A density-sensing factor controls development in *Dictyostelium*

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For an unknown reason, several genes expressed during *Dictyostelium* development are regulated by cell density. This is mediated by an 80-kD glycoprotein, conditioned medium factor (CMF), which is slowly secreted and simultaneously sensed by starved cells. To examine further this eukaryotic cell density-sensing mechanism, we have isolated a cDNA encoding CMF. The derived amino acid sequence of CMF shows no obvious similarity to any known protein and thus may represent a new class of eukaryotic intercellular signal. CMF antisense transformants do not aggregate, whereas normal development is restored by the addition of purified CMF protein. This suggests that CMF might synchronize the onset of development in *Dictyostelium* by triggering aggregation when a majority of the cells in a given area have starved, as signaled by CMF secretion.

[Key Words: Cell density; *Dictyostelium*; ligand; differentiation factor]

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Dictyostelium discoideum normally exist as unicellular amoebas that, in the wild, eat bacteria on decaying leaves and soil. The amoebas divide by fission and eventually overgrow their food supply. When they therefore become starved, the amoebas aggregate together using relayed pulses of cAMP as the chemotactic signal to form a multicellular, multi-cell-type structure. The aggregate of typically 10^5 cells first forms a slug that exhibits both phototaxis and thermotaxis. When it finds a location favorable for spore dispersal, the slug transforms itself into a fruiting body consisting of a mass of spores at the top of a ~2-mm-high stalk (Loomis 1975, 1982).

Gene expression at the various developmental stages is generally regulated by external cues rather than timers within cells (Devreotes 1989). The differentiation of cells into the precursors of stalk and spore cells (called prestalk and prespore) is determined initially by the phase of the cell cycle at the time of starvation, rather than a preset lineage (Weijer et al. 1984; McDonald 1986; Gomer and Firtel 1987). The pulses of cAMP that mediate the chemotaxis of the starving amoebas to form aggregates repress the expression of some early developmental genes and induce the expression of others (Janssens and Van Haastert 1987; Mann and Firtel 1987, 1989). The high continuous levels of extracellular cAMP found in aggregates induce the expression of sets of prespore and prestalk genes (Barklis and Lodish 1983; Mehdy et al. 1983; Morrissey et al. 1984; Devine and

Loomis 1985; Mehdy and Firtel 1985; Datta et al. 1986; Gomer et al. 1986a,b; Saxe and Firtel 1986; Wang et al. 1986; Mann et al. 1988; Williams 1988). The levels of extracellular ammonia, adenosine, oxygen, and a set of secreted chlorinated hydrocarbons (DIFs) affect patterns of gene expression after aggregate formation; DIFs, for instance, are required for stalk cell differentiation (Gross et al. 1983; Schaap and Wang 1986; Brookman et al. 1987; Williams et al. 1987; Sternfeld 1988; Kwong and Weeks 1989; Xie et al. 1991).

Another parameter that regulates gene expression during *Dictyostelium* development is cell density. For instance, the expression of a variety of prespore and prestalk genes is induced by extracellular cAMP in cells starved at high densities (10^5 cells/cm²) in submerged monolayers. At low cell densities (5×10^3 cells/cm²), cells in otherwise identical conditions neither aggregate nor express the prespore and prestalk genes in response to cAMP (Mehdy et al. 1983; Mehdy and Firtel 1985). Although cells starved at the low density in buffer conditioned previously by high-density starving cells still do not aggregate, they express prespore and prestalk genes in response to extracellular cAMP (Grabel and Loomis 1976; Mehdy and Firtel 1985; Gomer et al. 1986a). This indicated that a secreted density-sensing factor, conditioned medium factor (CMF), rather than cell-cell contact, mediates the effect of cell density on gene expression. Using time lapse videomicroscopy, we observed starved cells that never touched another cell differentiate into prespore and prestalk cells in the presence of

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CMF, further suggesting that CMF concentration, rather than cell–cell contact, mediates the response of genes to cell density (Gomer and Firtel 1987).

CMF thus appears to allow *Dictyostelium* cells to sense whether there are other *Dictyostelium* cells nearby. Because very little is known about how cells in eukaryotes sense the local density or total number of cells of a given cell type during development, tissue regeneration, or for homeostasis, we have begun an examination of the *Dictyostelium* CMF. Using a bioassay for CMF (immunofluorescence staining of low-density cells in an eight-well slide to detect the expression of genes that require CMF for their induction), we found that CMF activity is exhibited by two size classes of molecules, which have activity independent of each other. The first class can be purified to an 80-kD glycoprotein that is secreted at an approximate rate of 12 molecules/cell per minute (Gomer et al. 1991), whereas the second class consists of polypeptide breakdown products of the 80-kD CMF (Yuen et al. 1991). For prespore and prestalk gene expression, cells respond to levels of CMF as low as 0.3 ng/ml, and the presence of CMF is necessary only during the first 6 hr of starvation, suggesting that CMF has a function as an intercellular messenger. We have hypothesized that the concentration of 80-kD CMF in the immediate vicinity of an isolated cell will never be able to build up to the threshold sensitivity level of ~0.3 ng/ml, whereas the concentration within and in the vicinity of a large cluster of cells would be sufficient to allow expression of the density-sensitive genes.

