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A density sensing mechanism in the eukaryote *Dictyostelium discoideum*

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Rice University, 1992
A DENSITY SENSING MECHANISM IN THE EUKARYOTE

*Dictyostelium discoideum*

by

ITA S. YUEN

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ABSTRACT

A DENSITY SENSING MECHANISM IN THE EUKARYOTE

*Dictyostelium discoideum*

by

ITA S. YUEN

I am interested in understanding a mechanism by which multicellular organisms sense number of cells of the same type. The model selected is the conditioned medium factor secreted by *Dictyostelium discoideum*. In submerged monolayer culture, *Dictyostelium* cells can differentiate into prespore and prestalk cells at high cell densities in response to cAMP but this process does not occur at low cell densities. However, cells at low densities will differentiate in medium taken from developing cells starved at a high density. The putative factor in the medium was designated CMF for Conditioned Medium Factor (Mehdy and Firtel 1985). In this report, we show that the CMF activity can be separated into high and low molecular weight fractions. The large conditioned medium factor can be purified to a single 80 kD protein with N- and O-linked glycosylation and has CMF activity at a concentration of ~4 pM (0.3 ng/ml). This 80 kD CMF can undergo size reduction to a ~100-fold more active set of smaller peptides with molecular weight less than 10 kD. Glycosylation is required for the activity of the low molecular weight CMF. Starvation triggers the release of CMF from a precursor pool already present in vegetative cells,
and diffusion calculations indicate that the CMF level in the vicinity of a single isolate will not accumulate to the threshold concentration ~0.3 ng/ml. CMF antisense transformants do not aggregate, whereas normal development is restored by the addition of purified 80 kD CMF. These results suggest that CMF is a secreted factor that functions *in vivo* as an indicator of cell density in starved cells. The developing cells simultaneously secrete CMF and monitor its extracellular level. When a majority of the cells in a given area have starved as indicated by the high level of CMF, aggregation is triggered to ensure the onset of development is synchronized. When present below a threshold concentration, the expression of genes required for early development is blocked or not induced. This factor plays an essential role in the regulatory pathway necessary for cells to obtain the developmental competence to induce prestalk and prespore gene expression in response to cAMP.
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<thead>
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<th>Description</th>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic 3’,5’-adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complemental deoxyribonucleic acid</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic 3’,5’-guanine monophosphate</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>CMF</td>
<td>Conditioned medium factor</td>
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<td>CMF-H</td>
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<tr>
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<tr>
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<td>dCTP</td>
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<td>ELISA</td>
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<td>Abbreviation</td>
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</tr>
<tr>
<td>g</td>
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<td>HEPES</td>
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<td>HPLC</td>
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<td>kD</td>
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<td>Sodium phosphate, dibasic</td>
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Neo  Neomycin
nl   Nanoliters
nM   Nanomolar
PAGE Polyacrylamide gel electrophoresis
PBM  Phosphate buffer plus MgCl₂
PMSF Phenylmethylsulfonylfluoride
PSF  Prestarvation factor
RNA  Ribonucleic acid
rpm  Revolutions per minute
SDS  Sodium dodecyl sulfate
SSC  NaCl/Na citrate solution
STI  Soybean trypsin inhibitor
TBS  Tris buffered saline
TBST Tris buffered saline plus Tween 20
TFA  Trifluoroacetic acid
Tris-HCl Tris[Hydroxymentyl]-aminomethane hydrochloride
µg   Micrograms
µl   Microliters
µM   Micromolar
UV   ultraviolet
v/v  Volume/volume
W    Watt
Acknowledgements

I would like to thank my advisor Dr. Richard Gomer for the help and support he provided during the 3.5 years I worked in his laboratory and for all the late night scientific discussions. I must also thank Dr. Maureen Price, the co-leader of the Gomer/Price group, for her help and cares. In addition, I would like to thank the members of my committee for the scientific rigor and suggestions that they supplied. All the staff in the Department of Biochemistry and Cell Biology has been especially kind and generous. I would like to especially thank Delores for her help throughout my graduate career.

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I would like to acknowledge the contribution of Dr. G. Schroepfer and members of his lab in my development as a scientist.
Finally I dedicate this thesis to my parents. I would like to thank my parents for their unfailingly love, support and encouragement and for their willingness to listen to my complaints, uncertainties, and frustration throughout my graduate career.
CHAPTER I

Introduction

Tissue Density and Mass Sensing in Biological Systems

One aspect of developmental biology that remains poorly understood is the molecular mechanism(s) by which multicellular organisms control the size of their tissues. This mechanism will determine how long cells of a given lineage should proliferate during embryogenesis, during tissue regeneration, or during wound healing. Disruption of such a density sensing mechanism would probably lead to tumor formation, i.e., transformed cells may have lost their ability to regulate the number of cells in that tissue and thus result in uncontrolled proliferation. One way cells can sense globally the number of other cells of the same type within a given tissue is for them to secrete a tissue-type-specific density sensing factor which functions through an autocrine mechanism. The concentration of such a density sensing factor could be used by cells to monitor the number of other cells in that tissue. Thus in the case of a tissue deficiency created by disease, injury or surgical removal, the total number of cells in that tissue would drop leading to a decrease in secretion of that tissue-specific density sensing factor. Once cells in the injured tissue sense a fall in the concentration of the density sensing factor, they can presumably become released from G0 arrest and multiply until the concentration of such a factor reaches normal levels. One would also predict that such a factor acts through surface receptors and plays a role in regulation of gene expression to perhaps effect cell proliferation. The biological precedents for
autocrine type factors necessary for growth and differentiation have been
described in mammalian cell culture systems; most of these secreted
molecules are peptide growth factors (Sizeland and Burgess 1991; Bernard
et al. 1991; Tosato et al. 1990; Böhmer and Burgess 1989; Karasuyama et
al. 1989). Similar autocrine-like factors are also used by prokaryotes to
detect cell density. Examples include the extracellular differentiation
factor secreted by Bacillus subtilis (Grossman and Losick 1988) and C-
factor present on the membrane of Myxococcus xanthus (Kim and Kaiser
1990a,b) for spore formation and the autoinducer secreted by Vibrio

**Biochemistry of Dictyostelium discoideum**

To investigate the feasibility of the proposed density sensing
mechanism, one needs to select a relatively simple developmental system
where regulation of developmental processes can be easily dissected by
both biochemical and molecular biological methods. The soil amoebae
Dictyostelium discoideum provides several advantages as a model system
for studies in development. The life cycle of this organism consists of
distinct growth (unicellular) and developmental (multicellular) phases. In
the natural environment, they feed on bacteria. However, strains that can
multiply in relatively inexpensive liquid media composed of glucose, yeast
extract and peptone have also been developed (the axenic strains). As long
as food is available, they divide by binary fission so that the population
increases exponentially. The generation time for axenic strains is about 8
hours, whereas that for the wild-type is 4 hours (Loomis 1982; Devreotes
1989). In shaking cultures, axenic strains are routinely grown to 0.5-1.0 x 10^7 cells/ml in our labotatory for biochemical manipulations.

The multicellular developmental phase is initiated by deprivation of nutrient, usually by shifting from nutrient-rich medium to low concentrations of phosphate buffers. The end products of development are multicellular fruiting bodies comprised of a mass of spores supported by a 2 mm column of stalk cells. If the parameters that may affect development, such as initial cell density, temperature, and surface wetness are held constant, the developmental program will take about 24 hours to complete, with reproducibility within 1 hour (Loomis 1982; Devreotes 1989). Cells can switch between growth and development readily; reintroduction of nutrients to developing cells will restore the ability of the cells to multiply, as long as terminal differentiation has not been reached. This reversibility provides a major advantage: developmental mutants can be selected, grown, maintained and the phenotype reexpressed by switching to nutrient-free buffers. Cells can be stored frozen or as spores indefinitely (Devreotes 1989).

The genome of Dictyostelium contains about 40,000-50,000 kb of DNA (1% of the size of the human genome) which are arranged in 7 chromosomes (Loomis 1986; Devreotes 1989). Although they have a true diploid phase, the major growth and developmental processes studied to date are expressed by haploid cells. Dictyostelium genes are also rich in A and T residues, limiting the possible number of making degenerate codons which makes the task of screening cDNA or genomic libraries more facile for a particular coding sequence (Loomis 1986). Common motifs shared
by *Dictyostelium* genes have also been reported, including a TATA box at about 30 to 40 nucleotides from the start of transcription and a stretch of T residues between the TATA box and the CAP site (Kimmel and Firtel 1987).

Transformation with exogenous DNA has also been well established with efficiency of approximately $10^{-5}$ to $10^{-3}$. The available plasmids contain a neomycin transferase gene flanked by the actin 6 promoter and a variety of termination sequences (Nellen *et al.* 1984b, 1987; Knecht and Loomis 1987; Knecht *et al.* 1990). Plasmids with additional markers have also been developed that can complement cells with thymidylate synthase deficiency (Dynes and Firtel 1989) or cells lacking uridine monophosphate synthase (Kalpaxis *et al.* 1990). Most vectors integrate into the genome in tandem repeats of 10 to 300 copies, although extrachromosomal plasmids have also been developed (Farrar and Williams 1988). These transformation vectors have been used for antisense and homologous recombination experiments, for expression of heterologous genes, and for analysis of promoter function (Devreotes 1989). The antisense and homologous recombination approaches in *Dictyostelium* have been proven to be especially powerful in mimicking mutations of specific genes, examples including the studies of discoidin gene and myosin heavy chain gene (Crowley *et al.* 1985; Knecht and Loomis 1987; Klein *et al.* 1987b; Rubino *et al.* 1989).

As will be described in later sections, *Dictyostelium* cells share many similarities of functions and genes with mammalian cells. The availability of transformation tools, easy and inexpensive maintenance, and relatively
simple developmental pattern make this organism attractive for studies of signal transduction, motility and chemotaxis, cell-cell communication, gene expression in development and pattern formation (Devreotes 1989). These studies will undoubtedly yield general insights for biological systems. It is also our hope that what we understand about the density sensing mechanism of *Dictyostelium discoideum* can be applied to higher eukaryotic organisms.

**Dictyostelium discoideum as a Developmental Model System**

*Dictyostelium discoideum* life cycle consists of simple but distinct developmental stages, studies of which may provide insights in pattern formation and induction of differentiation. As mentioned previously, *Dictyostelium* cells can be switched between unicellular (vegetative growing) and multicellular (developing) modes by simply removing food sources. Growth ceases within a few hours of development. The first development stage is completed when the previously solitary *Dictyostelium* cells become sociable and aggregate into a group of $10^5$ cells by means of chemotaxis towards cAMP-producing signalling centers. The aggregate normally found below the surface on the forest floor has then developed into a migratory slug and subsequently migrates to the surface where it culminates and forms a mature fruiting body containing only two terminally differentiated cell types: spore and stalk cells (Loomis 1982).

Aggregation of amoebae is directed by chemotaxis: upon receipt of cAMP stimulus, cells migrate towards the signalling center and synthesize and release cAMP to relay the chemotactic signal throughout the
Figure 1.1  The developmental cycle of *Dictyostelium discoideum*
aggregation territory. The local cell density affects aggregate formation, and the development is highly regulated over a very wide range of cell number (Loomis 1982; Williams 1988). At the end of aggregation, a tip is formed at the top of cell mound. The aggregate at this stage has become a discrete entity, surrounded by an extracellular matrix comprised of protein and cellulose. All cells within the aggregate behave cooperatively to facilitate spore formation. *Dictyostelium* cells develop normally only at an air-water interface; however, submerged cells can advance through tight aggregate stage under water without further development (Loomis 1982).

