Calcium mediates numerous cellular responses and plays a pivotal role in the regulation of cellular homeostasis (1). These diverse cellular activities of Ca\(^{2+}\) are mediated by cytoplasmic Ca\(^{2+}\) receptors of which calmodulin is the major one in non-muscle cells (2). Calmodulin is a highly conserved acidic monomeric polypeptide (M, \(\sim 16,700\)) that is present in eukaryotic cells as diverse as mammals (3-6), invertebrates (7-9), green plants (10,11), and fungi (12,13). The calmodulin gene has been studied primarily in the cDNA form in human (14), chicken (15), electric eel (16), rat (17), toad (18), trypanosome (19), and slime mold (20). The genomic form of the gene has been well analyzed in chicken (21), rat (22), and trypanosome (19). Chicken and rat calmodulin genes have 5 introns in the coding region whereas the Drosophila gene has 3 introns. The three tandemly repeated calmodulin genes of trypanosomes are identical and intronless; so it is not surprising, they have less resemblance to calmodulins from invertebrates such as Drosophila and trypanosomes when compared to mammalian calmodulins.

We have shown in a series of studies that Achlya klebsiana has a strong physiological dependence on calcium. Growth (23), energy-linked transport of amino acids (24), nucleosides (25), and sugars (26), and the process of sporulation (27) were all shown to be dependent on the availability of Ca\(^{2+}\); Ca\(^{2+}\) therefore, must play a pivotal role in cellular homeostasis of Achlya. In support of the notion that Ca\(^{2+}\) is important for sporulation, Suryanarayana et al. (28) isolated calmodulin from Achlya ambisexualis and showed that its induction was associated with cell wall lysis during sporulation (29). I show here that A. klebsiana has a functional intronless calmodulin gene which is induced to produce an abundant quantity of calmodulin transcripts when it is undergoing sporulation.

**MATERIALS AND METHODS**

**Organisms**—A coenocytic freshwater mold, A. klebsiana, was used as the source of the calmodulin gene studied.

**Induction of Sporulation**—Hyphal cells derived from sporangiospores germinated and grown vegetatively for 20 h at 24 °C in defined medium, were harvested and resuspended in sporulation induction medium as described (30).

**Isolation of High Molecular Weight DNA**—About 20 g (fresh weight) of sporangiospores were homogenized using liquid nitrogen and ground to a fine powder with mortar and pestle. The powder was suspended in 50 ml of buffer of composition 0.1 M NaCl, 0.1 M Na\(_2\)EDTA, 50 mM Tris-Cl, pH 8, and 1% Sarkosyl. Proteinase K was added to a final concentration of 50 μg/ml and incubated for 1 h at 37 °C. An equal volume of 0.1 M NaCl, 0.1 M Na\(_2\)EDTA, 50 mM Tris-Cl, pH 8)-saturated "phenol" (redistilled phenol mixed with 0.1% 8-hydroxyquinoline and 0.5% 2-mercaptoethanol) was added. The solution was gently mixed by hand for 10 min. The mixture was centrifuged at 10,000 × g for 10 min at room temperature to separate the phases. The upper aqueous phase was recovered and phenol extraction of proteins repeated three times or until the interface was free of precipitate. The aqueous fraction was extracted twice with an equal volume of CHCl\(_3\). One-tenth volume of 3 M NaOAc, pH 5.2, was added to the aqueous phase and nucleic acids precipitated by layering 2.2 volumes of chilled absolute ethanol over the nucleic acid solution and DNA recovered by spooling with a clean glass rod. The DNA was redissolved in TE (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) buffer and resuspended. This process was repeated three times and the final spooled DNA was air-dried for 10 min before dissolving in a small volume of TE buffer to a concentration of 0.5-1.0 mg/ml.

**Construction of Genomic Library andScreening for Calmodulin Gene**—DNA isolated as described above was consistently greater than 80 kb.\(^1\) The DNA was partially digested with restriction endonuclease MboI and size-fractionated in sucrose density gradients. DNA fragments of 15-23 kb were selected and ligated into the BamHI site of pBR322. The library was screened with \(^32\)P-labeled calmodulin cDNA (pCM1166) provided by John Putkey and Anthony Davis of Baylor College, Houston, TX by the method of Benton and Davis (31).

