

## Identification of the Single Gene for Calmodulin in *Dictyostelium discoideum*

HARRY GOLDHAGEN AND MARGARET CLARKE\*

Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461

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**We report the isolation and sequence determination of a cDNA containing most of the coding sequence for *Dictyostelium discoideum* calmodulin. The cloned cDNA was used as a probe to examine the complexity of *D. discoideum* genomic DNA. These studies indicated that *D. discoideum* cells possess a single calmodulin gene.**

The calcium-binding protein calmodulin has been implicated in mediating the regulatory effects of calcium on a number of essential cellular activities (for a review, see references 9 and 20). We studied calmodulin from the eucaryotic microorganism *Dictyostelium discoideum*. This is one of the simplest eucaryotes as judged by the complexity of the genomic DNA (6). It possesses a calmodulin protein very similar but not identical to the calmodulins of higher organisms (1, 11). *D. discoideum* calmodulin is therefore potentially useful for exploring structure-function relationships of calmodulins. In addition, the possibility of combining genetic and biochemical techniques makes *D. discoideum* well suited to an analysis of the roles of calmodulin. This is particularly true with respect to the role of calmodulin in the regulation of motility because many of the *D. discoideum* proteins involved in the production of movement have already been characterized (for a review, see reference 18) and motility mutants have been isolated (4). Furthermore, as we show here, *D. discoideum* cells contain a single calmodulin gene, which should simplify a mutational analysis of the protein.

A cDNA library had been prepared from poly(A)<sup>+</sup> mRNA isolated from 8-h developing cells of *D. discoideum* and cloned into the expression vector bacteriophage λgt11 at its unique *EcoRI* site (2); this library was generously provided by S. Cohen and H. Lodish. The library was probed with oligonucleotides synthesized with an Applied Biosystems 380A DNA synthesizer (12) and purified as described elsewhere (J. Chase, manuscript in preparation). The oligonucleotide sequences were chosen from the amino acid sequence of *D. discoideum* calmodulin (11) by using preferred codons predicted from other *D. discoideum* genes (7). The library was screened by hybridization of end-labeled oligonucleotides to nitrocellulose filters containing replicas of the bacteriophage plaques. Standard methods were followed during the screening and subsequent cloning steps (10).

The first probe was a mixture of two oligonucleotides [5'-dGT(T/C)AAAATGATGATTGT] based on the amino acid sequence Val-Lys-Met-Met-Ile-Arg found near the carboxy terminus of *D. discoideum* calmodulin (Fig. 1). Hybridization was carried out at 40°C for 3 h. This probe identified four positive colonies among 35,000 screened.

Two of the four isolated clones contained DNA that hybridized (at 42°C) to a second probe mixture, [5'-dGA(T/C)AC(T/C)GAAGAAGAAT], based on the amino acid sequence Asp-Thr-Glu-Glu-Glu-Ile found near the center of the protein (Fig. 1). Restriction digests indicated that both of these strains contained lambda DNA that had an insert with a size of approximately 0.5 kilobase pairs (kbp); in neither case could the insert be excised by using *EcoRI* alone.

One of the calmodulin cDNA inserts was subcloned as an *EcoRI-PvuI* fragment into plasmid pBR322 that had been cut with the same two enzymes. The resulting plasmid, pDCM1, contained nucleotides 1 to 3737 from the pBR322 vector (19) and an insert composed of the calmodulin cDNA and part of the adjoining *lacZ* sequence (nucleotide pairs 1791 to 3016 [3]) from bacteriophage λgt11. The direction of transcription of the calmodulin sequence was opposite to that of *lacZ* transcription. The total fragment length (1.7 kbp as determined by agarose gel electrophoresis) minus the length of the expected *lacZ* sequence gave an estimate of 450 base pairs (bp) for the cDNA fragment, long enough to encompass all or most of the calmodulin gene.

The sequence of the calmodulin cDNA is shown in Fig. 1. The entire coding region is present, except the region coding for the 12 amino acids at the NH<sub>2</sub> terminal end, along with 23 bp of the 3' untranslated region. The predicted amino acid sequence confirmed the existence of 12 amino acids in this portion of the protein that are different from those in mammalian calmodulin, including an additional residue at the carboxy terminus (11).

The plasmid pDCM1 was labeled by nick translation (15) and used in an analysis of genome complexity by the method of Southern (17). Genomic DNA was purified essentially as described by Maizels (8) from axenically grown vegetative cells of *D. discoideum*, and 5-μg portions were digested with the restriction enzymes *XbaI*, *NdeI*, *EcoRI*, *EcoRV*, *BclI*, *HindIII*, *BglII*, *PvuII*, and *DdeI*. The digests were resolved on a 0.7% agarose gel, and the restriction fragments were transferred to nitrocellulose and probed with pDCM1. Hybridization was carried out at 65°C overnight under buffer conditions described by Maniatis and co-workers (10); the filters were washed in 30 mM NaCl-3 mM sodium citrate (pH 7.0)-0.1% sodium dodecyl sulfate at 65°C. Six of the enzymes yielded a single band (Fig. 2), indicating that there is only a single gene for calmodulin in *D. discoideum* cells.