In response to light, humidity, and ionic strength of the environment, the tip on the aggregate mound collapses onto the substratum and transforms into a cylindrical migratory pseudoplasmodium (slug). The slug is exquisitely sensitive to light and temperature gradients. These sensitivities direct the slug to migrate to light where it reaches up to form fruiting body. Precursors to spore and stalk cells begin to appear in the early slug stage. They are arranged spatially with the anterior 20-30% of slug occupied by prestalk cells while the posterior 70-80% is composed predominantly of prespore cells. However, approximately 10-15% of cells in the prespore region display the morphological and biochemical characteristics of prestalk cells and are thus termed anterior-like cells (Sternfeld and David 1981, 1982; Devine and Loomis 1985). At this stage, prespore and prestalk cells can easily be separated by centrifugation in percoll gradients (Ratner and Borth 1983). Although the decision to become a prespore or a prestalk cells is initially determined by cell cycle phase at the time of starvation (Weijer *et al.* 1984; McDonald 1986; Gomer
and Firtel 1987), cell fate during later development is position-dependent and can modulated by local diffusible molecules in the slug such as cAMP, a group of chlorinated hydrocarbons known as differentiation inducing factors (DIFs), ammonia, adenosine and oxygen (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). Furthermore, _Dictyostelium_ cells develop without further growth; a change in cell type reflects the conversion of one cell type to another rather than cell division (Loomis 1982, 1986). Thus, we have a traceable developmental system in the slug with an easily traceable fate map: the anterior 20% are destined to be stalk cells and the posterior 80% are destined to be spore cells; furthermore, the ratio of readily distinguishable prespore to prestalk cells can be induced to vary by local determinants. Ammonia which is required for spore cell formation acts antagonistically to DIF, an inducer of prespore-specific genes, and reverses the decision of a cell to become a prestalk cell (William 1988). The induction of a cell type switch by local diffusible factors resembles the mesoderm induction of the vertebrate embryo (Williams 1988; Jessel and Melton 1992).

Terminal differentiation takes place roughly 18 hours after starvation. At this culmination stage, the prestalk cells migrate backward through the prespore zone coupled with the swelling that accompanies their final differentiation into stalk cells. The swelling of prestalk cells creates a column of highly vacuolated, celluose-ensheathed dead cells to lift up the prespore cells. These eventually differentiate into ellipsoidal spore cells which are dormant and encapsulated in heat and stress resistant spore coats.
The development cycle is thus completed with the end product being a mature fruiting body. The construction of the fruiting body accomplishes two things: the stress-resistant spores can survive longer and, being raised above ground by stalk cells, have a better chance of being dispersed to a food supply-rich environment (Loomis 1982). Once scattered to food sources, spores germinate to amoebae and will begin another developmental cycle upon exhaustion of the food supply.

**Signal Transduction and Chemotaxis**

**Signal Relay by Aggregating Dictyostelium discoideum**

The entry of *Dictyostelium discoideum* into multicellular mode is the formation of the aggregate. For the initially dispersed cells to become aggregated, some type of signaling system must be utilized. This organism has developed a chemotactic apparatus that takes at least 7 to 9 hours to become fully active. By unknown mechanism, a few cells start to synthesize and release cAMP in pulses and become the signalling centers in response to starvation. The neighboring cells, upon receipt of cAMP signal begin to acquire the ability to move towards the cAMP source and then the capacity to relay the signal, and finally, after 7 to 9 hours, can emit the cAMP signal autonomously (Gingle and Robertson 1976). To generate and maintain cAMP in pulsatile waves requires concerted actions of the cAMP receptor (Henderson 1975; Klein *et al.* 1987a,b), adenylate cyclase (Devreotes and Steck 1979; Klein 1977; Roo *et al.* 1977), extracellular cyclic nucleotide phosphodiesterase, its inhibitor (Gerish *et al.* 1972; Klein 1975; Orlow *et al.* 1981; Franke and Kessin 1981) and cAMP secretory
apparatus (Loomis 1979). When cAMP is released from the signalling center, it binds to cell-surface cAMP receptors on the neighboring cells and transiently activates adenylate cyclase via a guanine nucleotide binding protein (G protein). As a result, ATP is converted to cAMP which is then released into the extracellular space (see Firtel et al. 1989 for review). The accumulation of cAMP to a high level around a cell (which would inhibit aggregation) is prevented through diffusion and the action of membrane-bound and extracellular phosphodiesterases (Malchow et al. 1972; Chang, 1968). To prevent the degradation of all the cAMP, Dictyostelium cells also secrete a glycosylated inhibitor that binds the extracellular phosphodiesterase with a stoichiometry of 1:1 and changes the $K_m$ of the enzyme for cAMP from 10 µM to 2 mM (Kessin et al. 1979).

The chemotactic apparatus of aggregating Dictyostelium cells allows cells to relay the signal outwards from and move inwards to the initial signalling center. Once cells become sensitized by the first wave of cAMP, the activities of the signal-producing system (adenylate cyclase) and the signal-destructive system (phosphodiesterase) oscillate spontaneously in a few of the initiating cells. Activation of adenylate cyclase is followed by a self-stimulating rhythmic mechanism to liberate micromolar quantities of cAMP every 5-10 minutes. These initiating cells act as the aggregation center. The surrounding cells which have not reached this self-perpetuating state of excitement are nonetheless highly excitable. They will secrete a pulse of cAMP in response to one received. This is how cAMP signals are relayed and transmitted outwards during aggregation. The signal is prevented from transmitting inwardly because the amoebae which
have just relayed become refractory for further stimulation for about 2.5 minutes (Newell 1986).

**Chemotactic Events**

Amoebae also respond to the cAMP signal by moving for about 10 seconds towards the signal center and stopping to await for the next signal that will come 5 to 7 minutes later. Meanwhile, cells to the rear of those that have just received a signal can still respond to it by moving inwardly and relaying the signal outwardly. This discontinuous but rhythmical movement enables up to $10^5$ cells to aggregate (Newell 1986; Kessin 1988). Chemotaxis towards the signalling center is associated with actin-mediated events. Within 3-5 seconds of binding of cAMP to the receptor, actin associated with the cytoskeleton is transiently polymerized. The major, most acidic isoactin A1 is involved in this process. The actin converts from globular, unassociated form to the filamentous form during polymerization which occurs at the cortical region beneath the plasma membrane. Since polymerization of actin is correlated with the rapid formation of a pseudopodium from the cell in the direction of the chemotactic stimulus, it acts as a direction-determining system. Cell elongation and translocation occurs 40-60 seconds later. In addition to polymerization, cAMP also induces a transient 5-10 fold elevation in intracellular cGMP level which peaks at 9-12 seconds after stimulation (Newell *et al.* 1988; van Haaster *et al.* 1989). Mutant streamers (*stmF* loci) presumably due to defective cGMP phosphodiesterase showed normal events of chemotaxis except for cell elongation (occurs normally at 40-120 seconds after stimulation).
Therefore, cGMP is proposed to regulate shape change. In addition to the changes described following the reception of the cAMP signal, a great uptake of Ca\(^{2+}\) to the low micromolar range can also be observed. This uptake of Ca\(^{2+}\) might be associated with the activation of enzymes of the citric acid cycle in the mitochondria leading to the increased ATP production required for subsequent cell movement (Newell et al. 1987).

**Cell-Surface cAMP Receptors**

All of the cell responses to the cAMP signal described so far are mediated through the cAMP surface receptors. In addition, extracellular cAMP, similar to peptide growth factors in vertebrate embryos, acts as a developmental regulator coordinating the expression of genes and morphogenesis via cell surface receptors (Melton 1991). The transmembrane signaling by cAMP in *Dictyostelium* cells, like hormone-mediated signal transduction in mammalian cells, is conducted through a G protein linked surface receptor (Devreotes 1989). The 40 kD *Dictyostelium* receptor contains the classical seven transmembrane-spanning domains and a serine- and threonine-rich carboxyl terminus, typical of those that interact with G-proteins (Saxe et al. 1988; Klein et al. 1988). The expression of the receptor responsible for effecting chemotaxis and signal relay during aggregation stage is undetectable in vegetative cells, reaches maximum levels 3 to 4 hours after starvation, and is induced by pulses of cAMP (Klein et al. 1988). The formation of a covalent bond between a nucleophilic residue of the receptor and the phosphate of cAMP molecule activates the receptor (van Haastert and Klein 1983).
The adaptation of *Dictyostelium* cells to further cAMP stimulation after they have responded to a signal is controlled at the level of receptor. It is observed that prolonged occupancy by ligands results in desensitization of the cAMP surface receptor (Newell 1977; Loomis 1982; Klein *et al.* 1985; Newell 1986; Gerish 1987; Janssens and van Haastert 1987; Klein *et al.* 1987b; Kessein 1988; Firtel *et al.* 1989) which is closely related to the phosphorylation of serine residues at the carboxyl terminus (Vaughan and Devreotes 1988). The association of phosphorylation with desensitization of a cell surface receptor is also observed in rhodopsin and β-adrenergic receptors (Lefkowitz *et al.* 1988). Resensitization to cAMP occurs concomitantly with dephosphorylation of receptors as the extracellular cAMP diffuses away or becomes hydrolyzed by the developmentally regulated membrane-bound and secreted forms of phosphodiesterase (Gerish 1987; Kessin 1988). The presence of continuous high levels of cAMP that saturate phosphodiesterase results in loss of binding sites and down regulation of receptor at the transcriptional level (Wang *et al.* 1988).

**Transmembrane Signal Transduction**

To effect differential responses to external cAMP signal, *Dictyostelium* cells utilize the connection of cAMP receptors via one set of G proteins to sensory and motility apparatus for chemotaxis and the linkage of the receptor via another set of G-proteins to the secondary messenger cascades for gene regulation. It appears that there are a multitude of subtypes of cAMP receptors that are encoded by separate but closely related receptor genes that are developmentally regulated (Saxe *et al.*
1991). However based on cAMP dissociation rate, only fast receptors (50-100 x 10^3/cell) and slow receptors (~4 x 10^3/cell) have been reported (Janssens and van Haastert 1987). These receptors are in turn coupled to different G proteins. One class of G proteins contains \( \alpha \beta \gamma \) subunits, similar to those found in mammals. Two genes for \( \alpha \) subunits have been found that share a large portion of sequence identity with each other and with those found in mammalian and yeast cells (Firtel et al. 1989). From complementation studies, a family of mutants designated Frigid A has been isolated and found to be defective in the \( G_2 \) subunit (Kumagai et al. 1989). Frigid A mutants do not aggregate even when provided with exogenous cAMP; in addition, the cAMP binding is unaffected by guanine nucleotides. A homolog of the G protein \( \beta \) subunit has also been found in yeast that participates in \( \alpha \)-a-mating factor response (Devreotes 1989).

