Inducible Expression of Calmodulin Antisense RNA in *Dictyostelium* Cells Inhibits the Completion of Cytokinesis

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The single gene encoding calmodulin in the eukaryotic microorganism *Dictyostelium discoideum* was cloned and sequenced. The gene was found to contain three introns, one lying immediately after the translation initiation codon. The deduced amino acid sequence indicated that *Dictyostelium* calmodulin contains 19 amino acid differences from vertebrate calmodulin, including extensions at both termini. Northern blot analysis showed that similar levels of calmodulin mRNA are present throughout growth and development of wild-type cells. A complete copy of the calmodulin cDNA was prepared, and an 87-base pair fragment complementary to the 5'-end of the calmodulin mRNA was subcloned into the *Dictyostelium* transformation vector pVEII, such that expression of the antisense transcript was driven by the discoidin I γ promoter. Transformed cells were selected and maintained at low cell density, a condition resulting in minimal activity of the discoidin I promoter. High level expression was induced by allowing the transformants to reach high cell density or by growing them in the presence of medium conditioned by high density cells. Under these conditions, in which calmodulin mRNA and protein levels were reduced about twofold, the calmodulin antisense transformants lost the ability to complete cytokinesis. A contractile ring formed and constricted, but the midbody linking daughter cells failed to break. The resulting cell population contained multinucleated cells and networks of cells connected by cytoplasmic bridges. Normal cell division was restored when the cells were diluted to low density. These observations have identified a new point at which calmodulin may regulate cell cleavage.

INTRODUCTION

Calmodulin is a highly conserved, multifunctional, calcium-binding protein found in all eukaryotic cells. It mediates the regulatory effects of calcium on a variety of cellular processes, including metabolic pathways, ion transport, cell proliferation, and cell motility (reviewed in Cohen and Klee, 1988; Means *et al.*, 1991).

Many studies have analyzed the interactions of calmodulin with its target proteins in vitro, and recent work has begun to probe calmodulin function in vivo. Expression vectors capable of inducibly synthesizing calmodulin sense or antisense RNA, introduced into cultured mouse cells, caused changes in calmodulin levels that affected cell-cycle progression through G1 and mitosis (Rasmussen and Means, 1989). Similar experiments in *Aspergillus nidulans* indicated that intra-

cellular calmodulin and extracellular calcium are interactive factors that regulate the nuclear division cycle of this organism (Lu *et al.*, 1992). The single calmodulin gene has been deleted from *Saccharomyces cerevisiae* (Davis *et al.*, 1986), *Schizosaccharomyces pombe* (Takeda and Yamamoto, 1987), and *A. nidulans* (Rasmussen *et al.*, 1990); in all cases, the deletion was lethal. A temperature-sensitive calmodulin mutant of *S. cerevisiae* was found to lose viability during mitosis; chromosome segregation and possibly cytokinesis were affected (Davis, 1992). Non-lethal mutations of calmodulin in *Paramecium* have demonstrated that calmodulin regulates the activity of Ca⁺⁺-gated Na⁺ and K⁺ channels (reviewed in Preston *et al.*, 1991). Site-directed mutagenesis has revealed a role for calmodulin in sporulation of *S. pombe* (Takeda *et al.*, 1989), and this technique also has been used in *S. cerevisiae* to analyze functional do-

mains of calmodulin (Geiser *et al.*, 1991; Persechini *et al.*, 1991).

The eukaryotic microorganism *Dictyostelium discoideum* is another system in which the methods of molecular genetics can help to elucidate calmodulin function in the living cell. *Dictyostelium* amoebae are haploid, and many genes, including calmodulin (Goldhagen and Clarke, 1986), that are present in multiple copies in mammalian cells are single-copy in *Dictyostelium*. Thus, for several *Dictyostelium* proteins, it has been possible to manipulate gene expression by deleting or altering the appropriate genomic sequences via homologous recombination (DeLozanne and Spudich, 1987; Witke *et al.*, 1987; Manstein *et al.*, 1989; Jung and Hammer, 1990; Doring *et al.*, 1991; Kumagai *et al.*, 1991; Sun and Devreotes, 1991; Cox *et al.*, 1992). Reduction of mRNA levels by the introduction of vectors producing antisense RNA also has proved effective. The *Dictyostelium* genes whose expression has been blocked by antisense RNA include discoidin I (Crowley *et al.*, 1985), myosin II heavy chain (Knecht and Loomis, 1987) and essential light chain (Pollenz *et al.*, 1992), cAMP receptor (Klein *et al.*, 1988; Sun *et al.*, 1990), D2 esterase (Rubino *et al.*, 1989), gp24 (Loomis and Fuller, 1991), V4 (McPherson and Singleton, 1992), and conditioned medium factor (CMF) (Jain *et al.*, 1992).