**Preparation of cDNA—Poly(A)\(^+\)** RNA was isolated from vegetatively growing and sporulation induced Achlya cells (32) and converted to double stranded cDNA by fast protein liquid chromatography.

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\(^1\) The abbreviation used is: kb, kilobase(s).
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phy-pure avian myeloblastosis virus reverse transcriptase, RNase H, E. coli DNA ligase, and DNA polymerase I combinations as recommended by Pharmacia (Uppsala, Sweden). [3H]dCTP was used during synthesis of the second strand to monitor the reaction and estimate yield.

Genomic Southern Blot Hybridization—High molecular weight DNA was digested to completion with a variety of restriction enzymes, and the nucleic acid fragments were separated in 0.7% agarose gel slabs by electrophoresis and then transferred to Hybond (Amer sham Corp.) nylon sheets by the alkaline method of Reed and Mann (33). Hybridization was carried out using, as probes, the 2.0- and 1.7-kb DNA fragments representing the 5' and 3' ends, respectively, of Achlya calmodulin. Achlya DNA probes were labeled with 32P by the random primer method (34).

RNA Blot Hybridization—Poly(A)⁺ RNA was isolated as described (32) and electrophoresed in 1.2% agarose denaturing gel containing 6% formaldehyde and transferred to Hybond. RNA on Hybond was probed with a 3.8-kb DNA fragment containing the full Achlya calmodulin gene. The probe was 32P-labeled by nick translation (35).

S1 Nuclease Mapping—The S1 nuclease transcript mapping procedure of Berk and Sharp (36) was used to evaluate whether the calmodulin gene isolated was transcriptionally active in vivo and to determine the approximate initiation and termination sites of transcription of the gene. Total RNA was isolated from calmodulin-induced cells and used to hybridize against the 2.0- and 1.7-kb ClaI DNA fragments representing the 5' and 3' ends of the fungal calmodulin gene. These two DNA fragments contain several hundred nucleotides of untranslated borders of the gene. A single ClaI site situated about 90 nucleotides from the 5' end of the coding sequence of the gene was used as the reference point in estimating the transcription initiation and termination sites. S1 nuclease-insensitive DNA-RNA hybrids as well as standards were analyzed in nondenaturing 12% agarose gels according to Maniatis et al. (37).

Preparation of Phagemid M13/Calmodulin DNA Recombinants—Phagemids M13⁺ and M13⁻ (Stratagene, San Diego, CA) were linearized with ClaI and ligated independently to 2.0- and 1.7-kb ClaI fragments recovered from a recombinant clone isolated from the genome, and known to contain the fungal calmodulin gene. The phagemid recombinants were used to transform E. coli JM107.

Isolation of DNAs from Gels—DNA in agarose gels were recovered by the "GeneClean" technique (Bio 101 Lab., La Jolla, CA).

Exonuclease III and Mung Bean Nuclease Deletions—Phagemid recombinants containing the 2.0- and 1.7-kb DNA fragments were linearized with ApaI to yield 3' extended ends; this was followed by Sall digestion which created a 5' extension at one of the two ApaI ends in both phagemids. In case of the 2.0-kb-containing M13 recombinant, a 0.2-kb fragment was removed from the insert by Sall resulting in a 1.8-kb/M13 recombinant phagemid. The 1.8-kb/M13 and the 1.7-kb/M13 phagemid were foreshortened unidirectionally by the combined action of exonuclease III and mung bean nuclease by the method of Henikoff (38). The deleted phagemids were ligated to either ApaI, DraI, EcoRV, or Sall restriction endonucleases, and the DNA fragments were separated in 0.7% agarose gels by electrophoresis and then transferred to Hybond (Amer sham Corp.) nylon sheets by the alkaline method of Reed and Mann (42). [35S]dATP was used as the radioactive label in DNA sequencing reactions.

Computer Analysis—The DNA sequence was analyzed by the Beckman MicroGenie program (Beckman Instruments Inc., Palo Alto, CA).

Biohazard Precautions—All cloning experiments and disposal of recombinant cells were performed in compliance with the Medical Research Council of Canada guidelines.