Chemotaxis induced by cAMP is closely connected to the signal transduction pathway that leads to the production of inositol phosphate (IP\(_3\)). It has been observed that IP\(_3\) and Ca\(^{2+}\) can mimic cAMP-induced actin polymerization and cGMP production (Small et al. 1986; Europe-Finner and Newell 1980). Stimulation with cAMP causes transient accumulation of IP\(_3\) which in turn mobilizes Ca\(^{2+}\) from intracellular stores (Europe-Finner and Newell 1986; Kimmel and Eisen 1988). Thus polymerization of actin and the production of cGMP in response to the activation of the surface cAMP receptor are probably effected via the formation of IP\(_3\) (by the action of phospholipase C) and subsequent release of Ca\(^{2+}\) from internal non-mitochondrial stores (Europe-Finner and Newell 1985;). Since permeabilized Frigid mutants cells do not produce IP\(_3\)
stimulation by cAMP, it has been suggested that the chemotaxis cAMP receptor (the slow receptor) is coupled to phospholipase C by $G_{o2}$ (Janssens and van Haastert 1987; Newell et al. 1988; Snaar-Jagalska et al. 1988; Kumagai et al. 1989).

The activation of adenylate cyclase which leads to the production of cAMP has been associated with the fast cAMP receptor (Janssens and van Haastert 1987). A series of mutants designated Synag show normal chemotaxis as long as cAMP pulses are supplied either by wild type cells or exogenous sources but their adenylate cyclase and cAMP signaling response are not sensitive to guanine nucleotides, indicating that chemotaxis and signal relay are separate pathways. Adenylate cyclase, G proteins $\alpha$ and $\beta$ subunits and receptor in these mutants show normal characteristics (Theibert and Devreotes 1986; Kumagai et al. 1989). Biochemical analysis with one of the mutants, Synag7, showed that the defect in the activation of adenylate cyclase by guanine nucleotides can be complemented in vitro with a cytosolic protein from wild-type cells (Theibert and Devreotes 1986).

*Regulation of Early Genes by cAMP*

Expression of many genes that are essential for the completion of aggregation is tightly linked to chemotaxis and morphogenesis since the common control is by extracellular cAMP through its cell surface receptor (Mann et al. 1987; Kimmel 1987; Mann and Firtel 1988; Kessin 1988). Pulsatile cAMP is responsible for induction of signal relay and chemotaxis. Thus, it is not surprising that expression of many early genes is regulated
by pulses of cAMP. Genes whose expression are induced by pulsatile cAMP include cAMP receptor itself, G\(_{\alpha}2\) that links the receptor to phospholipase C, contact site A that is important for cell adhesion during early development, and gene D2, a serine esterase, that is required for aggregation (Gerish 1987; Kimmel 1987; Klein et al. 1988; Kumagai et al. 1989; Mann et al. 1988). The transcripts of these genes are commonly not detected in vegetative cells, start to appear 2-3 hours after onset of development, and reach maximum levels during the peak aggregation. Periodic application of cAMP, with cAMP destroyed by phosphodiesterase between pulses, results in a precocious and higher induction of gene expression. Continuous stimulation with high levels of cAMP, which causes phosphorylation and thus desensitization of the cell surface cAMP receptor, substantially reduces or inhibits the expression of these genes. Analysis of mutants that carry known defects in the signal transduction pathways demonstrates that the expression of these genes is regulated through the inositol phosphate second messenger pathway linked to phospholipase C.

Pulses of cAMP also repress expression of some early genes. This class of genes is usually not expressed during vegetative growth. Starvation induces their expression; transcriptional activity reaches maximum levels at about 2.5 hours of development and is repressed during aggregation. Repeated stimulation with low concentrations of cAMP down-regulates the expression of these genes, while cAMP, at levels too high to be totally hydrolyzed, induces gene expression. Just like their counterpart (pulse-induced genes), the pattern of expression of one of these
genes is altered in mutants that have defective Gα2 protein believed to activate phospholipase C to initiate inositol phosphate second messenger cascade (Mann et al. 1987; Mann and Firtel 1988).

The expression of the second class of pulse-repressed genes is also down-regulated by pulses of cAMP, however the repression seems to require the production of intracellular cAMP as the second messenger. One of well studied genes in this class is the M4-1 gene. It is normally expressed in vegetative cells and is repressed during aggregation. Its expression can be repressed by repeated application of low concentrations of cAMP. However, in mutants that are defective in the cAMP receptor-mediated activation of adenylate cyclase, the gene expression cannot be repressed by pulses of cAMP, suggesting that the repression is mediated through the signal relaying (fast dissociating) receptors and cAMP-dependent kinase (Kimmel and Saxe 1986; Kimmel 1987).

There is another class of early genes, notably phosphodiesterase and its inhibitor, whose regulation is not subjected to the adaptation of the surface receptor in the presence of extracellular cAMP. Phosphodiesterase expression is induced by both pulses and continuously high levels of cAMP whereas phosphodiesterase inhibitor shows the opposite regulation (Coukell and Cameron 1987; Kessin 1988). The pathway controlling the expression of these two genes is not well understood at present. It has been postulated that a second messenger system not involving adenylate cyclase or phospholipase C must exist in which the phosphorylated receptor has no effect on an effector molecule producing yet another second messenger. In this way, the second messenger cascade would continue, resulting in
sustained synthesis of phosphodiesterase (and blockade of inhibitor synthesis). The advantage of such a system is that extracellular cAMP can be buffered efficiently to allow coordination between the processes of signal relay and chemotaxis and expression of genes that are involved in these processes. Since no late functions can be induced before the formation of the aggregate is completed, high amounts of extracellular cAMP would interfere with chemotaxis. Attaching the receptor to an effector molecule that is unaffected by the phosphorylated form allows induction of phosphodiesterase synthesis and blockade of inhibitor synthesis in the presence of high levels of cAMP, favoring rapid clearance of the ligand. Cells thus work to restore the conditions under which the cascade leading to the receptor and Gα2 induction can proceed with chemotaxis (Kessin 1988).

Control of Late Gene Expression by cAMP

Many prestalk-specific and prespore-specific genes are also regulated by cAMP. Using mutants defective in the signal transduction pathway, pharmacological agents such as caffeine (blocks activation of adenylate cyclase) or cAMP analogs, it has been demonstrated that the regulation of expression of these late genes by cAMP is mediated through the surface receptors (Mehdy and Firtel 1985; Schaap and van Driel 1985; Gomer et al. 1986a; Schaap et al. 1986; Kimmel 1987). Their regulation of expression does not require a rise in the intracellular cAMP level, suggesting that adenylate cyclase is not involved. Recent experiments using diacylglycerol and IP₃ with permeabilized cell membranes have
demonstrated great induction of prespore and prestalk gene expression, implying that the effector molecule coupled to the cell surface receptor is phospholipase C (Ginsburg and Kimmel 1989). In contrast to pulse-induced genes, the expression of these late genes requires micromolar concentrations of cAMP, which should result in the adaptation of the cAMP receptor found during early development. Recently several additional genes encoding surface cAMP receptors have been reported (Saxe et al. 1991). Based on the appearance of their transcripts during development, the most likely candidate encoding the late receptor is CAR2. Presumably, this late receptor is coupled to either Gα2 or a novel class of Gα protein expressed later in development to mediate the regulation of expression of these late genes.

*Model for the Investigation of Tissue Density and Mass Sensing*

The development of *Dictyostelium discoideum*, like many other developmental systems, proceeds through a temporal sequence of events. Five stages of differentiation characterized by the gene products can be described. As vegetative cell density reaches a threshold (i.e. approaching stationary phase), some early genes begin to be expressed. Starvation triggers the expression of another set of genes, including that for the cAMP receptor, and represses those required specifically for vegetative growth. In response to cAMP pulses, a third set of genes is induced to form multicellular aggregates; in addition, expression of some of starvation-triggered genes, including cAMP receptor CAR1 gene, can also be potentiated by pulses of cAMP. These aggregation-specific genes in turn
induce the fourth set of genes that prepare cells for terminal
differentiation. Many prespore- and prestalk-specific genes presumably
belong to this group. Cytodifferentiation to spore and stalk cells takes
place after the induction of the final set of genes that are regulated by local
diffusible molecules within the slugs, including ammonia. As the temporal
sequence is unfolding, the spatial pattern of cell-type specific gene
expression is also imposed by local diffusible determinants in the form of
DIFs, cAMP, oxygen and ammonia so that posterior cells can express
prespore-specific antigens while anterior cells can differentiate to prestalk
cells in preparation for the formation of the fruiting body (Loomis 1986).

During the analysis of prespore and prestalk gene expression by
cAMP, Mehdy and coworkers (Mehdy et al. 1983; Mehdy and Firtel 1985)
have observed that in addition to high levels of cAMP, another extra-
cellular factor is also required. It has been observed that in the submerged
monolayer culture, Dictyostelium cells can express cell type-specific
antigens (thus exhibiting differentiated phenotypes) at high density but not
at a density too low for aggregate formation; cells at this low density can,
however, differentiate in buffer in which high density cells have previously
been starved (Mehdy et al. 1983; Mehdy and Firtel 1985). Assuming the
temporal sequence of events is as described and differentiation into
prespore or prestalk cells requires aggregate formation, an explanation for
this phenomenon is that during development, Dictyostelium cells secrete
some factor(s) that enable them to sense whether they are far from an
aggregation center and thus need to continue expressing aggregation-
specific genes, or whether they are at an aggregation center and thus can begin expressing differentiation-specific genes.

To investigate this hypothesis, we first isolated and identified factor(s) in the conditioned medium that can induce submerged monolayers of low density Dictyostelium cells to express prespore- and prestalk-specific genes. Characterization of the factor(s) in conditioned medium indicated that an 80 kD glycoprotein and its degradation products were responsible for the activity described by Mehdy and coworkers (Mehdy et al. 1983; Mehdy and Firtel 1985). The regulation of production and secretion of these factors was also examined. Finally, utilizing nucleotide sequence predicted based on peptide sequence of the purified 80 kD protein, we were able to generate antisense transformants that did not produce conditioned medium factors and did not aggregate. Since normal development of the antisense transformants is restored by adding back purified 80 kD conditioned medium factor, we concluded that the conditioned medium activity originally described by Mehdy and coworkers is required for aggregate formation.
CHAPTER II

Purification and Characterization of Conditioned Medium Factors

I. Introduction

Very little is known about how cells in an organism sense how many cells of a particular type there are in a given tissue. One possible mechanism would be for cells to secrete cell-type-specific factors, the concentrations of which would be proportional to the relative number of that cell type. Examples of this type of autocrine factor that cells simultaneously secrete and sense are the C-factor secreted by high density *Myxococcus xanthus* (Kim and Kaiser 1990a,b), the autoinducer produced by the light organ symbiont *Vibrio fischeri* (Eberhard *et al.* 1981), and the extracellular differentiation factor A that stimulates spore formation of *Bacillus subtilis* (Grossman and Losick 1988). We are studying a factor of this sort that is apparently used by the eukaryote *Dictyostelium discoideum* to sense cell density during development.