Calmodulin is an attractive focus for gene targeting experiments in *Dictyostelium*. The calmodulin protein of *Dictyostelium* cells is similar to vertebrate calmodulin in structure and activity, although there are some amino acid differences (Marshak *et al.*, 1984). In contrast to cells of the lower eukaryotes described above, *Dictyostelium* amoebae closely resemble mammalian cells in mechanisms of cell locomotion, chemotaxis, phagocytosis, and cytokinesis. Thus, the properties of *Dictyostelium* cells make them especially appropriate for examining the roles of calmodulin in motility. Furthermore, for many aspects of motility, biochemical data, ultrastructural information, and even mutants are already available (reviewed in Kessin, 1988; Egelhoff and Spudich, 1991; Jones *et al.*, 1991; Van Haastert *et al.*, 1991).

cDNA clones spanning most of the *Dictyostelium* calmodulin coding region were described by Goldhagen and Clarke (1986) and Clarke (1990). Although we considered using those cDNAs for insertional inactivation of the calmodulin gene, it seemed likely that some calmodulin function is essential. We judged that a more informative course would be to construct a strain in which calmodulin expression could be conditionally suppressed. This report describes the construction of such a strain and the striking phenotypic consequences of reduced calmodulin levels.

METHODS AND MATERIALS

Strains and Culture Conditions

Dictyostelium discoideum strains AX2 and NC4 were maintained on SM nutrient agar plates (Loomis 1975) in association with *Klebsiella*

aerogenes. In addition, the axenic strain AX2 and its derivatives were grown in HL5 medium (Clarke *et al.*, 1980), either in suspension or on tissue culture plates. Cells transformed with plasmids carrying the neomycin phosphotransferase gene, which confers resistance to the antibiotic G418, were maintained in HL5 containing G418 (100 µg/ml). For preparation of conditioned medium, AX2 cells were allowed to grow in suspension on HL5 to a density of 5×10^6 cells/ml. The cells were removed by centrifugation, and the medium was clarified by passage through a 0.2-µm filter.

Cloning and Analysis of the *Dictyostelium* Calmodulin Gene

A genomic library was constructed by insertion of a partial *Sau3A* digest of DNA from AX2 cells into the *Bam*HI site of the plasmid pAT153 (Twigg and Sherratt, 1980). This unamplified library was screened with an *Eco*RI-*Hpa*I fragment of *Dictyostelium* calmodulin cDNA, appropriately subcloned from pDCM1 (Goldhagen and Clarke, 1986), gel purified, and labeled by nick translation. Three independent clones were isolated. Using standard chain termination protocols (Sanger *et al.*, 1977), the sequence of each clone was determined from the cloning site upstream of the calmodulin gene through the gene and its introns. Identical sequences were obtained for all three genomic clones. For one clone, the sequence from nucleotides 1–999 was additionally verified by sequencing both DNA strands. Primer extension experiments were performed by hybridizing a primer, end labeled with γ^{32} P-ATP and polynucleotide kinase, and corresponding to codons 25–32 of the cDNA (5'-GGTGATGGTTCAATTACCACC) to 50 µg of total *Dictyostelium* RNA. After extension by reverse transcriptase and isolation by ethanol precipitation, the reaction product was sized on a DNA sequencing gel using pUC DNA as a size standard.

Construction of Vectors Expressing Antisense Calmodulin RNA

The calmodulin gene contains an *Eco*RI site near its 5'-end (at nucleotide 40, counting from the translation initiation codon and excluding introns). An *Eco*RI fragment extending from that site through the 3'-untranslated region of the calmodulin cDNA was isolated from a λgt11 *Dictyostelium* cDNA library (Clontech, Palo Alto, CA) using methods previously described (Clarke, 1990). This fragment was placed in an antisense orientation in three different *Dictyostelium* transformation vectors. The promoters controlling expression of the antisense transcripts were the actin 6 promoter in pDNeoII (Witke *et al.*, 1987) and the D19 (Early and Williams, 1989) and CP2 (Pears *et al.*, 1985; Pears and Williams, 1987) promoters in vectors derived from pB10TP1 (Early and Williams, 1987).

A complete calmodulin gene without introns was constructed by combining the partial cDNA described above with an oligonucleotide encoding the 5' portion of the gene. Deoxyribonucleotides 5'-TGGACGAGCTCATGGCATCACAAGAAAGTTAACTGAACAAATTGCTG and 5'-AATTCAGCAATTTGTTTCAGTTAACTTTCTTGTGATGCTACGAGCTCG were synthesized and allowed to hybridize to each other, yielding a double-stranded DNA fragment that contained *Sal*I and *Sac*I sites just upstream of the calmodulin gene and ended at the *Eco*RI site within the gene. This fragment was cloned into pBluescript SK⁺ cut with *Sal*I and *Eco*RI. The *Eco*RI fragment containing the remainder of the calmodulin gene with its 3'-untranslated region was inserted at the *Eco*RI site, and clones with the correct orientation were identified by restriction mapping. The resulting construct (pDCaM1) contained the entire calmodulin gene flanked by *Sac*I restriction sites. A restriction fragment containing the complete gene was subcloned in an antisense orientation into the *Dictyostelium* transformation vectors pDNeoII (described above) and pVEII, in which expression was driven by the discoidin I promoter (Blusch *et al.*, 1992); the latter construct was termed pVC.

A construct containing only the 5'-portion of the calmodulin gene was also prepared. pDCaM1 was cut with *Hph*I (which cleaves at nucleotide 88 in the calmodulin gene) and treated with S1 nuclease

and then the Klenow fragment of *Escherichia coli* DNA polymerase I to produce a blunt end at nucleotide 87. The linear fragment was then cut with *Sac* I to liberate a restriction fragment containing the first 87 nucleotides of the calmodulin gene. This fragment was purified by gel electrophoresis. The *Dictyostelium* transformation vector pVEII was cut with *Bam*HI, treated with Klenow enzyme to produce a blunt end at the cleavage site, and then cut with *Sac* I. The large fragment of the vector was ligated to the 87-base pair calmodulin gene fragment, yielding the plasmid pVC87.