 Autoradiography—DNA sequencing gels were exposed, after drying, to Kodak X-Omat AR film for 1-3 days. Other autoradiograms were prepared with Kodak X-Omat RP films.

RESULTS

Isolation of Fungal Calmodulin Gene—About 50,000 recombinant bacteriophages constructed as described under "Materials and Methods" and representing more than five genome equivalents of the freshwater mold A. klebsiana were screened with 32P-labeled electric eel calmodulin cDNA as probe. Two positive clones were isolated. Both clones had DNA inserts of about 15 kb and identical restriction endonuclease digestion patterns (not shown). Consequently, one was selected for further study.

Restriction Endonuclease Map of Calmodulin Gene—DNA from the recombinant bacteriophage harboring the calmodulin gene was digested with several restriction endonucleases including BamHI, Clal, DraI, HindIII, Hpal, and Sall, the fragments electrophoresed, Southern blotted, and probed with 32P-labeled electric eel calmodulin cDNA. Although a single strongly hybridizing band of 3.8 kb was detected in the Sall digest products, two positively hybridizing bands of 2 and 1.7 kb observed in the Clal digest products were selected for subcloning and structural analysis of the gene. The rationale was that the two Clal DNA fragments probably represented the 5' and 3' ends of the gene, as proved to be the case.

A detailed restriction endonuclease map of the cloned 15-kb genomic fragment containing the calmodulin gene was obtained using the 2- and 1.7-kb subfragments as probes (Fig. 1, lower region). Location and orientation of the coding region in the 2- and 1.7-kb fragments was achieved by using as probes the 0.19-kb PstI DNA fragment from electric eel cDNA which represents the 5' end of the gene and the 0.75-kb PstI DNA fragment which represents the 3' end of the gene (16) (Fig. 1, upper region).

Evidence for Existence of Single Calmodulin Gene—Total genomic DNA and 15-kb DNA fragment containing the calmodulin gene were doubly digested with a combination of Clal and either ApaI, DraI, EcoRV, or Sall restriction endonucleases. Southern blots of these digests were probed with 2-kb Clal DNA fragment (representing the 5' end of the gene) and the 1.7-kb Clal DNA fragment (representing the 3' end of the gene). The results (Fig. 2) show that the genomic DNA and the cloned 15-kb DNA have the same hybridization patterns. Two minor hybridizing bands appearing in the Clal/ApaI digest of the cloned DNA probed with the 5' end of the gene are artifacts because no ApaI site exists in the 2- or 1.7-kb fragment; a feature that was exploited in preparing progressive unidirectional deletions for sequencing of the gene (see

![Fig. 1. Restriction endonuclease map of a cloned 15-kb DNA fragment containing Achlya calmodulin gene. The upper part is an exploded view of the gene and its border sequences. The lower part is the genomic fragment cloned in EMBL3 arms. A, Aval; B, BglII; C, Clal; D, DraI; E, EcoRI; F, EcoRV; H, HindIII; K, KphI; P, PstI; S, Sall; X, XbaI; Bam, BamHI. (Note: The short arm of EMBL3 is on the left and the long arm is on the right.)](image-url)
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FIG. 3. Nested deletions of A. klebsiana calmodulin genomic gene bisected by ClaI into 2-kb (a) and 1.7-kb (b) DNA fragments that were ligated and cloned separately in phagemid M13+. Clone A, in both cases, represents undeleted fragments. Clone B in a is SalI truncation of the 2-kb DNA insert. All other clones are exonuclease III/mung bean nuclease deletions. Arrow signifies region of each clone that was sequenced from either the T7 (○) or T7 (●) promoters. The rectangular boxes specify the coding regions of the gene.

fungal calmodulin gene was deduced from the DNA sequence and the information is incorporated into Fig. 4.

