucleotide kinase. Hybridization and washing were carried out under the indicated conditions.

Pulsed field gel electrophoresis. Agarose plugs containing 2 x 10^6 cells of chick embryo fibroblasts were digested with *Not*I or *Sse*8387I, and then the plugs were applied to 1% agarose gel. DNA fragments were separated by pulsed field gel electrophoresis using CHEF-DRII (Bio Rad) according to the instructions recommended by the manufacturer, transferred onto a nylon membrane, and hybridized with indicated random-primed ^{32}P-labeled probe.

RESULTS

Three nonoverlapping clones, λG102, λG211, and λB11 were isolated using DNA fragments amplified by the PCR method described under MATERIALS AND METHODS. λG102 carried the 5'-noncoding region (nucleotides -78) of gizzard *h*-CaD cDNA (4) (exon 1a-

1), and λG211 carried the 5'-noncoding and the short NH_2-terminal sequences (nucleotides 173-284) of the *h*-CaD cDNA (exon 1a-3). The 5'-noncoding and the short NH_2-terminal sequences (nucleotides -47) of brain *l*-CaD cDNA (5) (exon 1b) were encoded in λB11. Further screening using *h*-CaD cDNA fragments resulted in the isolation of three overlapping clones, λ12, λ1, and λ9 which carried the common exons of the gizzard and brain CaD subtypes (exons 2-14) (Fig 2). As a result, we have identified the exons of chicken CaD gene and found that their intron/exon junctions (Table 1) are compatible with the consensus sequence for splicing (22). However, it is still unclear whether the gizzard and brain CaD subtypes are encoded in a single gene or multiple copy genes because both λG211 and λB11 did not overlapped with λ12. We introduced pulsed field gel electrophoresis to answer this

Table 1. Exon organization of chicken CaD gene

3'-splice site	Exon	Size (bp)	5'-splice site
GTGTGCCTTT	1a-1	82	CACCGCTGAGgtaaggcat
ND TGATCACCTC	1a-2	94	TTGCTTTCCT ND
tcccgtttagGTTTGGATTG	1a-3	112	AAGCAGAGAGgtgaggttgc E A E23
AGCTAATAAT	1b	323	GTCTTTCCAAgtgaagttgt S L S15
tttgttgcagACTGTCCTAC R L S Y K	2	147	CCCAGAACAGgtaaatggtt A Q N72
ttttgtgtagTGTGGCAGAA S V A E	3	1039	AAGAAAACAGgtacagtaaa L R K Q419
cataacaaagGAGGAAGACA E E D	4	81	AAAAGATCAGgtaatttata K K D Q446
ttctgagcagGTAAAAGACA V K D	5	149	ATGCTTTTGGgtaagttcca N A F495
tctttcccagACGCTCCAAC G R S N	6	184	CAGAGAGGAGgtaagtgtaa I R E E557
ttaattccagGAGGAAAAGA E E K	7	141	ATCTCTCAAGgtaacctccc S S L K604
tctcttccagATAGAAGAAC I E E	8	44	CTCAGAAGAGgtaaaaaaaa A Q K618
ctccctgaagTGGTATGAAA S G M K	9	79	CGCAGTTGTGgtaagttaat S A V V645
tcttttgcagGGCAACAAGG G N K	10	138	ACCAAATAAGgtacgtattt T P N K619
tattctgtagGAAACTGCTG E T A	11	96	AAAACCTTCTgtaagttaat P K P S723
aaatacacagGATTTAAGAC D L R	12	84	TTCTTCAAAGgtaacacatt S S S K751
tttcaaacagGTAACAGCTA V T A	13	34	GAGACTAATGgtaagtaatt E T N762
tttctctcagCAGGTTTGAG A G L	14	ND	

Exon sequences are shown in capitals and intron sequences are in lowercase letters. The amino acid sequences encoded by each exon are indicated by one letter, and their position numbers at each 5'-splice site are from the chicken gizzard *h*-CaD sequence (4). The sequences and position number with underline are from the brain *l*-CaD sequence (5). Sizes of exons are given in base pairs. Exon 1a-2 is expected from Southern blotting (Fig. 1) and published cDNA sequence (4). ND; not determined.