\* Corresponding author.

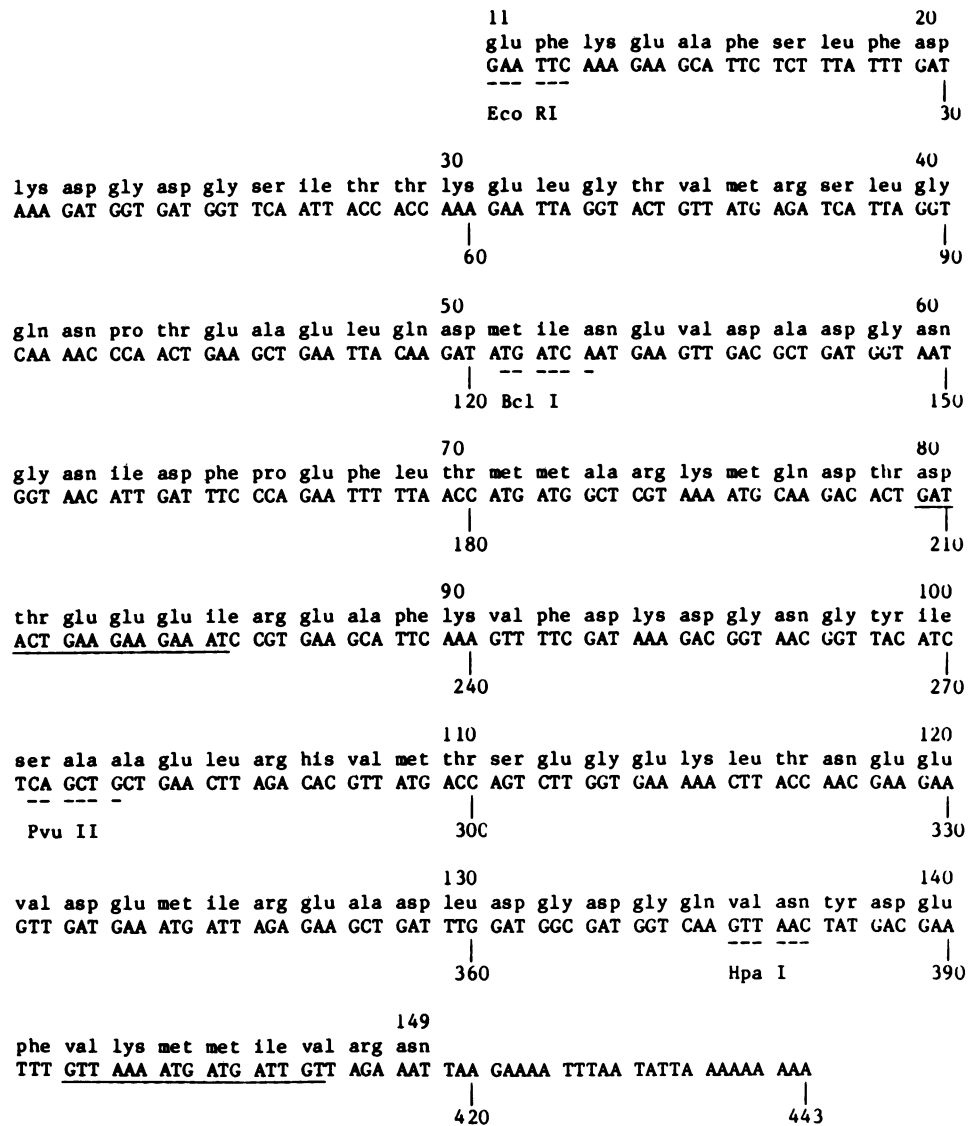


FIG. 1. Nucleotide sequence and restriction map of *D. discoideum* calmodulin cDNA. The translated amino acid sequence is shown above the nucleotide sequence and agrees exactly with the sequence of the protein determined previously (11). The numbering of the amino acids follows the convention used for numbering other calmodulins, although there are preliminary data suggesting that the *D. discoideum* protein has an extension at its amino terminus (11). Selected restriction sites are shown by broken lines. The portions of the sequence corresponding to the oligonucleotide probes are underlined. In each case, the probe mixtures included an oligonucleotide that agreed exactly with the actual cDNA sequence. Sequences were determined by the methods of Maxam and Gilbert (13) and Sanger and co-workers (16). Both DNA strands were sequenced in their entirety.