The mature fruiting body of *Dictyostelium discoideum* is composed of two cell types: spores and stalks. Precursors to spores and stalk cells (prespore and prestalk cells, respectively) can first be identified in the aggregate stage using monoclonal and polyclonal antibodies which react specifically with either prespore or prestalk cells (Krefft *et al.* 1984; Data *et al.* 1986; Gomer *et al.* 1986b; Jermyn *et al.* 1989; Williams *et al.* 1989; Haberstroh and Firtel 1990; Esch and Firtel 1991). Prestalk cells are
"randomly" scattered within the forming aggregate or slug and comprise ~15% of the cells. One to 2 hours later as the aggregate is forming a tip, prespore cells can be seen as an annulus in the middle of the aggregate. At this time, the prestalk cells then sort to the anterior region, thus forming the tip (Williams et al., 1989; Esch and Firtel 1991). In addition, some of the prestalk-specific gene products are also expressed in cells located at the extreme posterior of the slug. These include prestalk genes inducible by cAMP and those inducible by DIF, a factor necessary for stalk cell differentiation (see Morris et al. 1988). The pattern of expression of the prespore genes and both classes of prestalk genes has been characterized at the level of transcription (see Hjorth et al. 1989, 1990; Williams et al. 1989; Haberstroh and Firtel 1990).

The induction of prespore and cAMP-inducible prestalk gene expression in wild-type NC-4 cells and axenic strains such as KA3x-3, derived from NC-4, requires distinct developmental events regulated by at least two extracellular factors. One is cAMP, which induces the expression of both classes of genes via signal transduction pathways acting through cell surface cAMP receptors (Schaap and van Driel 1985; Gomer et al. 1986a; Oyama and Blumberg 1986; Haribabu and Dottin 1986). A continuous level of at least 30 nM cAMP after 4 to 6 hours of starvation is necessary for maximal gene expression (Mehdy and Firtel 1985; Gomer et al. 1986a), while cAMP added earlier blocks this expression, presumably by inhibiting the expression of genes that are expressed earlier and are required for establishing the necessary signal transduction systems (Mann and Firtel 1987, 1989; Mann et al. 1988a,b). This 4 to 6 hours of starvation required for NC-4
derived strains before high concentrations of cAMP can be added is not a requirement for V12M2, an independent wild-type isolate (Gross et al. 1981).

The second extracellular factor necessary for prespore and prestalk gene expression is conditioned medium factor (CMF). Mehdy and Firtel (1985) found that, in submerged monolayer culture, prespore and prestalk gene expression can be induced in NC-4 or KAx-3 cells at high cell densities (~10^5 cells/cm^2) by extracellular cAMP, but not in cells at low cell densities (~5x10^3 cells/cm^2). However, when cells at low densities were plated in buffer previously conditioned by starved cells developing at a high density [conditioned medium (CM)], the low density cells could then express prestalk and prespore genes in response to extracellular cAMP. The expression of earlier genes normally induced during aggregation and during the period between starvation and the onset of aggregation also appeared to require CMF. This result indicated that Dictyostelium cells use a factor present in conditioned medium (CMF), rather than cell-cell contact, to monitor cell density. It also suggested that the entry into development and the expression of selected genes is dependent upon the concentration of that factor, which is in turn proportional to number of cells. Using time lapse videomicroscopy, it was observed that starved cells which never touched another cell differentiate into prespore and prestalk cells in the presence of CMF, further suggesting that CMF concentration, rather than cell-cell contact, mediates the response of genes to cell density (Gomer and Firtel 1987). It was observed that for prestalk and prespore gene expression, CMF needed only to be present for 2-6 hours and could
be removed prior to the addition of cAMP. These data suggested that the factor functioned during the earliest stages of development and was no longer essential once cells were competent to be induced by cAMP (Mehdy and Firtel 1985; Gomer et al 1985; Mann and Firtel 1989).

In this chapter, the purification and characterization of factor(s) in the conditioned medium will be described. Size fractionation experiments indicate that two size classes of molecules secreted by developing Dictyostelium cells contain CMF activity. The high molecular weight CMF can be purified to an 80 kD glycoprotein, which is highly unstable (Gomer et al. 1991). It breaks down to smaller but more active molecules which are presumably the low molecular weight CMFs. The regulation and processing of CMF are also examined.

II. Materials and Methods

Cell Culture
The Dictyostelium axenic strain KAx-3 was used for all experiments. Cells were grown in axenic shaking culture in HL-5 (Firtel and Bonner 1972) with the following modifications in the recipe: 200 μl of a mixture of ampicillin (100 mg/ml), tetracyclin (25 mg/ml), and chloramphenicol (25 mg/ml) (Sigma, St. Louis, MO) and 0.8 ml of 50% glucose were added to 20 ml of a mixture of 14.3 g/l peptone (Oxoid Limited, Basingstoke, Hampshire, England), 7.15 g/l yeast extract (Oxoid), 0.4 g/l Na₂HPO₄, 0.2 g/l NaH₂PO₄, and 0.55 g/l KH₂PO₄ (Yuen et al. 1991). For CM produc-
tion, mid-logarithmic cells (<5 x 10^6) were used whereas for the CM assay, cells growing at a density of less than 1 x 10^6 cells/ml were used.

**Conditioned Medium**

We prepared CM from starving, developing cells using methods similar to the previously described protocol (Mehdy and Firtel, 1985). Log phase KAx-3 cells were collected by centrifugation at 200 x g for 5 minutes, and resuspended and washed in PBM (0.02 M potassium phosphate, 10^-2 mM CaCl₂, and 1 mM MgCl₂, pH 6.1) twice. The cells were then resuspended in PBM at a density of 9 x 10^6/ml and shaken at 110 rpm for 20 hours at room temperature. CM was also occasionally made with cells at 5 x 10^6/ml. The conditioned medium was clarified by centrifugation at 200 x g for 5 minutes. The supernatant resulting from a second centrifugation at 8,000 x g for 15 minutes at 4°C will be referred to as CM (Gomer et al. 1991; Yuen et al. 1991).

**Conditioned Medium Assay**

CM activity was determined using a modification of an immuno-fluorescence assay (Gomer et al. 1986a,b) which measured the expression of prespore-specific genes in *Dictyostelium* cells starved at low density. KAx-3 cells were washed as described above and resuspended in PBM at a density of 2 x 10^6 cells/ml. Two μl of cell suspension was mixed with 200 μl of 1:10 dilution of CM in PBM (positive control), PBM (negative control), or fractions to be tested for CM activity. This mixture was then placed in a well of a Lab-Tek 8-well glass slide (Miles Scientific,
Naperville, IL) and incubated at room temperature. Six hours after plating the cells, cAMP was added to each well to 300 μM final concentration. Eighteen hours after plating, cells were fixed for 10 to 20 minutes by aspirating the supernatant and adding 70 or 95% ethanol (room temperature) to fill the entire well. The slides were then air dried for 1 hour. Immunofluorescence was done as previously described using the anti-prespore antibodies against spore coat protein SP70 or anti-prestalk antibodies against the prestalk protein pst-cathepsin (CP2) (Gomer and Firtel 1987; Gomer et al. 1986b). Briefly, cells were stained first with 1:100 dilution of rabbit polyclonal anti-prespore or anti-prestalk antibody in TBST (10 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 4 drops of Tween-20 (Fisher Scientific, Pittsburgh, PA) for 1 hour, washed for 5 minutes and 30 minutes in TBST before staining with the fluorescein-linked goat anti-rabbit IgG antibody. The ratio of positively stained cells to total cells was determined by counting as described (Gomer et al. 1986b). For the discoidin CM assay, log phase cells at a density of 2 x 10^5 cells/ml were used; no cAMP was added and the cells were fixed at 7 hours after starvation. Rabbit polyclonal anti-discoidin I antiserum (a gift of Dr. Wayne Springer, UCSD) was fractionated by ammonium sulphate precipitation and used at a 1:100 dilution for immunofluorescence. The expression of SP 70 or discoidin was also quantitated by ELISA (enzyme-linked immunosorbant assay). A 96-well microtiter plate (Corning type 4818, Corning, NY) was used with all volumes reduced by a factor of four relative to the eight-chamber glass slides. Detection by ELISA also required higher cell number per area (7 x 10^6 cells/cm^2) so that after the second
wash in PBM, cells were resuspended to a density of $4 \times 10^6$ cells/ml. After fixing and drying, the plate was first preblocked with 10 mg/ml bovine serum albumin (BSA) (Sigma) for 30 minutes and then with 10% Carnation nonfat milk in TBS (TBST without Tween-20). Anti-SP 70 or anti-discoidin antibody was used as the first antibody and goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (Sigma, St. Louis, MO) was used as the second antibody. Phosphatase substrate (Sigma) was prepared freshly each time according to the manufacturer's instruction and 50 µl was added to each well. The reaction was carried out at room temperature until a detectable yellow color developed and was stopped by the addition of 50 µl of 1 M potassium phosphate, pH 10.5 and 30 µl of 1 M NaOH. The absorbance was read on an automated microplate plate reader (Perkin-Elmer Lambda Reader, Norwalk, CT) at 405 nm. Since the reaction times for the alkaline phosphatase-conjugated second antibody and its substrate were not fixed, absorbance at 405 nm should only be used for qualitative comparison. Triplicates were used for each sample when ELISA was performed.

*Quantitative Conditioned Medium Assay*

At least 3000 cells were scored per assay point. Initially, individual column fractions were assayed with at least two dilutions. Fractions containing CM activity were then pooled. Serial dilutions by a factor of 2 of the pooled fractions from each purification step were then assayed. We define maximum response to occur at the dilution where highest percentage of cells differentiated in that set of assays. Following Gherardi *et al.*
(1989), the unit of activity is then defined as the inverse of the highest dilution of the pooled material that showed 50% of maximum response; thus 100 units/ml of activity indicates that at a 100 fold dilution, the number of SP 70 positive cells is half of the maximum number in that assay of SP 70 positive cells.

**Size Fractionation**

Size fractionation and molecular weight determination was initially done using molecular sieve gel chromatography. One ml of CM or a CMF-containing fraction was loaded on a 26 cm high x 0.7 cm diameter column packed with Sephadex superfine G-50 resin (Pharmacia, Uppsala, Sweden). Separation was done at room temperature with PBM at a flow rate of 0.2 ml/minute and 1.1 ml fractions were collected. Each fraction was tested for CMF activity. Molecular weight standards (blue dextran, 2000 kD; BSA, 66 kD; aprotinin, 6.5 kD; and bromphenol blue, 0.67 kD) were also loaded onto the column for calibration before and after sizing the CMF.

Whole CM was also size fractionated by two additional methods. In the first method, it was pressure dialyzed against a PTGC 10 kD cutoff membrane (Millipore, Bedford, MA) using a Millipore minitan pressure dialyzer at a back pressure of 25 psi. The material retained by the membrane contained the high molecular weight conditioned medium factor (CMF-H), and the filtered material contained the low molecular weight conditioned medium factor (CMF-L). Another method of separating CMF-H from CMF-L was by centrifugation of a 400 µl sample in a Millipore
Ultrafree-MC ultrafiltration unit with a molecular weight cutoff of 10 kD. Centrifugation was done at 2,000 x g for 45 minutes at 4°C or until >99% of a sample was spun down. The > 10 kD fraction was resuspended in PBM to a final volume of 400 μl so that the concentrations for the large molecules would not be changed. The spin-through contained CMF-L and molecules smaller than 10 kD.