The biological function of CMF remains unclear. In addition to prespore and prestalk genes, cAMP pulse-induced genes such as I42 and M3 and a cAMP pulse-unaffected gene, discoidin I, are expressed at higher levels in the presence of CMF (Mehdy and Firtel 1985). However, unlike prespore and prestalk genes, I42, M3, and discoidin I are expressed at low levels in the absence of added CMF. The low level of discoidin I expression that occurs in the absence of CMF may be induced by a factor that is secreted by vegetative cells, prestarvation factor (PSF), which appears to allow cells nearing starvation to sense their density relative to the amount of available nutrients (Clarke et al. 1987; Rath et al. 1991). Although CMF and PSF are both protease sensitive, CMF is not secreted by vegetative cells and is heat stable (Gomer et al. 1991), whereas PSF has the opposite properties, suggesting that PSF and CMF are different molecules with somewhat different functions.

Because the behavior of cells lacking CMF might elucidate its function, we have isolated a CMF cDNA (that encodes a protein that does not appear to be similar to any protein in the data base) and used it to generate CMF antisense-transformed *Dictyostelium* cells. Even long after control-transformed and wild-type cells have formed fruiting bodies, the CMF antisense cells fail to aggregate. Addition of purified CMF restores these cells to a wild-type development, indicating that a CMF concentration of above ~0.3 ng/ml is not only required for the expression of prespore and prestalk genes but also for the earlier process of aggregation.

Results

CMF is not related to any known protein

To clone the cDNA encoding CMF, we first determined a partial amino acid sequence of CMF protein that was purified as described previously (Gomer et al. 1991). Attempts to sequence the intact protein revealed a blocked amino terminus. Approximately 5 μ g (~60 pmoles) of CMF protein was then digested with trypsin, and peptides were separated by high-performance liquid chromatography (HPLC). Four peptides that appeared as isolated peaks with OD₂₁₄ values >0.01 were sequenced. All four yielded ~40–60 pmoles of amino acids per cycle. The sequence of one peptide was used to make a degenerate oligonucleotide probe which was, in turn, used to screen a *Dictyostelium* cDNA library. Because CMF is secreted throughout development, we used a cDNA library made from RNA from early developing cells (Klein et al. 1988). The derived amino acid sequence encoded by the single open reading frame (ORF) in an isolated 1.4-kb cDNA fragment revealed a perfect match to three of the tryptic fragments (underlined in Fig. 1A). This suggested that the cDNA encoded the protein that we had sequenced. The 1.4-kb cDNA fragment (indicated between the two arrowheads in Fig. 1A) did not contain the entire 80-kD CMF sequence, based on its size and the absence of 5' AUG and 3'-stop sequences.

The rapid amplification of cDNA ends–polymerase chain reaction technique (RACE–PCR; Frohman et al. 1988) was then used to obtain the additional sequence information shown outside of the arrowheads in Figure 1A. The sequence of the DNA generated by 3' RACE overlapped and perfectly matched 54 nucleotides of the cDNA. It yielded 16 additional nucleotides of coding sequence, a TAA stop codon, a presumably untranslated region of 78 bp, and a poly(A) tail. A consensus AATAAA polyadenylation signal can be seen at nucleotide 1966. The sequence of the fourth peptide (Fig. 1A, underlined) was observed to be encoded by the additional ORF from the 3' RACE. The sequence of the DNA generated by 5' RACE overlapped and perfectly matched 51 nucleotides of the cDNA. It yielded 282 additional nucleotides of coding sequence, an AUG start codon, and 218 nucleotides of a 5'-untranslated region.

Analysis of the single large ORF indicates that the predicted molecular mass of the CMF polypeptide backbone is 62.6 kD. Because CMF is glycosylated (Gomer et al. 1991; Yuen et al. 1991), we would expect the polypeptide molecular mass to be <80 kD. Comparison of the predicted amino acid sequence with data base sequences showed no significant similarity to any known protein. The predicted pI is 4.87. Although there are more negatively charged than positively charged amino acids, there are no significant concentrations of either charge. The average hydropathicity (Kyte and Doolittle 1982) is –0.2. There are no large hydrophilic or hydrophobic domains (Fig. 1B), although a 130-amino-acid region beginning at residue 403 (near the carboxyl terminus) has slightly more hydropathicity (0.4). We have shown previously that CMF contains both N- and O-linked glyco-