question. Intact chicken chromosomal DNAs were digested with restriction endonucleases for genomic mapping, *Not*I or *Sse*8387I, and the resulting DNA fragments were analysed by pulsed field gel electrophoresis and Southern blotting. DNA fragments located 5'-upstream of exons 1a-1 and 1b were used as probes. The identical DNA fragments, 600 kilobases (kb) and 150 kb generating by *Not*I and *Sse*8387I digestion, were respectively hybridized with two probes (Fig 1A). We speculated from these results that the specific exons for gizzard and brain CaD subtypes would be in the same gene. *Sse*8387I fragment with 150 kb is the maximal length of chicken CaD gene. However, the positional relationships between the gizzard CaD subtype-specific exons and the brain CaD subtype-specific exon are still unclear and we could not isolate a genomic clone carrying a part of the 5'-noncoding sequence of the *h*-CaD cDNA (nucleotides 79-172). To reveal the relationships and the genomic character of such sequence, we carried out a series of Southern blotting analysis. First, we used the following DNA fragments as probes; the 5'-upstream DNA fragments of exons 1a-1 and 1b and the exon 2-specific DNA fragment amplified by PCR. An identical *Spe*I fragment (9 kb) was hybridized with two probes specific to the brain CaD subtype 5'-upstream region and the exon 2,

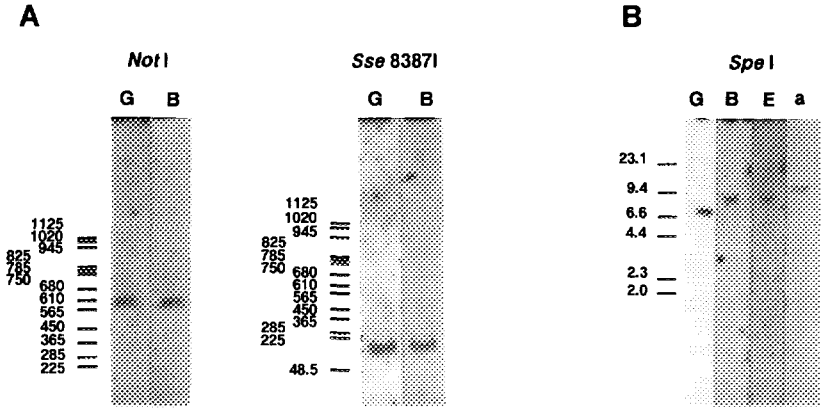


Fig. 1. **Southern blot analysis of the chicken genomic DNA by pulsed field gel electrophoresis (A) and normal gel electrophoresis (B).** A *Hin*dIII/*Spe*I fragment (0.65 kb) from λG102 spanning from the exon 1a-1 to its 5'-upstream region and a *Pst*I fragment (0.7 kb) from λB11 located 5'-upstream of exon1b were used as probes specific to the gizzard and the brain CaD subtypes, respectively. Exon 2-specific probe (0.1 kb) was prepared by PCR using λ12 as a template with a sense oligonucleotide (nucleotides 286 - 304 of the *h*-CaD cDNA (4)) and an antisense oligonucleotide (corresponding to nucleotides 361-384 of the cDNA). A synthesized DNA (probe a) corresponds to nucleotides 79-172 of the cDNA. Genomic DNA digested with *Not*I or *Sse*8387I and *Spe*I were separated by pulsed field and normal gel electrophoresis and then transferred onto nylon membranes. Hybridization and washing were carried out in 50% formamide solution as described elsewhere (5) except the exon 2-specific probe and probe a. Hybridization was done without formamide at 54°C (for the exon 2-specific probe) or 50°C (for probe a) and the membranes were washed in 2xSSC-0.1% SDS at 54°C (for the exon 2-specific probe) or in 6xSSC-0.1% SDS at 50°C and then in 2xSSC-0.1% SDS at 45°C (for probe a). G, B, and E indicate probes specific to the gizzard CaD subtypes, brain CaD subtype and exon 2, respectively. a indicates probe a. After removal of probe according to protocol recommended by the manufacturer, the nylon membranes were reused for hybridization with different probes. Size markers indicated in kb are from chromosomal DNA of *S. cerevisiae* (A) and *Hin*dIII-digested λ DNA (B).