To examine the kinetics of CMF-H and CMF-L secretion, log phase cells were centrifuged and washed twice in PBM. They were then resuspended in PBM to a density of 5 x 10^6 cells/ml. This point was considered to be the beginning of development. CM was made as described except that at t=10 hr, 1 ml of the medium was removed and clarified of cells as described above. CMF-H and CMF-L fractions were separated immediately at each time point by centrifugation of 400 μl of CM through a 10 kD cutoff Millipore Ultrafree-MC ultrafiltration unit for 45 minutes or until 99% of the liquid was spun through. PBM was added to the retained material (> 10 kD) to a final volume of 400 μl. The separated CMF-H and CMF-L fractions were stored at -20°C. After thawing, CM assays were done using serial factor of 2 dilutions for >10 kD fractions and serial factor of 3 dilutions for < 10 kD fractions.

Trypsin Sensitivity of Conditioned Medium Factors

Whole CM, CMF-L-containing samples (size fractionated by centrifugal ultrafiltration), or hydroxylapatite column-purified CMF-H fractions (purification procedures described later) were tested for trypsin sensitivity. The general procedure involved preincubating TPCK treated
trypsin (Sigma) in PBM in the presence and absence of soybean trypsin inhibitor (Sigma) for 5 minutes before adding to the sample to be tested. The final concentrations of trypsin and its inhibitor were each 1 mg/ml. The reaction was carried out at room temperature for two hours; after which trypsin digestion was terminated by the addition of soybean trypsin inhibitor (STI) to 1 mg/ml. CM activity was determined as described with series of two fold dilutions for CMF-H and series of three fold dilutions for CMF-L. Since both STI and trypsin were present in the CM assays, STI and boiled trypsin were also included as controls.

_Gel Electrophoresis and Protein Staining_

SDS-polyacrylamide gel electrophoresis and Coomassie staining were done as previously described (Gomer et al. 1986a). Gels were 1.5 mm thick and contained 15% acrylamide. Morrissey's procedure (Morrissey 1981) for silver staining was done with the following modification: Gels were fixed for 1 hour in 50% methanol/10% acetic acid, washed in several changes of water for 1 hour, and then soaked in dithiothreitol (DTT) (Sigma) omitting the glutaraldehyde step. For gel purification of CMF, the sample was not boiled prior to loading on the gel.

_High Molecular Weight Conditioned Medium Factor Purification Procedures_

Whole CM was concentrated with a Minitan pressure dialyzer and 10 kD cutoff polysulfone membrane (Cat.# PTGC, Millipore, Bedford, MA). Typically, 2 to 3 liters of CM was concentrated to 150 to 200 ml. The
concentrated material (molecular weight > 10 kD) was sealed in a 12-14 kD cutoff Spectrapor dialysis bag (Spectrum Medical Industrial, Los Angeles, CA) and dialyzed against 2 liters of PBM for 12 hours at 4°C. Preswollen DEAE-Sephacel (Pharmacia) was prepared by washing, equilibrating, and removing small particles in PBM. The concentrated and dialyzed CM was applied to a 2.8 x 12.5 cm DEAE column and then eluted from the column stepwise with one bed volume each of 0, 0.1, 0.2, 0.3, 0.4, 0.5 M NaCl in PBM at 4°C. DEAE fractions containing CM activity were pooled and loaded on a Bio-gel HTP hydroxylapatite column. Hydroxylapatite (Biorad Laboratories, Richmond, CA) was equilibrated and fines were removed in PBM. Columns (3-ml bed volume, 2.8 x 0.5 cm) were run at room temperature. The column was eluted stepwise with 5 ml each of 20, 50, 100, 150, 200, 250, 300, 400 mM K-PO₄ pH 6.1 containing 1 mM MgCl₂ and 10⁻² mM CaCl₂. The fractions were assayed for CM activity. The CM-containing hydroxylapatite fractions were pooled and sealed in a Spectrapor dialysis bag (molecular weight cutoff 12-14 kD) which was coated with Aquacide II (Calbiochem, La Jolla, CA) at 4°C. The Aquacide-concentrated materials were mixed without boiling with modified Laemmli sample buffer (10 μM DTT instead of 10 mM) prior to electrophoresis. After protein separation, the gel was evenly divided into 10 slices from top to bottom, crushed, and protein eluted passively into PBM at 4°C with constant mixing for four hours. CM activity was assayed for each slice. In order to determine further the molecular weight of CMF-H, a similar slice containing CM activity was further divided into 1 mm thick pieces and passively eluted into PBM. The
eluate from the gel slice was assayed and the molecular weight determined by SDS-polyacrylamide gel electrophoresis and silver staining as described. All protein concentrations were measured with the Biorad protein assay (Bradford, 1976).

**HPLC Chromatography of Low Molecular Weight Conditioned Medium Factors**

One ml of CMF-L-containing fractions from the size fractionation experiment were also loaded individually on a Dynamax 5-μm particle size, 4.6 x 250 mm HPLC C8 column (Rainin, Emeryville, CA) and eluted with a linear gradient of 0.1% trifluoroacetic acid (TFA) in water to 0.08% TFA in acetonitrile over a period of 60 minutes at a flow rate of 1 ml/minute. Absorbance was read at a wavelength of 214 nm and 1 ml fractions were collected. Because of the presence of TFA, HPLC fractions to be assayed for CMF activity were dried by Speed-vac (Savant Instruments, Inc., Farmingdale, NY), resuspended in 1 ml of water, redried, and finally resuspended in 1 ml of PBM. Peaks of CMF activity from the Sephadex column were also loaded on a 4.6 x 250 mm C18 reverse phase HPLC column (Custom LC Inc., Houston, TX) and a 4.6 x 250 mm HPLC phenyl column (Vydac, Hesperia, CA).

**Deglycosylation**

Various endoglycosylases were used to determine the effect of deglycosylation on CMF activity. As summarized in the data sheets from the suppliers listed below, endoglycosidases F and H specifically hydrolyze
high mannose N-glycans leaving one molecule of N-acetylglucosamine bound to asparagine, N-glycanase catalyzes hydrolysis of asparagine-linked oligosaccharides at the β-asparty1-glucosylamine bond, neuraminidase removes α-(2,3), α-(2,6), and α-(2,8)-linked sialic acid and O-glycanase cleaves only the Galβ(1,3)GalNac core disaccharide attached to serine or threonine residues of glycoproteins; any substitutions (e.g. sialic acid) will inhibit cleavage. CMF-L was produced by centrifuging whole CM that had degraded at room temperature for 35 hours through a 10 kD cutoff Millipore Ultrafree-MC ultrafiltration unit. Fifty µl of CMF-L produced this way was then incubated with 1 µl of either endoglycosidase F (50 mU; N-glycosidase F-free; Boehringer Mannheim Biochemicals, Indianapolis, IN), endoglycosidase H (1 mU; Boehringer Mannheim), N-glycanase (250 mU; Genzyme Corporation, Boston, MA), O-glycanase (1 mU; Genzyme), neuraminidase (20 mU; Genzyme), water, or 2 µl containing neuraminidase (20 mU) and O-glycanase (1 mU) at room temperature for 16 hours. The reactions were terminated by separating the enzymes from the substrates using Millipore ultrafiltration units as described. The spin-through fractions were assayed for CMF activity using a series of 3-fold dilutions. To examine the glycosylation of CMF-H, 16 µl of DEAE and hydroxylapatite-purified CMF-H (Gomer et al. 1991) was mixed with 1.6 µl of 5x Laemmli sample buffer, 1.6 µl of 200 mM EDTA and 52.8 µl of water. After boiling for 2 minutes, 8 µl of 10% NP-40 and 1 µl of 50 mm PMSF, 50 mm TAME, 62 mm O-phenanthroline and 25 mm benzamidine were added to the mixture. One µl of the enzymes as described above (or 2 µl of the neuraminidase/O-glycanase mix) was then added to 10 µl
aliquots of the CMF-H/detergent mixture and incubated at 37°C for 11 hours and 15 minutes. Five μl of the digest mixture was mixed with 15 μl of 1x Laemmli sample buffer and separated by SDS-polyarylamide gel electrophoresis that was then silver stained as described.

III. Results

Optimization and Development of Quantitative Conditioned Medium Assay

Dictyostelium cells in low density submerged monolayer culture require a factor (CMF) secreted by high-density starved cells in order to initiate differentiation (Mehdy and Firtel 1985; Gomer and Firtel 1987). As shown in Figure 2.1 in this assay, prespore and prestalk gene expression is not induced in dilute, starved cells (as determined by immunofluorescence using polyclonal antibodies against a prestalk and a prespore protein) in response to cAMP, unless these cells are first cultured in the presence of conditioned medium from developing cells which have been starved at high cell density (see Materials and Methods). This assay was subsequently utilized to measure CM activity. Initially many CM assays were done qualitatively, i.e. an assay was determined positive when it contained about 2-3 fold more positive cells than the PBM (negative) control. Using such criterion, CMF activity can only be detected but not quantitated. To purify CMF activity, quantitation was necessary to follow the yield of CMF activity through a purification protocol.

The first attempt in the development of a quantitative assay of CMF
Figure 2.1. **Conditioned medium assay.** Immunofluorescence of cells starved in the absence (A and C) or presence (B and D) of a 1:10 dilution conditioned medium (see Materials and Methods). Cells were starved at a density of 5,000 cells per cm$^2$ for 18 hours in submerged monolayer culture; six hours after starvation cAMP was added to 300 μM. A and B show immunofluorescence of cells stained with anti-prespore antibody, C and D are corresponding phase images. No cells differentiated in the absence of conditioned medium (A and C); 31% of the cells differentiated in the presence of conditioned medium. Note that some of the positive cells are in small aggregates. Bar in (D) is 100 μm. Experiments using the anti-prestalk antibody give similar results, although, as expected, the fraction of cells expressing the prestalk protein pst-cathepsin (cysteine protease 2) is lower (see Gomer and Firtel, 1987).
activity was to replace immunofluorescence staining with ELISA which is routinely used to quantify the amount of antigens. To increase the sensitivity of the assay, antibody against another antigen, discoidin, was used. CM had been shown to modulate discoidin expression (Gomer and Firtel, unpublished observation) which, when maximally induced, is 1% of the total soluble proteins (Siu and Lerner 1976). To reduce the variability of the assay, various assay conditions were optimized such as number of cells per assay, incubation time, light or dark during development. The percentage of ethanol for fixing cells and the washing and blocking conditions for ELISA were also examined. Even after repeated optimization of the CMF assay conditions, considerable variation was still observed. Therefore CM discoidin assay in conjunction with ELISA was only used to detect the presence of CMF in gel filtration column fractions.