A

ACAAAGCCAATTGAAAAATTCATTTGAATGGTCAGGAGTTGTACCAGTTGATTTCAGAAGAAGAATTTACATTAACATTCTTTTCAAGTTT 90
 TCCATTAAGTGAATTTAAAGTTGAAGCATCACCAAAAACTTCATTATCATCTTCCAAATCAGAATCAGAACATAAATCAAAATTTCTATAA 180
 1 10
 M G S F G I D G A T T P S F S L T
 GGTGATTAAATCAATATATCAAACACCAACCGTTACCAATGGGTTTCATTTGGTATTGATGGTGCAACAACCTCCATCATTTAGTTTAACAT 270
 20 30 40
 W D K P V V G D W N V V I T A T S S L R K N E K F Q K M V E
 GGGATAAACAGTTGTTGGAGATTGGAATGTTGTAATCACAGCCACATCATCATTAAGAAAGAATGAAAAATTCCAAAGATGGTAGAAG 360
 50 60 70
 D S T P Q L L M L V Q N P S D T H I Y S Y V S S Y N N L F T
 ATTC AACACCTCAATTGTTAATGTTGGTTCAAATCCAAGTGACTCATATCTACTCTTATGTATCATCATACAATAATTTATTCACAG 450
 80 90 100
 G Q K V S V L A M L H K K S E F I K K S S A N R P L N W K P
 GTCAAAAAGTATCAGTTT TAGCAATGCTCCACAAGAAGTCAGAATTCAATTAAGTCTTCTGCCAATCGTCCACTCAATTGGAAACCAT 540
 110 120 130
 S P I L L S D V S A E M I L G L P D G S K E T I P M F D D G
 CACCAATTTTATTCAGATGTTTCAGCAGAGATGATTTTAGGTTCACAGATGGTTCAAAGAAACTATCCCAATGTTTGATGATGGTC 630
 140 150 160
 L H D D E Q A N D G L F G G Y I N V S E L G N H D L Q V V Y
 TTCATGATGATGAACAAGCAAATGATGGTCTTTTCGGTGGTTACATCAATGTAAGTGAATTGGGTAATCATGACCTTCAAGTAGTTTACA 720
 170 180 190
 K G S Q N G N G V I R S N Q H L I P I T S Q Y L E L T G E V
 AAGGTTCTCAAATGGTAATGGTGAATTCGTTCAAATCAACATCTCATTCCAATAACTTCTCAATATTTAGAATTAAGTGGTGAAGTTC 810
 200 210 220
 Q S V Q D G D A N L N I Y F I V N S P N Q T T V D Q T P V H
 AATCAGTTCAAGATGGTATGCAAAATTTAAATATCTATTTTCATTGTCAATAGTCCAAACCAACCACTGTTGATCAAAACACAGTACATG 900
 230 240 250
 V Y S E V Y G T D D D G K K V A I A W V A G V T S A Q P I Q
 TTTATTCAGAAGTTTATGGTACTGATGATGATGGTAAAAAGTTGCAATTGCTTGGGTGCGGTGTTACATCAGCTCAACCAATTCAAG 990
 260 270 280
 G S T T T F A L S A V L N E R W I A K V G A T A P F F V K N
 GTTCAACCACTACTTTTGCCTCTCTGCAGTTCTTAATGAACGTTGGATCGCCAAAGTTGGTGCAACCGCTCCATTCTTTGTAAGAATG 1080
 290 300 310
V O V S D L D T F I P L S N T T S T S N V K M V G E Y K D V
 TTCAAGTCTCTGATTTGGATACTTTTATTCCTCTTCAAATACAACATCAACATCAAATGTTAAATGGTTGGTGAATATAAAGATGTTTC 1170
 320 330 340
R T I V H S P P L H E I T K E M R D G K M P K E L A D R I G
 GTACAATTGTTTCATTCTCCACCACTTCATGAAATCACCAAGAAATGAGAGATGGTAAATGCCAAAGAATTGGCTGATCGTATTGGTA 1260
 350 360 370
 K S T G N G K L I L T H G Y C S E G V W P I E D F E N S V E
 AATCAACTGGTAATGGTAAATTAATCTGACACATGGTTATTGTAGTGAAGGTGTTTGGCCAATTGAGGATTTTGAAATTTCTGTAGAGT 1350
 380 390 400
 F Q D F N Q N R G N D E F A Q I L A N F G S Q Y T D G F H L
 TCCAAGATTTTAAATCAAATCGTGGTAATGATGAATTTGCTCAAATCTTCCAATTTCCGTAGCCAATATACCGATGGTTTTCATTGTT 1440
 410 420 430
 L L I V K V V N A A L H L V T F Y F S G L D L S Q K L E G R
 TGCTCATAGTCAAGGTGGTAAACGCTGCTCTTCACCTCGTAACATCTATTTTCAGTGGACTTGATCTTTCTCAAAACTTGAAGGTGCTG 1530
 440 450 460
 V I Q S M G T P Y Q G T A L A G T W A S I G S A V G V G C S
 TCATTCAATCAATGGGTACTCCATACCAAGTACTGCTCTCGTGGTACTTGGGCTTCTATTGGTAGTGCTGATAGGTGTTGGTTGTTCTG 1620
 470 480 490
 A N D D L T V D G A A L W L K S I P A D K R A L V Y Y T T T
 CAAATGATGATCTCACTGTTGATGGTGCCGCTTATGGTTAAATCAATTCAGCAGATAAAAGAGCTTTAGTTTATTTATCAACTACTC 1710
 500 510 520
 Q Y S T G S I L N Y C N L A S N A D L R M A E M M V L W I M
 AATATAGTACTGGTTCTTTAATTAATTATTGTAATTTGGCTTCCAATGCAGATCTTGAATGGCCGAATGATGGTGTGTGGATAATAG 1800
 530 540 550
 R H T P L E G G V Y L N N F K D W C H S D G M H S P Q Q T T
 GGCATACTCCACTCGAAGGTGGTGTATATTTAAATAACTTTAAAGACTGGTGTCACTCTGATGGTATGCACTCACCACAACAACTACAA 1890
 560 570
N T E Y N K E M S S N S V W *
 ATACTGAATATAACAAAGAATGAGTTCCAACCTCTGTTTGTAAACTACTTTGTATTATTTTTTTTTTCTCTACAATAATTGTAATAAAAT 1980
 CGATTTATATTAAATTTATTGTCACTTATAATTAAAAA

Figure 1. (Continued on facing page.)

sylation (Yuen et al. 1991); an examination of the derived amino acid sequence indicates that there are three potential N-glycosylation sites located on the asparagines at amino acid positions 154, 217, and 301. A possible internal signal sequence (von Heijne 1985; Zerial et al. 1987; Gierasch 1989) starts at amino acid 240 with a sequence of KKVAIAWVAGV. This is the largest consensus signal sequence in the first half of the cDNA in all three reading frames.