respectively (Fig. 1B, lanes B and E) and the gizzard CaD subtype 5'-upstream region-specific probe hybridized to a 7 kb DNA fragment (Fig. 1B, lane G). These results suggest that the exons 1b and 2 are present in the same *Spe*I fragment which locates downstream of the gizzard subtype CaDs-specific exons. Second, a synthesized DNA, which is composed of 94 nucleotides (nucleotides 79-172 of the *h*-CaD cDNA), was used as a probe (probe a). Probe a hybridized to a single DNA fragment (10 kb) generating by *Spe*I digestion (Fig. 1B, lane a). A single DNA fragment, 2.5 kb *Pst*I fragment or 5 kb *Sph*I fragment, was also hybridized to this probe (data not shown). From these analysis, the 5'-noncoding sequence (nucleotides 79-172 of the *h*-CaD cDNA) is postulated to be encoded in a single exon (exon 1a-2). Considering the results of Southern blottings and the estimated molecular sizes of the isolated genomic clones, we have concluded that the chicken CaD isoforms are generated from a single copy gene composed of 17 exons with 100-150 kb long. Genomic organization of chicken CaD gene is schematically shown in Fig. 2.

We have already published a unique structure of exon 3 of human CaD gene (16). The same structure is found in exon 3 of chicken CaD gene; the common domain of the CaD isoforms (amino acid residues 74-199 for gizzard residues*h*-CaD and residues 66-191 for brain *l*-CaD) is encoded in exon 3a, whereas the central repeating domain specific to *h*-CaD (amino acid residues 200-419 for the *h*-CaD) resides in exon 3b. The two consensus sequences for 5'-splice site are found in the borders between exons 3a and 3b, and exon 3b and intron (indicated by underlines in Fig. 3A). Bryan *et. al.* have reported the primary structure of chicken smooth muscle *h*-CaD (3). One repeating unit based on a glutamic acid-rich sequence, EEER (or K) KAAEERERAKA (4), within the central repeating domain was deleted in their sequencing data. This discrepancy has been clarified by genomic analysis, in which the sequence of exon 3b is completely identical with our cDNA data. Exon 4 encodes the sequence specific to *h*-CaD. The human *l*-CaD isoforms with the sequence of exon 4 (*l*-CaDs I) are identified by molecular cloning and reverse transcription PCR (16), while these *l*-CaD isoforms

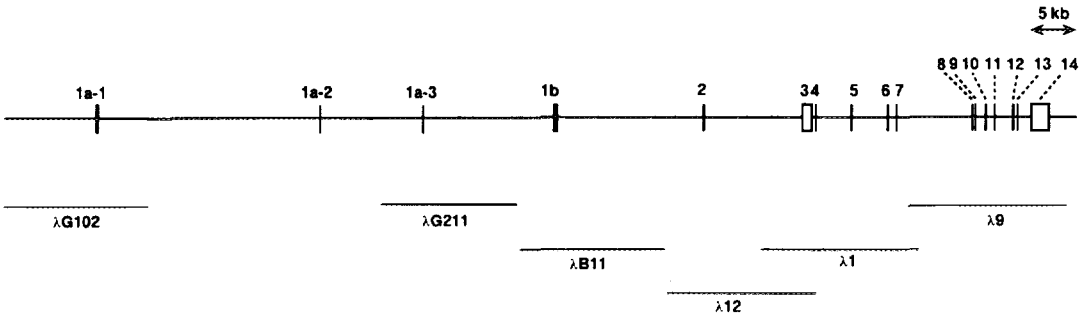
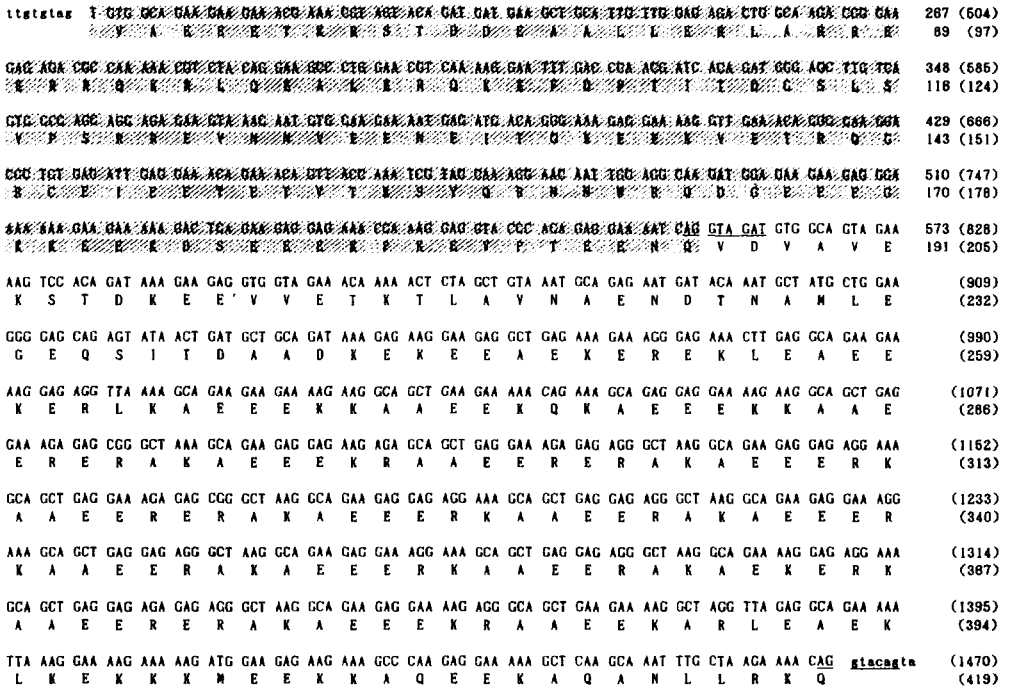