The percentage of cells differentiating into prespore cells was used as a quantitative measure of CMF activity. This measurement also allowed the determination of whether there was a heterogeneity in the response of starved cells to CM. A dose-response curve with at least 8 dilutions was performed for each sample, and the expression of anti-prespore antigens was measured using immunofluorescent staining as described. At least 3000 cells were counted to give the percentage of differentiated cells. The activity was then defined as the inverse of dilution that gave 50% of the maximum response of that assay. It is important to note that cells expressing SP 70 in this assay arise from only one of each pair of sister cells that happened to be in a ~ 6 hour quadrant of the ~ 8 hour cell cycle at the time of starvation; thus the maximum number of SP 70 positive cells is
1/2 (6/8) = 37.5% (Gomer and Firtel 1987). The absolute number of differentiated cells varies greatly from assay to assay and starving cells at approximately the same growth phase (i.e. early logarithmic phase) does not reduce this variability. The maximum number observed previously of isolated cells that differentiate into prespore cells in low density cultures was approximately 38% (in migrating slugs, approximately 47% of the cells differentiate into prespore cells) (Gomer and Firtel 1987); in the course of this thesis project, the maximum percentage of differentiation varied between 8 and 38%, similar to what was observed previously. The reason why more cells differentiate into prespore cells in migrating slugs compared to low density culture is unknown. The variation of the maximal number of cells that differentiate in low density culture appears to be due to variations in culture conditions that we and others (Mehdy & Firtel, unpublished observations) have not been able to control. Interestingly, comparisons of assays in which a maximum of 8% of the cells differentiated with assays in which 38% of the cells differentiated shows that there is no difference in the strong fluorescence of the positive cells in either assay, or of the low fluorescence of negative cells.

Two Classes of Conditioned Medium Factors Observed Based on Their Molecular Weight

In the initial characterization of CM, it was observed that there were two classes of molecules which exhibited CMF activity, and these did not need to be combined for activity (Gomer et al. 1991). My results based on size fractionation of whole conditioned medium on a Sephadex G-50
Figure 2.2 Two molecular weight classes of factors are contained in the conditioned medium. Size fractionation of medium conditioned by starving *Dictyostelium discoideum*. One ml of whole CM was loaded on a 0.7 x 25 cm Pharmacia superfine Sephadex G-50 column. The column was eluted with PBM; 1-ml fractions were collected, diluted 1:10, and assayed for CM activity. The column was calibrated with blue dextran (MW 2,000 kD), BSA (MW 66 kD), lysozyme (MW 14.3 kD), and bromophenol blue (MW 670 D). The ability of fractions to make cells competent for prespore cell differentiation is shown. The arrows indicate the fractions containing the peaks of the molecular weight standards.
column confirmed this initial observation. As shown in Figure 2.2, multiple peaks of CMF activity were observed. The high molecular weight peak contained two subpeaks. The presence of the second subpeak (fractions 11 and 12) was variable in appearance from experiment to experiment and thus might be a degradation product of the first peak (see below). When compared to known standards, the large peak has an approximate molecular weight of $> 66$ kD and the second set of peaks (fractions 27-40) was composed of molecules $< 14$ kD. The high molecular weight CMF activity (G-50 fractions 8-12) has been designated CMF-H while the lower molecular weight fractions have been categorized together as CMF-L.

Dr. Gomer verified that the low molecular weight material was not a retention artifact of the gel filtration chromatography by doing the following experiment: 20 ml of unfractionated CM were dialyzed against 1 liter of water at 4°C overnight, using a Spectrapor dialysis bag (12-14 kD MW cutoff). The liter of water, which contained material that passed through the dialysis membrane, was lyophilized and resuspended to a final volume of 20 ml with water. This material had CMF activity at dilutions of up to 1:5. Conversely, the material remaining in the dialysis bag after 5 changes of PBM over 3 days also had CMF activity.

*CMF-H Is Secreted Earlier Than CMF-L.*

Mehdy and Firtel (1985) observed that for cells developing at an air-water interface, CMF activity is secreted throughout development, with a higher rate during the aggregation stage. To determine if CMF-L is
Table 2.1  Time Courses for Secretion of CMF-H and CMF-L

<table>
<thead>
<tr>
<th>Hour after starvation</th>
<th>CMF Activity (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;10 kD fraction</td>
</tr>
<tr>
<td>10</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>20</td>
<td>7 ± 2</td>
</tr>
</tbody>
</table>

Cells were starved in PBM at a density of 5 x 10^6 cells/ml for 10 or 20 hours. After clarification, CM made for the different time periods were centrifuged through a Millipore Ultrafree-MC ultrafiltration device to separate materials > and <10kD. After bringing the final volume to 400 µl for both fractions, the spin-through material (<10 kD) and the retained material (>10 kD) were diluted serially and assayed for CMF activity. The data were based on four sets of experiments.
secreted throughout development, CMF-L and CMF-H activities were separately measured for CM collected at 10 hours and 20 hours of starvation. The >10 kD fraction of 10 hour CM contained 5 ± 2 units/ml of activity and the > 10 kD fraction of 20 hour CM contained 7 ± 2 units/ml of activity (Table 2.1). CMF-L activity was not detected in 10-hour CM; at 20 hours, CMF-L activity was 4 ± 2 units/ml (Table 2.1), thus indicating that CMF-H appears before CMF-L.

*Conditioned Medium Factors Are Trypsin Sensitive*

Both CMF-H and CMF-L were examined for trypsin sensitivity. Preliminary results indicated the presence of an inhibitor for trypsin in the CM (Gomer, R.H., unpublished observation). To remove the trypsin inhibitor in the CM from CMF, CMF-H was first separated from CMF-L by ultrafiltration. CMF-H was subsequently purified by DEAE chromatography and hydroxylapatite chromatography (see below). When BSA and trypsin were added to the hydroxylapatite-purified CMF-H, we found that the BSA was proteolysed. As shown in Table 2.2, we found that trypsin addition also eliminated the activity of CMF-H. The CMF-H activity was not eliminated if the trypsin was boiled or if trypsin inhibitor was added to the trypsin before incubating with CMF-H. The presence of trypsin inhibitor alone seemed to slightly increase CMF activity. The fraction containing CMF-L was treated with either trypsin, trypsin and soybean trypsin inhibitor (STI), or STI alone. At the end of a 2 hour room temperature incubation, STI was added to the reaction containing trypsin. The samples were then assayed for CMF activity. When CMF-L was
Table 2.2  High molecular weight CMF is a protein.

<table>
<thead>
<tr>
<th>Trypsin</th>
<th>STI</th>
<th>CMF Activity (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>2 hour</td>
<td>7762</td>
</tr>
<tr>
<td>0 hour</td>
<td>0 hour</td>
<td>4169</td>
</tr>
<tr>
<td>0 hour</td>
<td>2 hour</td>
<td>0</td>
</tr>
<tr>
<td>0 hour (boiled)</td>
<td>2 hour</td>
<td>5821</td>
</tr>
</tbody>
</table>

Trypsin and/or soybean trypsin inhibitor (STI) were added to hydroxylapatite column purified CMF-H at the times indicated, to a final concentration of 1 mg/ml. After 2 hours at room temperature, the reactions were assayed for CM activity with series of two-fold dilution. Identical results were obtained with 50 μg/ml BSA in the reactions. SDS-gel electrophoresis of reactions that contained 50 μg/ml BSA showed that the BSA was degraded only when trypsin was added at 0 hour and STI added at 2 hours.
Table 2.3  Low relative molecular mass CMFs are polypeptides.

<table>
<thead>
<tr>
<th>Trypsin</th>
<th>STI</th>
<th>CMF activity (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>6</td>
</tr>
<tr>
<td>None</td>
<td>2 hr</td>
<td>50</td>
</tr>
<tr>
<td>0 hr</td>
<td>2 hr</td>
<td>0</td>
</tr>
<tr>
<td>0 hr</td>
<td>0 hr</td>
<td>45</td>
</tr>
<tr>
<td>0 hr (boiled)</td>
<td>None</td>
<td>7</td>
</tr>
</tbody>
</table>

CMF-L was isolated from whole conditioned medium as described. Ten units of CMF-L was treated with trypsin and/or trypsin inhibitor (final concentration of 1 mg/ml, respectively) at the time indicated for 2 hours at room temperature. At the end of the reaction time, CMF activity was determined as described using serial factor of 3 dilutions.
treated with trypsin for 2 hours, CMF activity was destroyed (Table 2.3). However, if trypsin was inhibited with STI during the reaction time, CMF activity could still be detected (Table 2.3). As observed before, the presence of STI enhanced CMF activity significantly, suggesting that STI may either inhibit proteolysis of CMF or an action of a CMF inhibitor. These results indicate that CMF-H and CMF-L are both polypeptides.

*High Molecular Weight CMF Can Be Purified to an 80 kD Protein.*

To understand and delineate the function of CMF, it was necessary to have purified protein as a tool. Since larger proteins contain more amino acids and yield more information on peptide and thus nucleotide sequence, purified CMF-H was used in order to search for the CMF gene. From preliminary studies, it was found that an approximately 1:10 dilution of this whole CM induced the highest percentage of cells to differentiate. In order to separate CMF-H from CMF-L, minitan ultrafiltration was used to retain and concentrate materials larger than 10 kD. Dialysis against PBM was performed using Spectrapor (MW cutoff 12-14 kD) to further ensure no retention of CMF-L. The concentration and dialysis step did not detectably affect the protein composition (Figure 2.3, lane 3). This material was then loaded on a DEAE-Sephacel column and most of the CMF activity was eluted by 200 mM NaCl. The DEAE ion exchange chromatography purified CMF-H 12 fold (based on the decrease in total amount of protein; Table 2.4) and probably removes an inhibitor of the CMF since the total units of activity were increased ~1.3 fold (Table 2.4). Fractions containing CMF activity were pooled and further fractionated on
Figure 2.3. SDS-polyacrylamide gel of conditioned medium. The gel was silver stained to show proteins. Lane 1, molecular weight standards which from the top are 205, 116, 97, 66, 45, and 29 kD; lane 2, whole conditioned medium (16 μl out of 2.4 liter); lane 3, concentrated (16 folds by minitan ultrafiltration system) and dialyzed CM (1.6 μl out of 150 ml); lane 4, pooled fractions eluted from DEAE by 200 mM NaCl (8 μl out of 46 ml); lane 5, pooled fractions eluted from hydroxylapatite by 250 mM potassium phosphate (16 μl out of 4.9 ml); lane 6, SDS-polyacrylamide gel purified CMF that was passively eluted in PBM at 4°C for 15 minutes; lane 7, the same as in lane 6 except that the elution time was 30 minutes; lane 8, the same as in lane 6 except that the gel slice containing CMF was subjected to electrophoresis immediately. For lanes 6, 7 and 8, 16 μl out of 1.6 ml was loaded. The material loaded in lane 8 contained ground-up gel; the material loaded in lanes 6 and 7 did not. In lane 5, * indicates the 80 kD band from the pooled hydroxylapatite column fractions that have CMF activity after excision and elution from a SDS-polyacrylamide gel. The 65 kD band appears to be a degradation product of 80 kD CMF since the intensity of the 80 kD band decreases and that of the 65 kD band increases as the elution time increases.
a hydroxylapatite column. Elution with various concentrations of phosphate was done at room temperature and CMF activity determined every third fraction. The amount of potassium phosphate required to elute CMF-H varied from 150 mM to 250 mM. The hydroxylapatite column effectively purified the CMF an additional 15 fold as judged by the increase in specific activity (Table 2.4). Subsequently, the fractions containing CMF activity were pooled, concentrated with Aquacide, and subjected to electrophoresis on SDS-polyacrylamide gels. Purification using SDS-polyacrylamide gel electrophoresis has been demonstrated successfully in the purification of several growth factors (Matsushima et al. 1985; Singh and Bonin 1988; Parnell et al. 1990). After the completion of electrophoresis, the gel was evenly divided into 10 slices, which were ground (to increase total surface area and facilitate elution) and the proteins contained in each slice eluted into PBM at 4°C overnight. The polyacrylamide fragments were removed by centrifugation at 15,000 xg for 10 minutes and the supernatants were then assayed for CMF activity using dilutions of 10^{-3} to 10^{-4} (see Table 2.4). We detected two peaks of activity, one having a molecular weight between 70 and 97 kD and the other between 50 and 70 kD. A second gel was then run and the region between the 45 and 97 kD protein marker bands was divided into 1 mm thick slices and similarly eluted and the eluates then assayed for CMF activity. The two CMF activities were located to protein bands of MW 80 kD and of 65 kD and resulted in a total recovery of 32% of the activity and a 1300-fold purification as compared to the crude CM (Table 2.4).