Transformation Methods and Cloning of Transformants

Transformation of AX2 cells was carried out as described by Nellen *et al.* (1987), with minor modifications. The HL5 medium in which the cells were transformed was buffered with bis(2-Hydroxyethyl) imino-tris(hydroxymethyl)methane; 2-bis(2-Hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol (Bis-Tris) (pH 7.1). Each 100-mm plate was incubated with a calcium phosphate precipitate of 12 μ g of DNA, and the cells were permeabilized in 15% glycerol for 7 min. Transformants were selected in HL5 containing 10 μ g/ml G418. After 5 d, colonies of growing cells could be detected. Individual colonies were picked and spread with *K. aerogenes* on an SM plate at a dilution appropriate to yield single plaques. Cells from single plaques were transferred to wells of 24-well plates containing HL5 plus 10 μ g/ml G418; as the clones grew, the G418 concentration was increased to 100 μ g/ml.

Northern Blot Analysis

For analysis of possible developmental regulation of calmodulin mRNA levels, NC4 cells were washed free of their food source (*K. aerogenes*), suspended in development buffer (10 mM 2-(*N*-Morpholino)ethanesulfonic acid (MES), pH 6.2, 20 mM KCl, 20 mM NaCl, 1 mM CaCl_2), plated on filters (5×10^7 cells/25 mm HABP filter; Millipore, Bedford, MA), and incubated in a humid environment at 22°C. At selected times, cells were collected by rinsing a filter with 10 ml of development buffer. Total RNA was isolated, size fractionated on a formaldehyde-containing agarose gel, transferred to a nylon membrane (Hybond-N⁺, Amersham, Arlington Heights, IL), and probed with radiolabeled RNA from pBD13, all as described in Rath *et al.* (1991).

For analysis of the effects of the calmodulin antisense vector on calmodulin mRNA levels, strains AX2 and AX2/pVC87 (a pVC87 transformant of AX2) were grown in HL5 to a density of 5×10^6 cells/ml. For each cell type, a total of 1×10^8 cells was harvested, and mRNA was purified by oligo(dT) chromatography using a PolyA Tract kit (Promega, Madison, WI). Each mRNA sample (1.25 μ g) was loaded into one broad well of a formaldehyde-containing agarose gel (1.5% agarose), electrophoresed, and transferred as described above. Each membrane was cut into strips, and adjacent strips were probed for calmodulin and hisactophilin mRNA levels. (Hisactophilin was used as a control to check the quantity of mRNA loaded for each cell type.) The probe for calmodulin mRNA was pDCaM1; the probe for hisactophilin mRNA was HCB175, a subclone of cDH175 (Scheel *et al.*, 1989) in pGEM4, provided by Drs. Noegel and Schleicher (Max-Planck Institut für Biochemie, Martinsried, Germany). In both cases, RNA probes were prepared. Hybridization conditions were as recommended for Hybond-N⁺ membrane by the manufacturer (Amersham). Relative mRNA levels were determined by scanning densitometry.

Electrophoresis and Immunoblotting Procedures

For quantitation of calmodulin, axenic cultures of AX2/pVC87 and AX2/pVEII (i.e., AX2 transformed with the empty pVEII vector) were grown to high density (5×10^6 cells/ml). A low density AX2/pVC87 culture (8×10^4 cells/ml) was also prepared. Duplicate samples containing 5×10^4 cells and 1×10^5 cells were taken from each culture, and lysates were prepared by suspending the cells in sodium dodecyl

sulfate-containing denaturation buffer (Laemmli, 1970) and immersing them in a 100°C water bath. The denatured lysates were electrophoresed and immunoblotted as previously described (Hulen *et al.*, 1991). Quantitation was by scanning densitometry.

Microscopy

Living cells on plastic tissue culture dishes were viewed and photographed using an inverted microscope (Diaphot; Nikon, Garden City, NY) equipped with Hoffman Modulation Contrast optics (Modulation Optics, Greenvale, NY) and a Nikon UFX 35 mm camera. For imaging nuclei, cells growing on glass cover slips were overlaid with agarose, then fixed in HL5 containing 2% formaldehyde (5 min, room temperature) followed by 1% formaldehyde in methanol (5 min, -15°C), washed in TBS (150 mM NaCl, 20 mM Tris-HCl, pH 7.5), stained with 4'-diamidino-2-phenylindole $\cdot$ 2 HCl (DAPI) (0.1 μ g/ml in TBS, 10 min, room temperature), and washed in TBS. The cells were mounted as described (Yumura *et al.*, 1984) and were photographed using an epifluorescence microscope (Axiophot; Zeiss, Thornwood, NY) equipped with a 100 $\times$ planneofluar phase objective.

RESULTS

Cloning and Sequence of the *Dictyostelium* Calmodulin Gene

Three independent clones of the *Dictyostelium* calmodulin gene were isolated from an unamplified AX2 genomic library. Southern blots indicated that in each case the 5'-end of the calmodulin coding sequence lay $\sim$ 0.5 kilobase from the cloning site, whereas different extents of 3'-flanking sequence were present in the three clones. A restriction map of pDCL1, the genomic clone containing the longest stretch of 3'-flanking sequence, is shown in Figure 1.