Fig. 2. **Genomic organization of chicken CaD gene.** Boxes and lines indicate exons and introns, respectively. 17 exons were totally identified in chicken CaD gene, exons 1a-1, 1a-2, and 1a-3 are specific to the gizzard CaD subtypes and exon 1b is specific to the brain CaD subtype. Genomic clones isolated from the chicken library are shown under the alignment map. Three clones, λ12, λ1, and λ9 are overlapped, but other clones are not. Exon 1a-2 is expected from Southern blotting (Fig. 1) and *h*-CaD cDNA sequence (4).

A



B

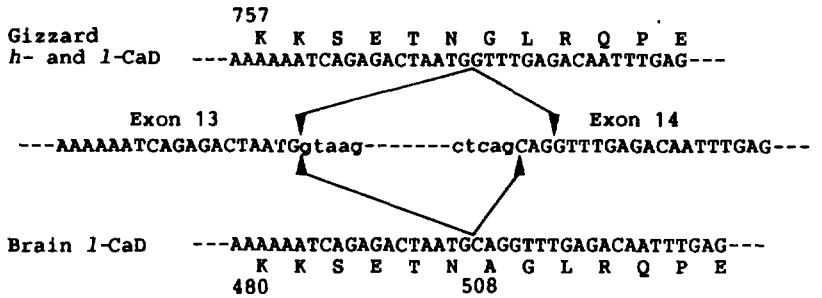


Fig. 3. **Nucleotides sequence of exon 3 with flanking introns (A) and alternative splicing mechanism between exons 13 and 14 (B).** A; The sequences in the exons and introns are shown by capitals and lowercase letters, respectively. The sequence in exon 3a is indicated with shaded box. The consensus sequence of 5'-splice sites for exons 3a and 3ab are underlined. The nucleotides and the deduced amino acid sequences from the brain *l*-CaD cDNA (5) are numbered at the right hand of the each line, and the parenthesized numbers are from the gizzard *h*-CaD cDNA (4).

have not been identified in chicken cells. Insertion of alanine at residue 508 in the brain *l*-CaD (5) would depend on selection of the two 3'-splice sites at the intron/exon junction of exon 14; the 3'-splice site at downstream is functional in the gizzard CaD, in contrast, the upstream site is preferentially selected in the brain CaD (Fig. 3B). We have sequenced about 2.0 kb from the 5'-end of exon14 to 3'-end (data not shown). Four polyadenylation signals, AATAAA, are

present in this DNA fragment, but it is not well elucidated whether a fixed signal is preferentially selected or multiple signals are functional.

Comparison of nucleotide identity and size of exons between chicken and human CaD genes (16) shows that their structures well resemble each other (Table 2). Exon distributions in the both genes are also highly conserved. Exons 2-14 of the chicken gene and exons 2-13 of the human gene are concentrated in a range of 40-50 kb, whereas the upstream exons, exons 1a-1, 1a-2, 1a-3, and 1b of the chicken gene and exons 1 and 1' of the human gene, diverse in a wide range. Exons 1 and 1' of the human gene correspond to exons 1b and 1a-3 of the chicken gene, respectively. The 5'-terminal sequences of exon 13 of the chicken and human genes are 5'-GTAACAGCTA--- and 5'-GTTTGAGACG---, respectively. In the human exon, translational termination codon, TGA, indicated by underline is present in this exon. Exons of the human gene corresponding to exons 1a-1, 1a-2, and 14 of the chicken gene have not been found.