We made the following observations that delineated the relationship
Table 2.4 Recovery of protein and CMF activity after various purification steps for CMF-H.

<table>
<thead>
<tr>
<th></th>
<th>Volume, ml</th>
<th>Units per ml</th>
<th>Units</th>
<th>%</th>
<th>Protein</th>
<th>Specific activity</th>
<th>fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>μg/ml</td>
<td>Units/μg</td>
<td></td>
</tr>
<tr>
<td>Whole CM</td>
<td>2400.0</td>
<td>$1.6 \times 10^2$</td>
<td>$3.8 \times 10^5$</td>
<td>100</td>
<td>12.3</td>
<td>$3.0 \times 10^4$</td>
<td>$1.3 \times 10^1$</td>
</tr>
<tr>
<td>Concentrated, dialysed CM</td>
<td>150.0</td>
<td>$1.9 \times 10^3$</td>
<td>$2.9 \times 10^5$</td>
<td>76</td>
<td>142.9</td>
<td>$2.1 \times 10^4$</td>
<td>$1.4 \times 10^1$</td>
</tr>
<tr>
<td>DEAE fractions</td>
<td>46.0</td>
<td>$1.1 \times 10^4$</td>
<td>$5.1 \times 10^5$</td>
<td>134</td>
<td>75.1</td>
<td>$3.5 \times 10^3$</td>
<td>$1.5 \times 10^2$</td>
</tr>
<tr>
<td>Hydroxyapatite fractions</td>
<td>4.9</td>
<td>$1.0 \times 10^5$</td>
<td>$4.9 \times 10^5$</td>
<td>129</td>
<td>47.3</td>
<td>$2.3 \times 10^2$</td>
<td>$2.1 \times 10^3$</td>
</tr>
<tr>
<td>SDS-gel purification</td>
<td>1.6</td>
<td>$7.7 \times 10^4$</td>
<td>$1.2 \times 10^5$</td>
<td>32</td>
<td>4.2</td>
<td>6.7</td>
<td>0.02</td>
</tr>
</tbody>
</table>
between the 80 and 65 kD CMF-containing protein bands. When the supernatant from overnight elution or the acrylamide gel fragments from the 80 kD band prepared as describe above were sized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), we observed a 65 kD band but no 80 kD band (data not shown). However, after a 15 or 30 minute elution of the proteins from crushed gel slices (see Fig. 2.3, lanes 6 & 7), some 80 kD protein was detected. The amount of 80 kD protein was greater when the elution time was shorter. Reelectrophoresis of the crushed acrylamide immediately after excising the 80 kD band showed even a greater percentage of 80 kD material and less 65 kD protein (Fig.2.3, lane 8). Thus, under the conditions of elution, the 80 kD CMF protein appears to be degraded to a 65 kD protein that still retains CM activity. The 65 kD band was present in all purification steps. Presumably, other proteins present in cruder fractions inhibit the degradation of the 80 kD protein. These results suggest that the 80 kD band isolated from the SDS-PAGE gel slice is the native CMF-H activity. Interestingly, assays of serial dilutions (by a factor of 2) of crude CM and gel-purified CMF showed that the range of concentration in which the highest percentage of differentiated cells was induced is narrow (Figure 2.4). Too much or too little CMF present resulted in submaximal response. Dose-response curves for each purification step look similar to Figure 2.4 (summarized in Table 2.4).

Other chromatographic properties of CMF-H examined by Dr. Gomer have further demonstrated that the 80 kD protein had CMF activity. One hundred μl of the hydroxylapatite purified fraction was loaded on a
Figure 2.4 Dose-response curve for the unpurified and purified 80 kD CMF. Various amounts of whole CM (A) and SDS-polyacrylamide gel electrophoresis purified CMF (B) were added to induce a monolayer of submerged low density cells. Immunofluorescence was used to detect the expression of prespore antigen, as a measure of differentiation. The dilution for each assay point was converted to protein concentration based on the final specific activity in Table 2.4. The protein concentrations were plotted against the percentage of differentiated cells. A half of maximum response for the gel-purified 80 kD CMF occurred when 9.6% of the cells differentiated, corresponding to about 0.3 ng/ml of protein (B).
HPLC Biosil TSK-125 column, and the column was eluted in PBM. Fractions were collected and aliquots were assayed for CM activity and subjected to electrophoresis on SDS-polyacrylamide gels and silver stained. CM activity and the 80 kD protein both peaked in the same single fraction. In the elutions from DEAE, hydroxylapatite and other chromatography resins tested, the 80 kD band always coeluted with the high molecular weight CM activity; when CM activity was in two or more fractions, the 80 kD protein was also found in these fractions and the amount of the 80 kD band paralleled the amount of CM activity in the fractions. On the Biosil column, this 80 kD band eluted shortly before BSA. Similar results, although with decreased resolution of the protein peaks, were obtained with a Pharmacia FPLC Superose-12 sieving gel. A variety of other column resins gave no further improvement in purification after DEAE and hydroxylapatite chromatography. These included Biorex-70, heparin-agarose, phenyl sepharose, affigel blue, mono Q, Mono S, and zinc agarose. The 80 kD CMF-H was found in the flow through of all the resins except Affigel blue, Mono Q, and Mono S.

80 kD CMF and CMF-L Have Similar Types of Glycosylation

CMF-H has been shown by Dr. Gomer to bind to the lectin concanavalin A (Con-A) indicating that it contains high mannose oligosaccharides (Gomer et al. 1991). To characterize further the types of oligosaccharide groups associated with the 80 kD CMF, the DEAE and hydroxylapatite-purified CMF-H-containing fractions were prepared as described above and treated with various deglycosylating enzymes or water
before electrophoresis on an SDS-polyacrylamide gel. Proteins were visualized by silver staining. To monitor for nonspecific degradation of the 80 kD CMF, an equal amount of the untreated fraction was also run on SDS-polyacrylamide gel (Figure 2.5, lane Fr). As previously described, (Gomer et al. 1991) both the 80 kD and 65 kD proteins partially degraded at room temperature (compare Figure 2.5, lanes Fr and W) to produce small peptides that had run off the gel. As seen in Figure 2.5, treatment of the CMF-H-containing hydroxylapatite fraction with endoglycosidases F and H or N-glycanase (lanes F, H and G) resulted in the reduction of the 80 kD protein band (arrow, Figure 2.5) and the appearance of many protein bands with sizes ranging between 66 to 80 kD, indicating that CMF-H contains high mannose oligosaccharides attached to an asparagine residue on the protein backbone. Changes in protein band patterns suggest that other proteins in CMF-H-containing hydroxylapatite fractions were also deglycosylated. Interestingly, deglycosylation with endoglycosylase H and N-glycanase also resulted in the disappearance of the 65 kD bands (lanes H and G). Treatment with neuraminidase did not affect the 80 kD band (Figure 2.5, lane N). Neuraminidase appears as the band immediately above 80 kD CMF in Figure 2.5 lanes N and N/O. Treatment with O-glycanase alone did not cause the disappearance of the 80 kD band (Figure 2.5, lane O). Deglycosylating with neuraminidase in combination with O-glycanase (Figure 2.5, lane N/O) did cause breakdown of the 80 kD CMF-H, implying the presence of sialic acid-containing oligosaccharides linked to serine or threonine. BSA was treated with all the deglycosylases described above and showed no degradation, suggesting that the
Figure 2.5  The 80 kD CMF has both N- and O-linked glycosylation. A column fraction of partially purified 80 kD CMF was divided into 8 aliquots. Seven of the aliquots were treated with enzymes or water overnight. Samples were then separated by electrophoresis on an SDS-polyacrylamide gel which was then silver stained. Lane M, molecular weight markers; the molecular weights in kD are indicated. Lane Fr is an aliquot of the crude 80 kD CMF that did not sit out overnight. Overnight digestions were F, endoglycosidase F; H, endoglycosidase H; G, N-glycanase; N, neuraminidase; N/O, neuraminidase and O-glycanase combined; O, O-glycanase; W, water alone.
disappearance of the 80 kD CMF after treatment with deglycosylases was not a result of degradation by other proteases contained in the reaction mixtures (data not shown).

Kerri Halfant in the laboratory has characterized CMF-L and found that the small molecular weight CMFs are heterogeneous in size and properties. Some of the CMF-Ls are retained in the Con-A column indicating glycosylation, but others do not bind Con-A (Yuen et al. 1991). Since there is heterogeneity in the glycosylation of CMF-L, we examined whether the sugar moieties are important for CMF activity. CMF-L was incubated with endoglycosidases F or H, N or O-glycanase, neuraminidase, a combination of neuraminidase and O-glycanase, or water. As shown in Table 2.5, removal of sugar moieties by any of the above enzymes greatly reduced the activity of CMF-L, indicating that CMF-L contains glycosylations that are cleaved by the above enzymes and that these glycosylations are important for its activity. The removal of sialic acid from CMF-L by neuraminidase did not reduce CMF activity as drastically as the other deglycosylation enzymes. The CMF activity of the deglycosylated CMF-H was not tested due to difficulty in separating deglycosylating enzymes from CMF-H after treatment. Thus CMF-L, like the 80 kD CMF, appears to contain both N- and O-linked glycosylation.

Low Molecular Weight CMFs May Originate from the 80 kD CMF.

The results described above indicate that CMF-H and CMF-L are both glycosylated polypeptides. To test whether the CMF-L's originated from the 80 kD CMF, whole CM was size fractionated on a Sephadex
Table 2.5  Effect of deglycosylation on CMF-L activity.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Activity (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (water)</td>
<td>173</td>
</tr>
<tr>
<td>Endoglycosidase F</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Endoglycosidase H</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>N-Glycanase</td>
<td>10</td>
</tr>
<tr>
<td>O-Glycanase</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>30</td>
</tr>
<tr>
<td>Neuraminidase/O-Glycanase</td>
<td>10</td>
</tr>
</tbody>
</table>

50 μl of CMF-L containing fraction was incubated with 1 μl of various enzymes at the concentrations specified in Materials and Methods for 12 hours at room temperature. At the end of the incubation, the reaction mixtures were centrifuged using a 10 kD cutoff Millipore Ultrafree-MC filter unit to separate CMF-L from the enzymes. The filtrates were then assayed for CM activity.
superfine G-50 column. Fractions were assayed for CMF activity with ELISA. As observed previously, one peak of activity (fraction 8) coeluted with blue dextran and BSA whereas multiple peaks are seen having molecular weights smaller than aprotinin (6.5 kD) (Figure 2.6A). CMF activity can be detected in fractions eluted after bromphenol blue (0.65 kD) presumably due to nonspecific interactions between some CMFs and the Sephadex resin, as has been observed for other polypeptides (Savage and Cohen, 1972). When fraction 8 was immediately refractionated by the same column, only one peak of activity was observed, coeluting again with blue dextran and BSA (Figure 2.6B). In order to detect low molecular weight breakdown products of CMF-H, fraction 8 was allowed to sit at room temperature for 24 hours and was then rechromatographed. An increased number of peaks of activity were observed, some of which coeluted with CMF-L from whole CM (Figure 2.6C). This suggested that there were molecules of the same sizes found both in crude CM and the breakdown products of CMF-H.