The nucleotide sequences of all three clones, determined between the cloning site at map position 5.2 and the *Pvu* II site at map position 3.4, were identical. The nucleotide sequence of the *Dictyostelium* calmodulin gene, *calA*, and its flanking regions is shown in Figure 2 (GenBank accession number M64089). The region upstream of the calmodulin gene was found to include a consensus TATA box (underlined) and long oligo(dT) stretches that are also features of other *D. discoideum* genes (Kimmel and Firtel, 1982). The presumptive translation initiation codon is also underlined. Three small introns, bounded by consensus splice donor (GT) and acceptor (AG) sites, lay within the calmodulin gene; an intron immediately after the translation initiation codon is a characteristic feature of calmodulin genes (see DISCUSSION). Potential transcription initiation sites are marked with arrowheads; these correspond to the 5'-termini of calmodulin mRNAs, determined by primer extension.

Calmodulin mRNA Levels in Growing and Developing Cells

The relative level of calmodulin mRNA throughout growth and development of *Dictyostelium* cells was determined by Northern blot analysis. Exponentially growing wild-type cells were washed free of food and placed on a membrane in a humid chamber. Under these

Figure 1. Restriction map of pDCL1. This plasmid, which contains the *Dictyostelium* calmodulin gene (wide black segments, with introns hatched) and its flanking sequences (narrow black line), was isolated from a genomic DNA library prepared in pAT153 (Twigg and Sherratt, 1980). The thicker black line denotes pAT153 sequences, with heavy vertical bars marking the site (*Bam*HI/*Sau*3A) at which genomic DNA fragments were inserted. The restriction map of the 3'-flanking region of the calmodulin gene was consistent with data from Southern blot analysis of genomic DNA (Goldhagen and Clarke, 1986, and unpublished observations), extending from the *Eco*RI site in the calmodulin gene to the *Hind*III site at map position 1.

conditions, the cells proceeded synchronously through development, completing the process of fruiting body formation in ~24 h. Cells were harvested from one filter immediately after plating and from others at successive 3-h intervals, up to 24 h. Northern blot analysis indicated that there was little or no variation in calmodulin mRNA levels throughout growth and development of wild-type cells (Figure 3). In multiple experiments, the only consistent change detected was a reduced level of calmodulin mRNA in the 24-h sample, and this was probably a consequence of incomplete breakage of the mature spore cells present at this stage.

Construction of Strains Producing Antisense Calmodulin RNA

To reduce the level of calmodulin in *Dictyostelium* cells, several types of antisense RNA constructs were prepared and tested. Initial experiments made use of an *Eco*RI-*Hpa*I restriction fragment of the calmodulin cDNA. The promoters driving expression of this fragment in an antisense orientation included actin 6, CP2, and D19 (see METHODS AND MATERIALS). Although appropriate temporal expression of the antisense transcripts could be detected in transformed cells by Northern blot, none of these constructs caused a significant reduction in calmodulin mRNA or protein levels or any obvious phenotypic effects.

Inferring that an antisense transcript complementary to the 5'-end of the mRNA might be needed for effective inhibition, we constructed a complete calmodulin gene lacking introns. Although it had not been possible to obtain a full-length cDNA from the cDNA library we screened (Clarke, 1990), an oligonucleotide based on sequence information from the genomic clones was synthesized and combined with a partial cDNA to yield a complete gene.

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- 270 GGATCATTCTCTGATCTGGTAAAAAATTCATATTTAATTTAAATTAATAAAAAAAAAA
- 210 AAAAAAAAAATTACACAATTTTGTGAGTGAATTAATAAACTGAACAAAGGGTTACTTTTA
- 150 AGCCATTTTATTATAATTTTTTTTTTTTTTTTTTTGAAATTTTTTTTTTTTTTTTTT
- 90 ATTTTTTTTTTTTTTTTGAAGGATATTTTTTTTTTTTTTTTTTTTTTTTATTTAT
- 30 TCTTCCACCAATTCATAAATAAATGTAAGGACCTTATCAATTAATCATTAT
31 TATTTAATAAACTTTCAACTTTTAATTTAATTTTGTGAATTAATAAATAAATAAAT
91 TAAAAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAAT
151 AATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAAT
211 AATAATAAAAAAAAAAAAAATTTTAAATACTAATAAACAATAATAATAATAAT
271 AATAATAATAATAATAATAATAATAG CCA TCA CAA GAA AGT TTA ACT GAA
    ala ser gln glu ser leu thr glu