DISCUSSION

In this study, we demonstrate the genomic structure of chicken CaD gene and that the gene is a single copy. Intron/exon structures of CaD genes between chicken and human well resemble each other. The unique structure of exon 3 in chicken and human CaD genes is highly conserved. As demonstrated previously (10), the expressional change of *h*- and *l*-CaDs is closely associated with phenotypic modulation of smooth muscle cells. The present genomic analysis indicates that the expressional regulation of *h*- and *l*-CaDs depends on selection of the two 5'-splice sites within exon 3. Therefore, the unusual splicing mechanism involving the expressional regulation between *h*- and *l*-CaDs in association with phenotypic modulation of smooth muscle cells might be a common molecular event without distinction of vertebrates. The selection of alternative 3'-splice sites at the intron/exon junction of the exon 14 determines one amino acid insertion. Neuron-specific *c-src* protein also has a short insertional sequence resulting from alternative splicing (23). These evidences suggest a unique splicing mechanism specific to neuronal cells. Considering the genomic structure and published cDNA sequences, the respective 5'-upstream regions of exons 1a-1 and 1b would be distinctive promoters,

Table 2. Comparison of exons of CaD genes between chicken and human

Exon No.	1a-1	1a-2	1a-3	1b	2	3a	3b	4	5	6	7	8	9	10	11	12	13	14
Chicken [size (bp)]	82	94	112	323	147	380	660	81	149	184	141	44	79	138	96	84	34	ND
Human [size (bp)]	ND	ND	112	ND	147	403	687	78	146	262	141	44	82	138	96	81	ND	ND
Nucleotide identity (%)			74		72	70	65	77	71	62	85	84	77	79	82	72		

ND; not determined.

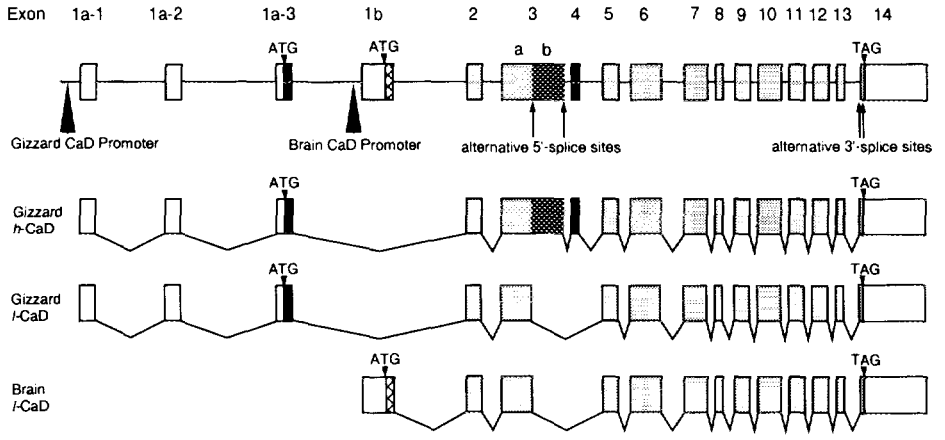


Fig. 4. **A model for generation of three CaD isoforms by combination of differential RNA transcription and alternative splicing.** Expression mechanism of three chicken CaD isoforms, the gizzard *h*- and *l*-CaDs and the brain *l*-CaD, are schematically shown. Shaded boxes are the common exons in all CaD isoforms, and the exons encoding the short NH_2-terminal sequences of the gizzard CaD subtypes and the brain CaD subtype are indicated by hatched and cross hatched boxes, respectively. Solid boxes are specific exons to *h*-CaD. 5'- and 3'-noncoding sequences are shown by open boxes. Two putative promoters and the initiation and termination codons for translation are indicated by large and small arrow heads, respectively.

respectively. We summarize alternative splicing pathways of the chicken CaD gene (Fig. 4). Gizzard and brain CaD subtype mRNAs would be transcribed from independent promoters and exons 2 and 5-14 are spliced in the mRNAs for all CaD isoforms. Exons 1a-1, 1a-2, and 1a-3 are specific to the mRNAs for gizzard CaD subtypes, and Exon 1b are specific to the brain CaD subtype mRNA. Exons 3ab and 4 are spliced in the *h*-CaD mRNA, whereas only exon 3a is selected for the *l*-CaDs mRNAs.

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