To examine the copy number of CMF genes, blots of restriction-digested genomic DNA were probed with a portion of the CMF cDNA (Southern 1975). In six digests, each with a different enzyme, only one band was observed to hybridize to the CMF cDNA (Fig. 2). This suggests but does not prove that there is only one CMF gene. Even at very low stringencies only one band was observed on Southern blots of *EcoRI*-digested *Dictyostelium* DNA (data not shown).

CMF protein is secreted during development and can be detected in a membrane fraction from vegetative cells (Yuen et al. 1991). To examine the developmental regulation of CMF mRNA, we used a portion of the CMF cDNA to probe Northern blots of RNA from different *Dictyostelium* developmental stages. As shown in Figure 3A, a single 2.1-kb RNA band hybridizes with the CMF cDNA in vegetative cells and in cells developed in standard conditions on filter pads for 2.5 and 5 hr after starvation. Assuming the poly(A) tail to be 100 bp, the observed mRNA size matches the size of the isolated cDNA. No CMF mRNA is detected in cells developed for 7.5, 10, 12.5, 15, or 17.5 hr (when migrating slugs begin to form fruiting bodies).

Having observed CMF mRNA in vegetative cells and knowing that CMF is involved in cell density sensing, we were interested to see whether there was an effect of vegetative cell density on CMF mRNA levels. Northern blots of RNA from vegetative cells growing in shaking culture at low density, mid-log phase, and saturated density were probed with a portion of the CMF cDNA (Fig. 3B). A single 2.1-kb band was seen with approximately the same intensity in all three samples, indicating that

vegetative cell density in axenic shaking culture does not affect CMF mRNA levels.

Antisense transformants do not secrete CMF

To begin to examine the function of the CMF gene, antisense constructs were transformed into *Dictyostelium* cells. In this technique, transcripts from backwards orientation gene fragments apparently hybridize to the endogenous transcripts and prevent their translation (Crowley et al. 1985). Although antisense transformation does not repress gene expression well in higher eukaryotes, it represses expression of the selected gene to below detectable limits in *Dictyostelium* (Crowley et al. 1985; Knecht and Loomis 1987; Klein et al. 1988). Antisense constructs were made by placing fragments of the CMF cDNA in a backwards orientation downstream of actin promoters in the *Dictyostelium* transformation vectors pA6NPT II (Knecht et al. 1986) and pD Neo II (Witke et al. 1987). Under selection for neomycin resistance, these vectors form 50–200 copy tandem repeats integrated into genomic DNA (Knecht et al. 1986; Witke et al. 1987). The actin promoters direct high levels of expression during vegetative growth in axenic shaking culture but not during vegetative growth on bacteria. For cells grown under either condition, the actin promoters also direct high levels of expression in starved cells for the first 6 hr of development and at lower levels thereafter. To determine whether the antisense constructs were able to repress the expression of CMF mRNA, Northern blots of vegetative RNA from wild-type-, antisense-, and vector-alone-transformed cells were probed with a portion of the CMF gene. As seen in Figure 4, wild-type- and vector-alone-transformed cells express the CMF gene. An antisense construct-transformed clone does not contain RNA that hybridizes with the CMF probe, indicating that it does not express CMF. The growth rate and light microscope morphology of the vegetative cells of this transformant were indistinguishable from those of vector-alone-transformed cells.

The antisense-transformed cells were used to verify

Figure 1. The sequence of CMF. (A) The CMF cDNA sequence is shown with arrowheads indicating the beginning and end of the 1.4-kb cDNA; sequence outside of the arrowheads is derived from RACE-PCR. A *PstI* restriction site used to cut the 1.4-kb cDNA into two fragments is at nucleotide 1014. The amino acid sequence of the single, large ORF is also shown, with underlining indicating the embedded tryptic fragment peptide sequences. (B) A Kyte and Doolittle (1982) hydrophobicity plot shows no large hydrophobic regions. Sequence data were submitted to EMBL/GenBank data libraries under accession no. Z11691.

Figure 2. There appears to be a single CMF gene. A genomic Southern blot of *Dictyostelium* DNA was probed with the 0.9-kb *Pst*I–*Eco*RI fragment of CMF cDNA. Approximately 10 μ g of genomic DNA was digested with *Bgl*III (B), *Cl*aI (C), *Eco*RI (E), *Hind*III (H), *Pst*I (P), or *Sal*I (S). (Bars) Positions of molecular mass markers; from top: 6, 5, 4, 3, 2, 1.6, and 1 kb. Only one hybridizing band can be seen in each digest.

that the cloned gene either encoded CMF or was involved in CMF secretion. Wild-type-, vector-alone-transformed, sense, and antisense cells were starved in buffer and the resulting conditioned medium assayed for the presence of CMF. As shown in Figure 5 and as seen previously for wild-type cells (Gomer et al. 1991), wild-type-, sense-, and vector-alone-transformed cells all secrete an activity that allows low-density starved cells to express a prespore-specific antigen. A clone of cells transformed with the pA6N5 Δ P antisense construct secrete much less of this activity. Roughly one in three clones of cells transformed with either antisense construct both did not express detectable amounts of CMF mRNA and secreted barely detectable amounts of CMF activity. The remaining clones contained CMF mRNA and secreted CMF activity. The very low levels of CMF activity secreted by the antisense transformant and the existence of CMF activity secreted by control transformed cells were observed for cells starved in a variety of conditions, including unshaken submerged monolayers, shaking suspension, or cAMP-pulsed shaking suspension. Interestingly, some antisense-transformed clones that showed a partial reduction of CMF message levels also secreted partial amounts of CMF activities (data not shown).