323 GAA CAA ATT GCT GAA TTC AAA GAA GCA TTC TCT TTA TTC GAT AAA
    glu gln ile ala glu phe lys glu ala phe ser leu phe asp lys
368 GAT GGT GAT GGT TCA ATT ACC ACC AAA GATGTATTATTTTTTTTGAAG
    asp gly asp gly ser ile thr thr lys
419 GTTAAATAAAGGAATCAATTTAAATTAATAATTATTTAATTACATTTTAAAT
479 GAAG GAA TTA GGT ACT GTT ATG AGA TCA TTA GGT CAA AAC CCA ACT
    glu leu gly thr val met arg ser leu gly gln asn pro thr
525 GAA GCT GAA TTA CAA GAT ATG ATC AAT GAA GTT GAC GCT GAT GGT
    glu ala glu leu gln asp met ile asn glu val asp ala asp gly
570 AAT GGT AAC ATT GAT TTC CCA GAA TTT TTA ACC ATG ATG GCT CG
    asn gly asn ile asp phe pro glu phe leu thr met met ala ar
614 GTATGTTTTTTTAAAAATTAATTTTATCATTTTTTCAGTTTTTCAAATTTGAAGTGG
674 TATATTAACTTTTTTTTTTTTTTTTTTCTCTCTTTTTTTTATTTATTTTAAAAATA
734 ATAG T AAA ATG CAA GAC ACT GAT ACT GAA GAA GAA ATC CGT GAA
    g lys met gln asp thr asp thr glu glu glu ile arg glu
778 GCA TTC AAA GTT TTC GAT AAA GAC GGT AAC GGT TAC ATC TCA GCT
    ala phe lys val phe asp lys asp gly asn gly tyr ile ser ala
823 GCT GAA CTT AGA CAC GTT ATG ACC AGT CTT GGT GAA AAA CTT ACC
    ala glu leu arg his val met thr ser leu gly glu lys leu thr
868 AAC GAA GAA GTT GAT GAA ATG ATT AGA GAA GCT GAT TTG GAT GGC
    asn glu glu val asp glu met ile arg glu ala asp leu asp gly
913 GAT GGT CAA GTT AAC TAT GAC GAA TTT GTT AAA ATG ATG ATT GTT
    asp gly gln val asn tyr asp glu phe val lys met met ile val
958 AGA AAT TAA GAAATTTAATTAATAAATAAATAAATAAATAAATAAATAAATAA
    arg asn
1011 AAAAAACAAAAATAATATATTATAAA

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Figure 2. Nucleotide sequence of the *Dictyostelium* calmodulin gene, *calA*, and its flanking regions. The marked features include a consensus TATA box and the presumptive translation initiation codon (both underlined) and potential transcription initiation sites (arrowheads). The deduced amino acid sequence agrees with the previous biochemical determination of the primary structure of the protein, which identified residues 6–151 and determined the composition of additional residues at the blocked amino terminus (Marshak et al., 1984). The sequence of *Dictyostelium* calmodulin is 87% identical to that of vertebrate calmodulin.

Figure 3. Northern blot analysis of calmodulin mRNA levels during growth and development. Wild-type *Dictyostelium* cells (strain NC4), developing on membranes, were harvested at the indicated hours of development. RNA was prepared and analyzed by Northern blot, using a fragment of radiolabeled calmodulin cDNA as a probe. Each lane of the gel received 5 μ g of RNA. Similar levels of calmodulin mRNA were observed in growing and developing cells. (Although the 12-h sample appears lower in this experiment, that difference was not reproducible.)

This was placed into pDNeoII (Witke *et al.*, 1987) in an antisense orientation; in this vector, expression of the antisense RNA is driven by the actin 6 promoter, which is active in axenically growing cells (Knecht and Loomis, 1987). *Dictyostelium* cells were transformed with this pDNeoII construct, and transformants capable of growth in 100 μ g/ml G418 were isolated. Northern blots detected calmodulin antisense RNA in these transformants. However, calmodulin mRNA levels did not appear to be reduced, and cell behavior was normal. Although no transformants were identified in which calmodulin expression was blocked, it was possible that such transformants had been created but had not survived.

The best means of examining the effects of a potentially lethal antisense RNA transcript would be a vector in which the selectable marker (G418 resistance) was expressed constitutively, but the antisense RNA was inducible. We obtained such a vector, pVEII, from Dr. Wolfgang Nellen (Max-Planck Institut für Biochemie). In pVEII, G418 resistance is driven by the actin 15 promoter, whereas any sequence inserted at the multiple cloning site is driven by the discoidin I γ promoter (Blusch *et al.*, 1992). Because the discoidin I γ promoter is regulated by a secreted factor that accumulates in proportion to cell density (see DISCUSSION), little or no expression is expected from this promoter in low density cells. We prepared two types of constructs in pVEII. The first (pVC) contained the full-length calmodulin cDNA oriented to yield an antisense transcript. The second (pVC87) contained only the 5'-portion of the gene, such that a transcript complementary to the first 87 nucleotides of the calmodulin mRNA, including the translation initiation codon, would be synthesized (Figure 4).

Dictyostelium cells were transformed with both types of constructs. Drug-resistant transformants were selected in 10 μ g/ml G418, and clones capable of growth in high G418 (100 μ g/ml) were identified. The transformed cell lines were maintained at low cell density, either on tissue culture plates or in suspension. For cells transformed with pVC87, several clones capable of

growth in high G418 were obtained. For cells transformed with pVC, only a single such clone was obtained.

To observe the effects of expression of the antisense RNA transcript, the transformants, growing axenically in the wells of multititer plates, were allowed to grow to high cell density. In the pVC transformant and several of the pVC87 transformants, giant multinucleated cells appeared as the cultures reached high density. A pVC87 transformant that manifested this phenotype was transferred to suspension culture, and the cells were sampled at intervals as they grew from 5×10^4 to 1×10^6 cells/ml. Each cell sample was stained with the DNA-binding fluorochrome DAPI to label cell nuclei. The cells were found to contain single nuclei at cell densities up to 2×10^5 cells/ml. When the culture density reached 4×10^5 cells/ml, multiple nuclei were observed in many cells; this condition persisted and worsened as the cell density increased.