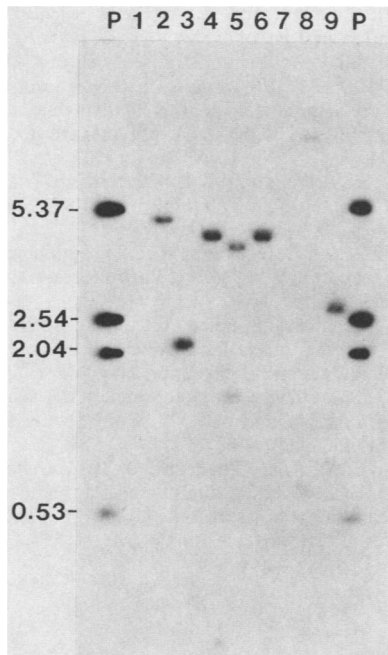


FIG. 2. Detection of DNA fragments containing calmodulin sequences in genomic DNA of *D. discoideum*. Genomic DNA was purified from vegetative amoebae of *D. discoideum*, digested with the indicated restriction enzymes, and analyzed by the method of Southern (17). The probe was plasmid pDCM1, labeled by nick translation. The enzymes used were *Xba*I (lane 1), *Nde*I (lane 2), *Eco*RI (lane 3), *Eco*RV (lane 4), *Bcl*I (lane 5), *Hind*III (lane 6), *Bgl*II (lane 7), *Pvu*II (lane 8), and *Dde*I (lane 9). Single bands were seen for all of these enzymes except *Pvu*II, *Bcl*I, and *Dde*I, which cut within the coding region of the calmodulin cDNA. The size standards (lanes P) were restriction fragments of pDCM1. One aliquot of plasmid DNA was digested with *Eco*RI, and another aliquot was digested with *Eco*RI and *Pvu*II; the products were then mixed. The calculated sizes of the restriction fragments in kilobase pairs are indicated at the left.

Three enzymes cut within the coding region of the cDNA, yielding multiple bands (Fig. 2). Of these, *Pvu*II and *Dde*I each yielded two fragments that hybridized to the probe, as expected (*Dde*I recognizes two sites, but they are separated by only 13 base pairs). Surprisingly, *Bcl*I, which cuts once within the coding region, generated three fragments, suggesting the presence of an intron containing a *Bcl*I site. Accordingly, genomic DNA was digested with *Pvu*II and *Eco*RI, both of which cut within the coding region of the cDNA. If the genomic DNA were colinear with the cDNA, a fragment of 272 bp would have been obtained. Instead, the size of the smallest fragment detected was approximately 530 bp (Fig. 3). Therefore, the genomic *Eco*RI-*Pvu*II fragment was larger than the corresponding cDNA fragment by approximately 260 bp, indicating the presence of at least one intron in this region. This was confirmed by additional double digests with *Pvu*II and other enzymes that generated fragments spanning this region (data not shown).

In summary, these studies confirmed the unusual amino acid sequence differences between *D. discoideum* calmodulin and other calmodulins. In addition, an intron was detected in the calmodulin gene. Introns are unusual in *D. discoideum* genes, although others have been identified (5). Most importantly, there appears to be a single *D. discoideum* gene for calmodulin. The presence of a single calmodulin

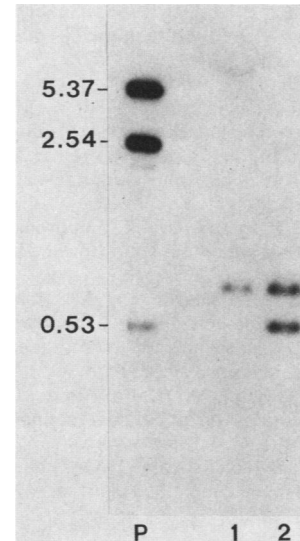


FIG. 3. Evidence for an intron. *D. discoideum* genomic DNA was digested with *Pvu*II (lane 1) or *Pvu*II and *Eco*RI (lane 2). The blot was probed with the small *Eco*RI-*Sac*I fragment of plasmid pDCM1, which contained the calmodulin cDNA and part of the  $\beta$ -galactosidase gene (base pairs 1948 to 3016 [3]). The size standards (lane P) were the same restriction fragments of pDCM1 described in the legend to Fig. 2; the calculated sizes of the restriction fragments in kilobase pairs are indicated at the left. The 2.04-kbp fragment seen in Fig. 2 was not detected by this probe. The *Pvu*II-*Eco*RI double digest yielded two fragments, one with a size of approximately 0.7 kbp and the other identical in size to the 530-bp marker. The expected size of the latter fragment, based on the nucleotide sequence of the cDNA, was 272 bp.

gene, coupled with the recent development of an efficient transformation system for *D. discoideum* (14), should make it possible to examine the *in vivo* effects of selective modifications of that gene.

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