The above results show that the gene we have cloned encodes CMF or is involved in its secretion. Fractionation of cells into a crude cytosol and a sucrose gradient of the pelletable fractions showed previously that CMF is sequestered in vegetative cells in a crude plasma membrane fraction (Yuen et al. 1991). A similar fractionation

of transformed cells showed that the antisense transformants had no detectable CMF activity associated with any fraction of vegetative cells. This suggests that the gene we have cloned or a closely related gene encodes the CMF sequestered in vegetative cells.

CMF is required for aggregation

The CMF antisense transformants allowed us to examine the function of CMF during development. Untransformed, vector-alone-transformed, sense, and antisense orientation-transformed cells were grown in axenic shaking culture and were then starved and allowed to develop on filter pads (Sussman 1987). Aggregate morphology was examined throughout the developmental cycle. We observed consistently that the first three types of cells showed normal development. To our surprise, the antisense transformants just as consistently did not aggregate for at least 2 and as many as 4 days after starvation (Fig. 6B). However, when grown and allowed to starve on a bacterial lawn, the antisense transformants formed normal fruiting bodies. This result was expected

Figure 3. CMF mRNA is present in vegetative cells and cells early in development. (A) RNA was isolated from vegetative (V) and filter pad-developed cells collected at the indicated hours after starvation. Approximately 4 μ g of RNA was loaded in each lane, electrophoresed, and blotted. The blot was probed with random primer-labeled 1.4-kb *Eco*RI fragment of CMF cDNA and washed at a high stringency. Upper and lower bars, respectively, indicate the positions of the 4.1- and 1.9-kb rRNA bands, visualized by methylene blue staining to verify that approximately equal amounts of undegraded RNA were loaded in each lane. (B) RNA was isolated from vegetative cells growing in shaking culture and similarly electrophoresed, blotted, and probed. The vegetative cell densities were very low, 1×10^4 cells/ml (L); mid-log, 1×10^6 cells/ml (M); and high, 1×10^7 cells/ml (H).

Figure 4. CMF mRNA is not detectable on a Northern blot of RNA from antisense-transformed cells. RNA was prepared from mid-log phase vegetative cells growing in axenic shaking culture, and electrophoresed, blotted, and probed as in Fig. 3. The RNA was isolated from untransformed Ax-4 cells (Wt), the same cells transformed with the pA6N5ΔP antisense transformation vector (antisense), or from cells transformed with the parental pA6NPT II vector (vector).

because the actin promoters used for the antisense constructs do not induce expression during growth on a bacterial lawn (Cohen et al. 1986; Knecht et al. 1986). This also indicated that the absence of aggregation by the axenically grown antisense transformants was not the result of the insertion of the vector into the genome or some other mutation.

A necessary prediction of the antisense phenocopy is that wild-type development should be restored by the addition of exogenous purified CMF. To test this, wild-type and transformed cells were developed on filter pads in the presence or absence of purified CMF. As shown in Figure 6, A and C, wild-type cells aggregate in the presence or absence of CMF; the antisense transformants do not aggregate in buffer alone (Fig. 6B) but do aggregate in the presence of CMF (Fig. 6D). The developmental morphology and timing of the antisense transformant starved in the presence of purified CMF had no consistent difference from that of wild-type cells. This indicated that the absence of aggregation in the antisense transformants is the result of a lack of extracellular CMF and not a lack of sequestered CMF or some secondary effect of the expression of the antisense gene.

Discussion

We used a degenerate oligonucleotide encoding a CMF peptide sequence to obtain a cDNA encoding the *Dictyostelium* density-sensing factor. Quantitation indicates that the sequenced peptides, which are all found in the single, large ORF, are from the 80-kD band identified previously as having CMF activity (Gomer et al. 1991) and not from a minor contaminant at this molecular mass. The AUG at the beginning of the ORF is preceded by an A, similar to virtually all *Dictyostelium* genes examined. The ORF ends with TAA, the heavily favored stop codon in *Dictyostelium* (Warrick and Spudich 1988). Analysis of the derived amino acid sequence indicates that CMF is an acidic hydrophilic protein with a polypeptide molecular mass ~78% that of the purifiable 80-kD glycoprotein, indicating that the latter has a large glycosylation content. The predicted acidic pI of the derived amino acid sequence of CMF is in agreement with our observations that CMF protein binds strongly to the anion exchanger DEAE at pH 6.1 (Gomer et al. 1991). Repeated data base searches have failed to demonstrate any significant similarity between the predicted amino acid sequences of CMF or subregions of CMF and those of any known protein. In filamentous bacteria, an extracellular glycoprotein, Sap B, is required for the development of aerial mycelia and thus would appear to have a function similar to that of CMF. However, Sap B has a much lower molecular mass (2 kD) and is present in much higher amounts compared with CMF and thus does not appear to be related to CMF (Willey et al. 1991). Another protein that has an effect somewhat similar to

Figure 5. CMF antisense transformants secrete very little CMF. Cells transformed with the pA6NPT II vector alone (open circle), the vector containing a fragment of the CMF cDNA in the sense orientation (+), or the same vector containing a fragment of the CMF cDNA in an antisense orientation (shaded circle) were starved in high-cell-density shaking culture for 20 hr. The conditioned media produced by these cells were assayed at four dilutions for CMF activity (the ability to induce low-density starved cells to express a prespore-specific antigen). The conditioned medium produced by CMF antisense-transformed cells induces a very small number of cells to express the prespore antigen and thus contains very little CMF activity.