The same effect was seen when calmodulin antisense transformants were grown at low density in the presence of medium conditioned by high density cells. Control cells, transformed with the vector alone, were not affected in this manner by either high cell density or conditioned medium. Figure 5 shows AX2 cells transformed with pVC87 (A) or with the empty pVEII vector (B). Both cultures were grown axenically on the surface of tissue culture dishes; both cultures had been exposed to conditioned medium for 2 d before being photographed. Note the larger size of the pVC87 transformant cells and the presence of cytoplasmic bridges connecting some of these cells.

Figure 4. Schematic representation of the structure of pVC87. The first 87 nucleotides of the calmodulin cDNA sequence were inserted in an antisense orientation into the *Bam*HI site of the multiple cloning site (MCS) of pVEII. The structure of pVEII resembles that of pDNeoII (Witke *et al.*, 1987) except that the discoidin I γ promoter (Vauti *et al.*, 1990) has been substituted for the actin 6 promoter and the MCS contains restriction sites for *Xba* I, *Bam*HI, *Sma* I, *Kpn* I, and *Sac* I (Blusch *et al.*, 1992).

Figure 5. Cultures of AX2/pVC87 and AX2/pVEII growing in conditioned buffer. AX2/pVC87 (the calmodulin antisense transformant, A) and AX2/pVEII (the empty vector control, B) were grown on tissue culture dishes in HL5 containing 100 $\mu\text{g/ml}$ G418. The HL5 was replaced with HL5 that had been conditioned by high density cells, and 2 d later, the living cells were photographed. Note the presence of linked cells and very large cells in the AX2/pVC87 culture. Magnification, 140 $\times$; bar, 50 μm . The lower panels show AX2/pVC87 (C) and AX2/pVEII cells (D) grown under the same conditions on a glass cover slip; the cells were fixed and stained with DAPI to reveal nuclei. Approximately one-third of the AX2/pVC87 cells contained multiple nuclei. Magnification, 580 $\times$; bar, 10 μm .

The large cells contained multiple nuclei, as revealed by staining of fixed cells from a similar culture with DAPI (C); control cells mostly contained single nuclei (D). These effects could be reversed by replacing conditioned medium with fresh medium or by diluting high density cells back to low density growth conditions.

A pVC87 transformant was chosen for detailed characterization; this strain was designated AX2/pVC87. This has proved to be a stable cell line; it has been maintained in culture for many months and also has been recovered from frozen storage. However, the single pVC transformant capable of growth in high G418 was unstable; it reverted to wild-type behavior before it could be fully analyzed.

Reduction of Calmodulin mRNA and Protein Levels in AX2/pVC87 Cells

Messenger RNA was prepared from AX2/pVC87 cells and from AX2 cells, both grown to high cell density. Northern blots were probed to detect calmodulin and hisactophilin mRNAs (Figure 6, top). The hisactophilin gene was chosen as a control because its mRNA is similar in size to that of calmodulin, and it is expressed at a constant level throughout growth and development (Scheel *et al.*, 1989). The results for calmodulin were normalized to the amount of hisactophilin mRNA detected for the two strains. These measurements indicated that the calmodulin mRNA level in AX2/pVC87 cells was approximately half (47%) of that in AX2 cells.

Figure 6. Calmodulin mRNA and protein in AX2/pVC87 and control cells. Upper, Northern blot of mRNA from AX2 and AX2/pVC87 cells probed for calmodulin and hisactophilin. Messenger RNA was purified from high density (5×10^6 cells/ml) axenic cultures of AX2 and AX2/pVC87 cells, and 1.25 μ g of mRNA from each cell type was electrophoresed in a broad lane of an agarose gel and transferred to a membrane. Adjacent strips of membrane were probed for calmodulin (CaM) and hisactophilin (His) mRNAs. Lower, Western blot of cell lysates from high density cultures of AX2/pVEII and AX2/pVC87, and a low density culture (8×10^4 cells/ml) of AX2/pVC87. Each lane shown contained 5×10^4 cells.

Calmodulin protein levels were also examined. For these experiments, AX2/pVC87 cells were compared with AX2 cells that had been transformed with an empty pVEII vector; both cultures were grown in suspension to high cell density in the presence of 100 μ g/ml G418. A low density AX2/pVC87 culture was also examined. Total cellular calmodulin was detected by immunoblot as described in Hulen *et al.* (1991). The lower portion of Figure 6 shows the calmodulin content of an equal number of cells from low and high density AX2/pVC87 cultures and from a high density AX2/pVEII culture. Scanning densitometry indicated that total calmodulin in the high density population of AX2/pVC87 cells was $\sim 60\%$ of that in the AX2/pVEII control cells. (The measured averages were

56% and 62% for pairs of duplicate samples containing 5×10^4 and 1×10^5 cells, respectively.)

Effect of Reduced Calmodulin Levels on Cytokinesis

Observation of living cells indicated that the occurrence of giant multinucleated cells was due to the failure of cytokinesis in AX2/pVC87 cells as they reached high cell density. Individual AX2/pVC87 cells growing on the surface of tissue culture dishes were observed by light microscopy as they passed through mitosis. The early stages of mitosis and cytokinesis appeared to proceed normally. In particular, contractile ring formation and constriction occurred as in normal cells (Figure 7, first five panels). However, the final step of cytokinesis, the severing of the narrow cytoplasmic bridge (midbody) linking the two daughter cells, did not take place. Instead, the daughter cells remained linked, and this linkage persisted indefinitely (Figure 7, remaining panels).