The assumption is made that like other calmodulins, the first amino acid of the mature protein succeeds the initiation codon AUG. This means that the protein is 148 amino acid residues long with alanine at the amino end and lysine at the carboxyl end. The protein sequence obtained is 92% homologous to electric eel calmodulin. A comparison of the amino acid sequence of calmodulins from human, electric eel, trypanosome and Achlya (Fig. 5) shows that compared to human, electric eel calmodulin has a single amino acid difference. Achlya calmodulin has 11 amino acid replacements and trypanosome has 11 also. Assuming that Achlya calmodulin has a tertiary structure that is similar to those of other eukaryotic calmodulins, four structural Ca2+ binding domains are recognizable (Fig. 6). An interesting feature is that single EcoRI and ClaI sites are present at identical positions of the coding sequences (codons 67-68 and 130-131, respectively) of Achlya and Drosophila (21) calmodulin genes. This feature is not observed in the calmodulins of chicken (15), Dictyostelium (20), electric eel (16), and rat (17), which reflects, to some extent, differences in the nature of codons used by these organisms.

Within the 5' and 3' flanks of the coding region presented are several TATA box homologies and many CAAT-like (CAAT and CAAAT) consensus sequences at the 5' end and two AAATAA-like homologies at the 3' end. Whether these sequences serve as transcription signals for the gene identified cannot be affirmed with the existing data.

Accumulation of Calmodulin mRNA during Development—Suryanarayana and Thomas (29) have shown that during sporulation in A. ambisexualis, the cells accumulated high levels of calmodulin which were localized by immunofluorescence to sporangial discharge papillae and the spores themselves. Achlya cells induced to develop sporangia and sporulate were analyzed for calmodulin mRNA content during the induction phase (starvation). Poly(A)+ RNA was isolated from starving cells at different times, electrophoresed, transferred to nylon filter and probed with a 3.8-kb SalI cleaved DNA fragment containing the full length of the calmodulin gene and hundreds of nucleotides of its 5' and 3' end flanks. The results (Fig. 7a) show that there was a single poly(A)+ RNA hybridizing band whose intensity increased with time. The most dramatic increase occurred within the first 2 h. When
L-glutamine, which arrests sporangial development (30), was added to starving cells the level of calmodulin poly(A)+ RNA recovered from the cells diminished markedly (Fig. 7b). This implies that enhanced transcription of the calmodulin gene may be linked to asexual differentiation in this organism.

Isolated Calmodulin Gene Is Transcribed—Although the cloned calmodulin gene hybridized to blotted poly(A)+ RNA recovered from the cells, it was necessary to show that it represents a gene that is transcribed in vivo. Indirect evidence for this was obtained by S1 nuclease transcript mapping. Total cell RNA was hybridized to (a) the 2-kb ClaI DNA fragment labeled at the 5' end and represented the 5' end of the gene and (b) the 1.7-kb ClaI site to the sole transcribed in the 3' end of the gene. Following S1 nuclease hydrolysis of unprotected regions of the hybrids, the products were sized with neutral 1.2% agarose gel (Fig. 8). The approximate size of the fragment defining the transcriptional start site to the sole ClaI site in the coding sequence was 790 nucleotides, while the size of the fragment from the same ClaI site to the transcriptional termination point was about 370 nucleotides. These results are compatible with single transcriptional start and stop sites for the gene being expressed under nutrient starvation conditions.

DISCUSSION

The amino acid sequence of Achlya calmodulin gene was deduced from the DNA sequence shown in Fig. 4. When it is compared to the amino acid sequence of calmodulins from trypanosome (19), electric eel (16), and the uniform sequence found in mammals such as human (14), chicken (15), and rat (17), and the amphibian Xenopus (18), it is seen that the fungal protein is 92% identical to the mammalian calmodulin and 93% identical to that of trypanosome (Fig. 4). Whereas Achlya and trypanosome calmodulins have 11 amino acid calcium binding domains. Each domain is defined by a stretch of amino acids running from residues 12 to 39, 48 to 75, 85 to 112, and 121 to 148 (43), and each fits the helix-loop-helix test sequence motif proposed by Kretsinger (44). The deduced amino acid sequence of Achlya calmodulin can also be divided into four structural calcium binding domains (Fig. 6). Analysis of the homology that exists between the four domains (calcium binding) of the Achlya and trypanosome calmodulins has 11 amino acid sequence motif proposed by Kretsinger (44). The deduced amino acid sequence of Achlya calmodulin can also be divided into four structural calcium binding domains (Fig. 6). Analysis of the homology that exists between the four domains (calcium binding) of the Achlya and trypanosome calmodulins has been sequenced (21). Unlike the fungal (this report) and protozoan proteins, it is segmented into introns and exons. The amino acid sequence of the insect calmodulin differs from the mammalian protein at only 3 residue positions: 99, 143, and 147. Interestingly, the replacements at these positions are the same as found in the fungal and protozoan calmodulins. As Smith et al. (21) have noted, the presence of serine at position 147 seems, therefore, to be a characteristic of practically all invertebrates.