Figure 6. CMF is required for aggregation. Wild-type (A,C)- and CMF antisense (B,D)-transformed cells were developed on filter pads in the presence (C,D) or absence (A,B) of hydroxylapatite-purified CMF (identical results were obtained using vector-alone-transformed cells instead of wild-type cells or SDS-gel-purified CMF instead of hydroxylapatite-purified CMF). Aggregates appear as white dots. The CMF antisense-transformed cells do not aggregate when starved in buffer (B) but do aggregate when starved in buffer containing purified CMF (D). Bar, 2 mm.

that of CMF is C-factor, a plasma membrane-bound extracellular protein required for cellular aggregation, spore differentiation, and gene expression in *Myxococcus xanthus* (Kim and Kaiser 1990a,b). Comparison of the CMF and C-factor amino acid sequences shows no significant similarity. CMF may thus represent a new class of polypeptide ligand involved in cell-cell signaling.

Northern blots of RNA from different developmental stages probed with the CMF cDNA suggest that there is a single CMF mRNA, with a size matching that of the complete cDNA. Although CMF is not secreted by vegetative cells but is secreted throughout development (Mehdy and Firtel 1985; Gomer et al. 1991), the CMF mRNA is present in vegetative cells and during early development and then disappears. This suggests two things about the regulation of CMF secretion: (1) In agreement with previous observations (Yuen et al. 1991), there is a sequestered pool of CMF protein in vegetative cells; (2) the CMF secreted during later development must have been synthesized during early development or is encoded by mRNA not detected on the Northern blots, the latter being a possibility because the CMF secretion rate is quite low (12 molecules/cell per minute; Gomer et al. 1991).

All tested clones of cells transformed with a vector alone or a "sense" construct expressed CMF mRNA and were able to secrete normal levels of CMF. Cells transformed with antisense constructs did not express CMF mRNA and secreted barely detectable amounts of CMF

activity. This strongly suggests that the isolated cDNA encodes CMF and that the peptide sequences used for cloning CMF did not originate from a contaminant of the DEAE, hydroxylapatite, and SDS-gel-purified 80-kD CMF. The absence of membrane-associated CMF in the vegetative cells and the single band observed in the genomic Southern blot suggest that the sequestered vegetative CMF activity is either the product of the same gene as the secreted CMF or the product of a gene whose transcript hybridizes to the CMF antisense transcript and that the cDNA that we isolated encodes CMF rather than a protein necessary for its secretion. The ability of purified CMF protein to rescue the development of the CMF antisense-transformed cells indicates that the antisense transformation is not causing a block at some early step that is required for, among other things, CMF secretion. The viability of the antisense-transformed cells indicates that CMF is not necessary for vegetative growth in axenic shaking culture. Because the actin promoter used for the antisense construct does not cause expression in cells grown on bacteria (Cohen et al. 1986; Knecht et al. 1986), we do not know whether CMF is necessary for growth on bacterial cells. The normal fruiting bodies formed by antisense-transformed cells grown and allowed to starve in plaques on bacterial lawns indicate that the vegetative CMF is sufficient for aggregate formation, as these cells would have CMF mRNA in the vegetative cells but not during development.

One of the most puzzling aspects of CMF was that it seemed to have a redundant function. There appeared to be two signals that could indicate to a cell that it was in an aggregate, the first signal being CMF and the second signal being the high continuous levels of extracellular cAMP found in aggregates. The antisense experiments now allow us to separate the functions of CMF and high continuous cAMP. If a high CMF level signals to cells that they have completed aggregation successfully, we would expect to see the CMF antisense-transformed cells aggregate normally but then not express prespore and prestalk genes. Instead, the antisense transformants do not even begin to aggregate. This, together with the fact that cells need exposure to CMF only for the first 6 hr of starvation (early aggregation period) (Gomer and Firtel 1987), suggests several possible functions for CMF. One possibility is that CMF levels allow cells to sense their local density just before they begin to aggregate. This might then allow the region with the highest density of cells to initiate aggregation and form a single aggregation center. Without such a mechanism, one might envision cells throughout the aggregation field simultaneously beginning to secrete pulses of cAMP and form aggregation centers, resulting in the formation of many small clumps of cells rather than one large aggregate (Mackay 1978). Another possible function for CMF is to coordinate the decision of cells to aggregate: As cells in an aggregation field consume the last few bacteria, one can envision some cells starving earlier than others. Low extracellular CMF levels might then prevent these cells from aggregating until many more of the cells in the aggregation field starve, improving the chances of relay-

ing successfully the chemotactic cAMP signal and also allowing the formation of a larger fruiting body.