Figure 8 illustrates the two possible fates of cells that failed to complete cytokinesis. The daughter cells shown at the top of the first three panels ultimately succeeded in breaking their linkage after moving several cell diameters apart. Nearby, another pair of linked daughter cells eventually moved toward each other and merged to form a binucleate cell (shown in the bottom of the first three panels and in all subsequent panels). This seemed to be the more usual outcome. Cells with four and eight nuclei were commonly produced by the subsequent rounds of failed cytokinesis, as seen in Figure 5. When cells were cultured for several days at high cell

Figure 7. Failure of cytokinesis in cells expressing calmodulin antisense RNA. AX2/pVC87 cells growing on tissue culture dishes in the presence of conditioned medium were photographed at intervals. Two cells attempting to divide were captured in this sequence. The number on each frame indicates the elapsed time in minutes. Note the normal appearance of the cleavage furrows. During the period of observation, neither pair of daughter cells completed cytokinesis. The upper pair of cells was in the process of merging in the last frame. Magnification, 540 $\times$; bar, 10 μ m.

Figure 8. Two outcomes of failed cytokinesis. This field of AX2/pVC87 cells, growing as described in Figure 7, illustrates the possible outcomes of failed cytokinesis. After the upper pair of cells moved apart a distance of several cell diameters, the mid-body linking them broke and was resorbed by the cells. The lower pair of cells, seen in all frames, eventually merged to form a single cell with two nuclei. A portion of a large multinucleated cell is seen at the center of the field in the upper panels. The numbers indicate elapsed time in minutes from the beginning of observation. Magnification, 540 $\times$; bar, 10 μ m.

density or in the presence of conditioned medium, giant multinucleated cells were also present. A portion of such a cell is shown in the upper panels of Figure 8.

DAPI staining revealed that the giant cells gradually accumulated abnormalities in chromosome condensation and distribution (Liu and Clarke, unpublished data), suggesting that prolonged expression of the antisense RNA affected karyokinesis as well. However, the earlier effects were on cytokinesis, implying that this was the more sensitive target for reduced calmodulin levels, and these effects were fully reversible.

DISCUSSION

Characterization of the Dictyostelium Calmodulin Gene

Three independent isolates from an unamplified *Dictyostelium* genomic DNA library were found to have identical nucleotide sequences throughout both the coding and noncoding portions of the gene, consistent with earlier Southern blot data, suggesting the presence of a single calmodulin gene in *Dictyostelium* (Goldhagen

and Clarke, 1986). This small gene contained three introns, although many *Dictyostelium* genes contain no introns (Kimmel and Firtel, 1982). The first intron lay between the ATG translation initiation codon and the codon for the first amino acid, as is common in genes for calmodulin and related EF hand proteins in other organisms (Means *et al.*, 1988). An intron also has been found at this position in the *Dictyostelium* gene encoding the essential myosin light chain (Pollenz and Chisholm, 1991). Although most calmodulin genes contain multiple introns, the positions of the second and third introns in the *Dictyostelium* calmodulin gene did not correspond to intron positions previously described in other organisms (Means *et al.*, 1988).

The deduced amino acid sequence of *Dictyostelium* calmodulin indicated that this protein consists of 151 amino acids and has 19 amino acid differences from vertebrate calmodulin, including extensions at both the amino and carboxy termini. The presence of an additional amino acid at the carboxy terminus was known from earlier data (Marshak *et al.*, 1984; Goldhagen and Clarke, 1986). The presence of two additional amino acids at the amino terminus had been deduced from

amino acid composition, but their order was not known (Marshak *et al.*, 1984). Throughout the molecule, there are several conservative substitutions, as well as some nonconservative changes; the greatest divergence occurs at the carboxy terminus. Site-directed mutagenesis is being used to explore the significance of these changes.

Northern blot analysis showed that the level of calmodulin mRNA remains essentially constant throughout growth and development of wild-type *Dictyostelium* cells. This result is interesting in light of the profound changes in growth, metabolism, and differentiation that accompany development. It suggests that calmodulin has important (although not necessarily identical) functions in both growing and developing cells. Experiments are in progress to examine the roles of calmodulin in developing cells by expressing antisense RNA under the control of developmentally regulated and cell-type-specific promoters.

The Effect of Antisense RNA Inhibition of Calmodulin Gene Expression in Growing Cells

Several factors combine to make antisense RNA a very useful tool for examining gene function in *Dictyostelium*. *Dictyostelium* cells can be easily transformed, the developmental stage of the life cycle is separable from growth and thus can be studied independently, and several developmentally regulated and cell-type-specific promoters are available to provide conditional expression of antisense sequences. Furthermore, for nonessential genes, the antisense results can often be confirmed by gene knock-out experiments. See Loomis (1992) for a review of antisense studies in *Dictyostelium*.