All calmodulins studied so far are composed of four similar calcium binding domains. Each domain is defined by a stretch of amino acids running from residues 12 to 39, 48 to 75, 85 to 112, and 121 to 148 (43), and each fits the helix-loop-helix test sequence motif proposed by Kretsinger (44). The deduced amino acid sequence of Achlya calmodulin can also be divided into four structural calcium binding domains (Fig. 6). Analysis of the homology that exists between the four domains (calcium binding) of the Achlya and trypanosome calmodulins has been sequenced (21). Unlike the fungal (this report) and protozoan proteins, it is segmented into introns and exons. The amino acid sequence of the insect calmodulin differs from the mammalian protein at only 3 residue positions: 99, 143, and 147. Interestingly, the replacements at these positions are the same as found in the fungal and protozoan calmodulins. As Smith et al. (21) have noted, the presence of serine at position 147 seems, therefore, to be a characteristic of practically all invertebrates.
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**Fungal Calmodulin Gene**

<table>
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<th>Eel</th>
<th>Human</th>
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**FIG. 5.** Comparison of amino acid sequences of calmodulins from *A. klebsiana*, trypanosome, electric eel, and human. Sites where residue differences occur are boxed. * identifies boxed residues that have changed between the species but remain identical in *Achlya* and vertebrate calmodulins. * identifies boxed residues that have changed between the species but in a similar fashion in *Achlya* and trypanosome.

**FIG. 6.** Possible four Ca\(^{2+}\) binding domains of *A. klebsiana* calmodulin.

The amino acid sequence in the calcium binding regions of the loops are identical to those of vertebrate calmodulin except for Phe-99 in domain II and Ile-130 in domain IV. * identifies amino acid residues that can participate in calcium binding. * identifies amino acid replacement in *Achlya* protein when compared to mammalian calmodulin.

Domain I......

<table>
<thead>
<tr>
<th></th>
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Domain II......

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Domain III......

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<th>Ala</th>
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</table>

Domain IV......

|   | Ile | Asp | Gly | Asp | Gly | Glu | Ile | Asn | Tyr | Glu | Glu |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|

binding region only) at the nucleic acid and protein levels is summarized in Table I. Domains I and III are nearly 70% homologous whereas domains II and IV are less than 56% homologous at the nucleotide level. At the amino acid level, domains I and III are 58.3% homologous while domains II and IV are only 50% homologous. In most comparisons of the various domains, there is a higher homology between the various domains at the nucleotide than amino acid level. This is the general pattern that has been observed for calcium binding domain homologies in other calmodulin genes (16, 21, 43, 45). Although 2 of the amino acid residue replacements, Phe-99 and Ile-143 fall into the calcium binding domains (Fig. 6), their effect on the binding function and loop structure is negligible because phenylalanine can replace tyrosine in bind-
Fungal Calmodulin Gene

Homology between nucleotide and amino acid sequences of putative calcium binding domains I through IV of Achlya calmodulin

Homology implies presence of identical nucleotides and amino acids at corresponding positions in the domains.

<table>
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<tr>
<th>Domains</th>
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<td>I × II</td>
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<tr>
<td>I × IV</td>
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<td>58.3</td>
</tr>
<tr>
<td>II × III</td>
<td>47.2</td>
<td>58.3</td>
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<tr>
<td>II × IV</td>
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</tr>
<tr>
<td>III × IV</td>
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</tr>
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</table>

Table II

Amino acid composition of calmodulins from Achlya, trypanosome, eel, and human

| M, values: Achlya, 16,567; trypanosome, 16,709; eel, 16,680; human, 16,760. |

**A. ambisexualis**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>no. of residues/molecule</th>
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<tbody>
<tr>
<td>Alanine</td>
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</tr>
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<tr>
<td>Arginine</td>
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<tr>
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<td>6</td>
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<td>Glycine</td>
<td>17</td>
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<tr>
<td>Histidine</td>
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</tr>
<tr>
<td>Isoleucine</td>
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<tr>
<td>Leucine</td>
<td>12</td>
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<tr>
<td>Lysine</td>
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<td>Methionine</td>
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<td>Valine</td>
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* Based on amino acid analysis of protein.
* Based on deduced protein sequence obtained from gene sequence.