Materials and methods

Cell culture

Dictyostelium strain Ax-4 was used for all experiments; growth and development was at 21–22°C. Cells were grown in shaking culture in HL-5 medium as described previously (Gomer et al. 1991). For starvation in the presence of cAMP pulses, 7×10^6 cells/ml were shaken at 110 rpm in PBM (20 mM KH_2PO_4 , 1 mM MgCl_2 , 0.01 mM CaCl_2 at pH 6.1 with KOH). After 1 hr of starvation, a peristaltic pump was used to deliver 0.5 ml of 4×10^{-5} M cAMP in PBM to each liter of cells every 6 min.

Conditioned medium was made from cells starved in PBM for 20 hr as described previously (Gomer et al. 1991). CMF activity was assayed as described previously by starving cells in PBM or PBM containing CMF and then fixing, staining for the prespore-specific antigen SP 70 (also referred to as beejin) by indirect immunofluorescence, and counting the number of SP 70-positive cells in the well of an eight-well slide (Gomer et al. 1991).

To obtain RNA and to observe cells at different developmental stages, cells were developed in PBM on AABP 04700 black filters (Millipore, Bedford, MA) lying on two thicknesses of PBM-soaked Whatman 3 filter pads as described by Sussman (1987). For development in the presence or absence of CMF, this procedure was modified by using 4.7-cm-diam. black Millipore filters cut into quarter sections and covered with 2.5×10^7 cells. Four hours after starvation, filters were transferred to new pads soaked with PBM, 2 ng/ml of hydroxylapatite-purified, or 1 ng/ml SDS-gel-purified CMF (Gomer et al. 1991). Aggregates on the filter pads were photographed on T max 100 film (Kodak, Rochester, NY) using a Nikon 8008 camera with AF 60-mm lens. The growth rate of cells was measured by counting twice a day at least 100 cells from a 20-ml shaking axenic culture starting with cells at 1×10^4 /ml and counting until cells reached saturation. Vegetative cell morphology was examined using phase-contrast microscopy and a 40 $\times$ objective on a Nikon Microphot Fx microscope.

Peptide sequencing

CMF (80 kD) was purified as described previously (Gomer et al. 1991) with the modification that the starving cells were pulsed with cAMP for the first 10 hr of starvation to increase their CMF secretion rate (I. Yuen and R. Gomer, unpubl.).

Aquacide-concentrated hydroxylapatite column fractions were electrophoresed on a SDS-polyacrylamide gel as described previously (Gomer et al. 1991). Proteins were electroblotted to PVDF membrane (Millipore) following Yuen et al. (1989). Bands were visualized by staining with 0.1% amido black in 10% methanol and 2% acetic acid, destained with 45% methanol and 7% acetic acid, and the CMF band was cut from the blot. Protein concentrations were estimated by comparison to defined quantities of molecular mass standards (Sigma). Approximately 5 μg of 80-kD CMF in a cut band was used in an attempt to sequence the undigested protein.

Trypsinization of the CMF protein was done using a combination of the methods of Aebersold et al. (1987) and Bauw et al. (1989). Briefly, the membrane piece containing $\sim 5 \mu\text{g}$ of CMF protein was cut into $\sim 1 \text{ mm}^2$ pieces and incubated for 30 min at 37°C in 1.2 ml of 0.5% PVP-40 dissolved in 100 mM acetic acid. Excess PVP-40 was removed by extensive washing with water and finally with 0.1 M Tris-HCl (pH 8.5). Digestion was carried out for 4 hr at 37°C with 100 μl of a freshly prepared solution of

trypsin at 0.01 mg/ml in 0.1 M Tris-HCl (pH 8.5). The reaction was stopped by acidifying the suspension with 5 μl of 10% trifluoroacetic acid (TFA). The supernatant from the digestion was collected along with 100 μl of 0.1% TFA wash and two water washes of the membranes. The mixture of supernatant from the digestion and washes was loaded on a 4.6-mm $\times$ 25-cm Dynamax 300 Å pore, 5- μm bead size C8 reversed-phase column (Rainin, Woborn, MA) and the peptides were eluted at room temperature with a 1-ml/min linear gradient starting with 0.1% TFA in water and ending 60 min later with 0.08% TFA in acetonitrile. Eluted peptides were detected by absorbance at 214 nm and collected by hand in Eppendorf tubes. The major peptides were concentrated to $\sim 100 \mu\text{l}$ in a Speedvac and sequenced with an ABI 477A pulsed liquid sequencer in Dr. Richard Cook's laboratory at Baylor College of Medicine (Houston, TX). Amino acid quantities were estimated by comparison to amino acid standard mixtures loaded on the sequencer.

cDNA isolation and sequencing

The degenerate oligonucleotide 5'-AT(T/C)GT(A/T/C)CA(T/C)TC(A/T)CCACCA-3' was obtained from Oligos, Inc. (Guilford, CT) and labeled with ^{32}P using [$\gamma^{32}\text{P}$]ATP and T4 polynucleotide kinase following Sambrook et al. (1989).

A λ gt11 cDNA library made from *Dictyostelium* cells starved for 4 hr in shaking culture was obtained from Dr. Peter Devreotes. The library was plated with Y1090 (Young and Davis 1983) on L-amp plates at initially 40–50,000 PFU per 150-mm plate. Benton-Davis screens following Sambrook et al. (1989) were done on Magna nylon 66 membranes (MSI, Westboro, MA). Prehybridization and hybridization were done as described by Sambrook et al. (1989), with the hybridization in a buffer containing tetramethyl ammonium chloride. Blots were washed as described by Wood et al. (1985), with the exception that final washes were at 42°C.