We tested several types of antisense constructs in an attempt to inhibit expression of the *Dictyostelium* calmodulin gene. We detected no reduction in calmodulin mRNA levels upon expression of an antisense RNA complementary to mRNA nucleotides 37–307 (an *EcoRI*-*Pvu* II fragment), whether the antisense transcript was expressed in growing cells or in only a subset of developing cells. This is consistent with recent studies in other systems. For alpha- and beta-globin mRNAs, duplex formation close to the initiation codon (within 18–26 bases) was found to be necessary for antisense inhibition of translation, apparently because formation of an antisense duplex in this region blocked 80S ribosome assembly (Liebhaber *et al.*, 1992). In agreement with those results, we did observe a reduction in calmodulin mRNA levels when the cells expressed an antisense RNA sequence complementary to the translation initiation codon and the next 84 nucleotides of the mRNA.

The use of an inducible promoter to drive expression of the antisense transcript also proved to be important. We did not observe an effect of constitutively expressed full-length antisense RNA on either cell behavior or mRNA levels, although abundant antisense RNA could be detected by Northern blot. The mechanism that protected the mRNA in these transformants is not clear. It

could be argued that the full-length transcript was ineffective because of secondary structure; the predicted secondary structure of the full-length sequence (but not the short transcript from pVC87) includes several regions of potential intramolecular duplex formation. However, using an inducible promoter, we did obtain one transformant from pVC that manifested the same phenotypic effects as the transformants from pVC87, even though that transformant was unstable. Furthermore, transformation with pVC yielded many transformants capable of growth at low drug concentration but not high, suggesting that cells that had received high copy numbers of the transforming plasmid may not have survived. Thus, it seems likely that the full-length transcript was effective in an inducible context, but clearer results and more stable transformants were obtained from the shorter transcript.

The discoidin I γ promoter was used to control expression of the calmodulin antisense RNA. We previously have shown that discoidin I mRNA and protein are absent or minimal in low density cells but increase to high levels in high density cells or in low density cells grown in the presence of buffer conditioned by high density cells. This pattern of behavior indicates that the discoidin I genes are regulated by prestarvation factor (PSF), an autocrine factor that is secreted by growing *Dictyostelium* cells and accumulates in proportion to cell density (Clarke *et al.*, 1987; Rathi *et al.*, 1991; Rathi and Clarke, 1992). Wolfgang Nellen's laboratory has cloned the discoidin I γ promoter region, placed it upstream of a heterologous reporter gene in a *Dictyostelium* transformation vector, and shown that in transformed cells the reporter gene responds to the same regulatory signals that normally control discoidin I expression (Vauti *et al.*, 1990; Blusch *et al.*, 1992). Our collaborative experiments have demonstrated that high cell density, conditioned medium, or partially purified PSF are sufficient to induce expression of such a reporter gene (Clarke and Nellen, unpublished data). These PSF-mediated regulatory effects were exploited in the present study.

Dictyostelium cells transformed with pVC and pVC87 were selected for drug resistance while growing at low cell density, a condition that minimized possible deleterious effects of the antisense RNA and allowed isolation of clones resistant to high levels of G418. The antisense RNA was then induced either by adding PSF-rich medium to low density cells or by allowing the cells to grow to high density. Under either condition, cytokinesis failed, and linked cells and multinucleated cells appeared in the culture.

The large multinucleated cells superficially resembled those resulting from antisense inhibition of the expression of the myosin II heavy chain (Knecht and Loomis, 1987) or essential light chain (Pollenz *et al.*, 1992). However, there were important differences. Cells with insufficient or inactive myosin II are unable to form a

functional contractile ring and thus cannot divide. Consequently, they cannot survive in suspension, although, if grown on a substratum, the cells can fragment through a process that has been termed "traction-mediated cytofission" (Fukui *et al.*, 1990). This process is efficient enough that the myosin mutant cells are viable when grown on tissue culture dishes.

In contrast, *Dictyostelium* cells with reduced calmodulin levels were viable not only on plates but also in suspension culture where shear forces probably helped to split apart the interconnected cells. Furthermore, the formation and constriction of the contractile ring in mitotic cells appeared to proceed normally. Only the final step of cytokinesis, severing of the midbody, was seriously impaired, leaving the daughter cells connected by a persistent cytoplasmic bridge.

Little is known about the mechanism by which the midbody is normally severed. Presumably, cytoskeletal elements such as microtubules and intermediate filaments in the midbody must be depolymerized or broken up, and the plasma membrane bilayer must undergo rearrangement and fusion. Calcium/calmodulin could play a role in either or both of these processes. In mammalian (PtK1) cells, microinjection of a fluorescent analogue of calmodulin showed that calmodulin is concentrated in the midbody of telophase cells (Welsh and Sweet, 1989). Our preliminary immunofluorescence studies also have shown an enrichment of *Dictyostelium* calmodulin in the midbody of AX2/pVC87 cells (Liu and Clarke, unpublished observations). It will be important to determine for these cells whether cytoskeletal elements persist in the midbody as it elongates and whether local calcium levels are altered sufficiently that an effect on membrane fusion might be expected. Such studies are in progress.

It is noteworthy that a relatively small change in normal calmodulin levels was sufficient to reveal this previously unknown function of calmodulin and to suggest a new point at which cell cleavage may be regulated. Thus, the local concentration of calmodulin could determine whether mitotic daughter cells will complete cleavage or remain connected. These observations also emphasize the importance of examining the effects of systematically altering wild-type calmodulin levels rather than focusing exclusively on mutational analysis of the protein.

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