The occurrence of a single calmodulin gene in the genomes of widely disparate organisms such as Drosophila (21), chicken (15), and Dictyostelium (20) has been reported. As well, multiple tandem copies and two non-allelic calmodulin genes have been reported for trypanosome (19) and Xenopus (18), respectively.

Fig. 7. RNA blots of poly(A)* RNA from A. klebsiana and hybridization to electric eel calmodulin cDNA. a, poly(A)* RNA from cells that were incubated in sporulation medium for 1, 2, and 3 h, and from vegetatively (V) growing cells that were sampled at the time of transfer of the cells from growth to induction medium. b, same as for a, but the induction medium contained 5 mM L-glutamine. Five micrograms of RNA was applied in every lane. Arrowheads signify hybridizing transcripts.

Fig. 8. S1 nuclease protection analysis of Achlya transcripts. S1 nuclease protection reactions were carried out with approximately 20 µg of total RNA and either 5' end-labeled 2-kb Clai DNA fragment representing the 5' end of the calmodulin gene or 3' end-labeled 1.7-kb Clai DNA fragment representing the 3' end of the calmodulin gene. Lane 1, Bethesda Research Laboratories 1-kb DNA size markers electrophoresed in 1.2% neutral agarose gel. Southern blotted, and probed with 32P-labeled 1-kb ladder DNA. Lane 2, 2-kb Clai DNA fragment. Lane 3, S1 nuclease protected hybrid of total RNA and 2-kb Clai DNA. Lane 4, 1.7-kb Clai DNA fragment. Lane 5, S1 nuclease protected hybrid of total RNA and 1.7-kb Clai DNA. Lane 6, same as for lane 4. Samples in lanes 2-6 were electrophoresed in 1.2% neutral agarose gel. Southern blotted, and probed with [α-32P]dATP nick-translated 3.8-kb Sall DNA fragment containing the entire coding sequence of the gene and hundreds of nucleotides at the 5'- and 3'-untranslated ends. Small arrows indicate the positions of the resulting hybrids.
But with the exception of trypanosome and Achlya, the gene appears to be segmented into introns and exons in every other case, including humans. 

The DNA sequence results presented includes about 500 nucleotides of presumed 5′-untranslated region, 447 nucleotides encoding the calmodulin protein and about 320 nucleotides of presumed 3′-untranslated region (Fig. 4). The 5′-untranslated region has several TATA- and CAAT-like sequence homologies, but it is not known which (if any) function in transcription. An interesting arrangement of two CAAT-like sequence homologies in direct repeat mode is present between −441 and −457. Within the 5′-untranslated region are several in frame stop codons, one of which is only 6 nucleotides upstream from the assigned translation start codon. Such structural organization implies that (a) the protein is unlikely to be synthesized as a secretable entity, (b) Achlya calmodulin is not synthesized as a precursor protein, and (c) the translation start codon designated is probably correct. The 3′-untranslated end is replete with in-frame translation stop codons, three of them occurring within 40 nucleotides of the assigned translation stop codon. Two possible closely set stop codons, three of them occurring within 40 nucleotides of the translated region, have several TATA- and CAAT-like sequence homologies, but it is not known which (if any) function in transcription. The translation start codon designated is probably correct.

In summary, this communication has presented evidence showing that A. kleebsiiana has a single type of calmodulin gene that does not have any introns but does have transcription signals sequence homologies similar to those of eukaryotic genes. Evidence is also presented to show that the gene is probably expressed in vivo as a single mRNA species. The protein encoded by this gene is quite typical of calmodulins in general.

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REFERENCES


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