Lambda DNA was purified following a modification of the procedure described in Sambrook et al. (1989) and digested with *EcoRI*. The cDNA insert was subcloned into pBluescript KS(+) (Stratagene, La Jolla, CA). Double-stranded DNA was sequenced with Sequenase version 2.0, following the manufacturer's directions. Seventeen- to 18-mer oligonucleotides were synthesized and used for priming within the cDNA.

To obtain the 5' and 3' ends of the cDNA, RACE-PCR was performed as described previously (Jain et al. 1992). For the 5' end, the oligonucleotide 5'-CCT AAA ATC ATC TCT GC-3' was used as a primer for the reverse transcriptase, 5'-GGT ATG CAC TCA CCA CA-3' was used for the first round of PCR, and 5'-TGA GTG GAC GAT TGG CAG-3' was used for the second round of PCR. The oligonucleotide 5'-GGT ATG CAC TCA CCA CA-3' was used to obtain the 3' end. The PCR products were then cloned into pCR1000 (Invitrogen, San Diego, CA) following the manufacturer's directions and sequenced as described above.

The predicted protein sequence of the CMF gene product was compared with the protein information resource (PIR), SWISS-PROT, and GENPEPT data bases with the FASTA (Pearson and Lipman 1988) and SEARCH programs (Lawrence and Goldman 1988). The Chargepro (Intelligenetics, Mountain View, CA) program was used to predict the pI, hydrophobicity was determined with the algorithms of Eisenberg et al. (1984), and hydrophathy by the algorithms of Kyte and Doolittle (1982).

Southern blotting and hybridization

Minipreps of genomic DNA were done as described by Nellen et al. (1987). DNA was digested with various restriction enzymes,

separated on a 0.9% agarose gel, and blotted onto Duralon UV membrane (Stratagene) according to Southern (1975). Blotting, prehybridizing, and hybridizing were done following the Duralon UV manufacturer's directions. A probe for the CMF gene was made with the 0.9-kb *PstI*-*EcoRI* fragment of the CMF cDNA. Labeling was done with a [α^{32} P]dCTP using a random hexamer primer procedure (Boehringer Mannheim, Indianapolis, IN). Blots were hybridized at 42°C and washed twice with 5× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 0.5% SDS, for 15 min each at room temperature and then twice for 10 min each at 42°C with 2× SSC, 0.1% SDS. For low-stringency Southern blots, hybridization was at 32°C and washes were done as above with 5× SSC, 0.5% SDS, at room temperature and then with 5× SSC, 0.5% SDS, at 32°C.

Northern blot analysis

RNA was isolated from cells grown in shaking culture or from filter pads at the times indicated after starvation following Nellen et al. (1987). Electrophoresis of RNA was done following Lebrach et al. (1977); RNA was visualized by ethidium bromide staining (Sambrook et al. 1989) to verify that approximately equal amounts of RNA had been loaded and that the visible ribosomal RNA bands were substantially free of degradation. Blotting to Duralon UV (Stratagene) was done following the manufacturer's directions. The probe was the 1.4-kb *EcoRI* cDNA fragment prepared as described for Southern analysis. Blots were washed twice with 2× SSC, 0.1% SDS, at room temperature for 15 min each and then twice for 15 min each with 0.1× SSC, 0.1% SDS, at 55°C. Autoradiography on preflashed Kodak X-Omat AR5 film was done at −70°C for 24–48 hr. The blot was then stained with methylene blue (Monroy 1988) to verify that transfer of RNA had occurred for all samples.

Dictyostelium transformation

For antisense transformation and sequencing, the 1.4-kb *EcoRI* cDNA fragment isolated from the λ gt11 library was cut with *PstI*, and the two fragments were subcloned separately into *EcoRI*- and *PstI*-digested pBluescript KS(+). The two fragments thus had opposite orientations in the vector. The two cDNA fragments and a portion of the polylinkers were excised by digesting with *SalI* and *BamHI* and again ligated separately into *BamHI*- and *SalI*-digested pA6NPT II (Knecht et al. 1986). The two constructs were designated pA6N8ΔP (containing the 905-bp *PstI*-*EcoRI* cDNA fragment in a "sense" orientation) and pA6N5ΔP (containing the 502-bp *PstI*-*EcoRI* cDNA fragment in an orientation appropriate for antisense repression of the CMF gene). Because the location of the *SalI* and *BamHI* sites in pA6NPT II causes the insert to be expressed as a portion of the neomycin phosphotransferase mRNA located downstream from the stop codon, we would not expect the CMF fragment in pA6N8ΔP to be expressed in transformed cells. A second antisense transformation vector designated pDNeo II α R was constructed by ligating the 1.4-kb *EcoRI* CMF cDNA fragment into the *EcoRI* site of pDNeo II (Witke et al. 1987). After subcloning, constructs having the CMF cDNA in an antisense orientation were identified by digestion with *PstI*.

Dictyostelium cells were transformed using the calcium phosphate method (Nellen et al. 1987), as described by Knecht et al. (1990), and selecting for clonal transformants by plating in soft agar (Knecht et al. 1990) containing 10 μg/ml of G418 (Sigma). Colonies appearing were first transformed to SM/5 plates spread with *Klebsiella aerogenes* (Sussman 1987). Vegetative cells from clearings in the bacterial lawn were then

transferred to 10 ml of HL-5 containing 10 μg/ml of G418 and grown axenically.

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