Fraction 30 from the G-50 chromatography of whole CM and fraction 30 from the G-50 chromatography of the breakdown products (CMF-H incubated at room temperature for 24 hours) consistently contained CM activity. To compare further the CMF-L from whole CM and the degraded CMF-H, the two fraction 30's were rechromatographed individually using C8 reverse-phase HPLC (Figure 2.7). Each peak of absorbance at 214 nm and the two fractions on either side of it were assayed for CMF activity. Comparing the elution profiles of the two samples, at least seven peaks from the whole conditioned medium sample
Figure 2.6. Size fractionation of conditioned medium. One ml of CM (whole CM in PBM) was loaded on a 26 cm high x 0.7 cm diameter column packed with Sephadex superfine G-50 resin (Pharmacia). Separation was done at room temperature with PBM and 1.1 ml fractions were collected. The O.D. at 214 nm for each fraction is shown (gray line). Each fraction was tested for CMF activity using an ELISA assay (solid line with filled circle). The measurement of CMF activity with ELISA assay was qualitative since the reaction time for the phosphotase and its substrates was not the same for all the experiments. Panel A shows an activity profile for size fractionated whole CM. Fraction 8, corresponding to the 80 kD CMF, was immediately re-run on the column; CMF activity can only be detected in the same fraction (panel B). When fraction 8 is allowed to sit out at room temperature for 24 hours, CMF activity can be detected in the lower molecular fractions (panel C). (Standards: BSA, 66 kD; aprotinin, 6.5 kD; bromphenol blue, 0.6 kD).
(Figure 2.7A) eluted at the same acetonitrile concentrations as did peaks from the degraded CMF-H sample (Figure 2.7B). Only two peaks from whole CM had CMF activity (*, Figure 2.7A); degraded CMF-H also had only two peaks of activity (*, Figure 2.7B). In both samples, the two CMF activity peaks eluted at 24% and 35% acetonitrile, indicating that CMF-L polypeptides with the same Sephadex superfine G-50 and C8 HPLC elution properties can be found in whole CM and degraded CMF-H. These data suggest that CMF-L is a breakdown product of CMF-H.

As seen in Figure 2.6, there are at least two peaks of CMF activity with molecular weight less than ~6.5 kD. A Sephadex G-50 column equilibrated and eluted with 0.5% acetic acid also showed multiple peaks of CMF activity below 6.5 kD. HPLC C8, C18, and phenyl column also yield multiple active peaks, reinforcing the idea that CMF-L is a family of peptides. CMF activity is not retained on a Dowex 50W-X8 cation exchange column equilibrated in PBM, pH 6, indicating that CMF-L has an acidic isoelectric point. (All work described in this paragraph was done by Kerri Halfant.)

**Breakdown of CMF-H Increases Its Specific Activity.**

Half of the CMF-H-containing fraction 8 from a G-50 Sephadex column was frozen immediately and the other half was left out at room temperature for 35 hours. At the end of the incubation period, the sample was frozen. The two samples were then thawed and CMF activity was measured. The fresh-frozen CMF-H had an activity of 7.3 units/ml (Figure 2.8A), while the breakdown of CMF-H surprisingly increased the activity
Figure 2.7  Small CMF's from whole CM and degraded large CMF coelute from C8 reverse phase HPLC. Samples were loaded on a C8 HPLC column that was then eluted with a gradient from 0.1% TFA in water (5 minutes) to 0.08% TFA in acetonitrile (65 minutes). Panel A, Sephadex G-50 column fraction 31 of whole CMF; panel B, Sephadex G-50 column fraction 31 of degraded fraction 8; panel C, blank. All fractions were dried, resuspended in PBM and assayed; activity could only be detected in the whole CM and degraded large CM fractions indicated by *.
to 730 units/ml (Figure 2.8A). The activity of the fresh-frozen fraction 8 at dilutions below 1:50 is variable from experiment to experiment and may be due to a variable amount of breakdown during the assay. The peak of activity in the degraded CMF between dilutions of 1:50 and 1:5000 is extremely reproducible and appears to represent an increase in the specific activity of CMF upon degradation. To further analyze the composition of the degraded CMF, it was fractionated by ultrafiltration. The two size fractions were then assayed for CMF activity. As seen in Figure 2.8B, the majority of CMF activity in the degraded fraction 8 is in the < 10 kD fraction. The reduction in the amount of CMF activity after ultrafiltration (compare Figure 2.8B with Figure 2.8A solid dots) is variable and due to unknown causes, possibly proteins sticking to the filter units. The composition of the frozen fraction 8 was similarly analyzed and found to contain only 80 kD CMF activity (data not shown).

Purified 80 kD CMF Does Not Break Down to CMF-L by Itself.

Fraction 8 from the chromatography of whole CM on G-50 Sephadex contains, in addition to the 80 kD CMF-H protein, on the order of 100 other proteins. To determine if the breakdown of CMF-H to CMF-L is autocatalytic, 12.5 µl of a gel purified 80 kD CMF stock (Gomer et al. 1991) with an activity of 185 units/ml was incubated in the presence or absence of 1.6 µl of whole CM which had an activity of 25 units/ml. At the end of 72 hours, PBM was added to a final volume of 400 µl so that the activity of the gel purified 80 kD CMF was 5.8 units/ml whereas that due to the whole CM was 0.1 unit/ml (i.e., the activity from the purified 80 kD
Figure 2.8. Degradation of 80 kD CMF is accompanied by an increase in the effective specific activity. Fraction 8 from the Sephadex G-50 column was divided into two; one half was frozen immediately and the other half was incubated at room temperature for 35 hours. They were then thawed and a series of dilutions were assayed for CMF activity. Panel A shows fresh CM as open circles and degraded CM as closed circles. The room-temperature incubated fraction 8 was further analyzed by centrifugation through a Millipore Ultrafree-MC ultrafiltration unit with a membrane cutoff at 10 kD and similarly assayed for CMF activity (Panel B). Open squares are the > 10 kD fractions and closed squares are the CMF-L.
CMF was 58 times more than that from the whole CM). The two reaction mixtures were then fractionated by ultrafiltration. When the gel-purified 80 kD CMF was incubated with whole CM at room temperature for 72 hours, the < 10 kD fraction was found to have an activity of 161 units/ml. Assuming CMF-L to be 100 times more efficacious than 80 kD (from Figure 2.8), the activity of CMF-L in 1.6 µl of whole CM that was subsequently diluted into 400 µl of PBM should be 10 unit/ml. Thus the observed 16 fold increase in activity came from the breakdown of the purified 80 kD CMF. In the absence of whole CM, no activity was found in the < 10 kD fraction of 72 hour room temperature-incubated gel purified CMF and the >10 kD fraction still had the same activity. These results suggest that the breakdown of the 80 kD CMF to CMF-L is not autocatalytic, and that fraction 8 (the G-50 void volume) of whole CM contains an activity that causes purified 80 kD CMF to break down.

IV. Discussion

Upon starvation, *Dictyostelium* cells initiate a developmental program that controls the sequential expression of classes of developmentally regulated genes. Previous work showed that many of the genes involved in aggregation are not induced when starved cells are plated at low densities and that such low density cells do not become competent to induce prestalk and prespore genes in response to cAMP. Addition of CM to these cells allows these developmental processes to proceed (Mehdy and Firtel 1985;
Gomer et al. 1985), suggesting the presence of a secreted factor required for the entry into the multicellular phase of development. Here we have shown that an 80 kD protein factor, in addition to being required for developmental competence to express prestalk and prespore genes, can also induce discoidin I expression during development. Discoidin I is a developmentally regulated lectin required for proper development, whose function in Dictyostelium development is analogous to fibronectin in mammalian cells, i.e., involvement in cell-to-substrate adhesion. It is not expressed in low density log phase cells and is induced at ~2 hours of starvation and maximally expressed during aggregation (5-7 hours). In addition, discoidin I is induced in growing cell populations as the density increases (Rosen et al. 1973; Rowe kamp et al. 1980; Poole et al. 1981; Devine et al. 1982; Springer et al. 1984; Crowley et al. 1985). Recent experiments by Clarke et al. (1987) have shown that discoidin I can be induced in low-density vegetative cells that are grown in growth medium previously conditioned by high density cells which are expressing discoidin I. This group has also shown that this induction of discoidin I is in response to a secreted, non-dialyzable factor present in the growth medium of cells grown to a high density but not in medium from cells grown to a low density. It has been observed that CMF can potentiate the developmental expression of discoidin I genes (Mehdy and Firtel 1985) and Biosil-purified CMF-H can induce discoidin gene expression in vegetative growing cells (Gomer et al. 1991). Therefore it was suspected that the factors in the two types of conditioned medium may be the same. However, the two factors do not appear to be the same molecule. Clarke’s
conditioned medium was produced by vegetative cells and could not induce prespore or prestalk gene expression under our assay conditions (Gomer, unpublished observation). The factor described by Clarke's group can not be purified with our purification protocol and is heat labile whereas CMF, under the same conditions, is heat stable (Gomer et al. 1991). The term, prestarvation factor (PSF), has recently been assigned to the factor described by Clarke (Rathi et al. 1991).

From the size fractionation of whole CM, we have shown the presence of two distinguishable sets of molecules, CMF-H and CMF-L, both of which have CM activity (Figure 2.2) and allow the differentiation of prestalk and prespore cells in response to cAMP. The larger molecule CMF-H is a protein as shown by its trypsin sensitivity and purification to a single 80 kD band on silver-stained SDS-polyacrylamide gel. The time pattern of appearance of the 65 kD protein band which still retains CMF activity indicates that it is a degradation product of the 80 kD CMF. Which CMF size contains more CMF activity can not be determined since 80 kD breaks down within 5 minutes (Figure 2.3). Recent (preliminary) results showed that polyclonal antibodies specifically against CMF fusion protein bind both the 80 and 65 kD protein bands in the hydroxylapatite-purified CMF-containing fractions, further supporting the proposed relationship between the two proteins (Jain, unpublished observation). The sensitivity of cells to as low as 0.3 ng/ml of CMF-H (Figure 2.4) suggests that there may be a specific cell surface receptor and signal transduction and amplification system for CMF, such as exists in other systems for growth factors.
Renu Jain was able to utilize protein sequence to generate oligonucleotide that bound to a cDNA encoding the *Dictyostelium* conditioned medium factor. Analysis of the derived amino acid sequence indicates that CMF is an acidic hydrophilic protein with a polypeptide molecular weight about 78% that of the purifiable 80 kD glycoprotein, indicating that the latter has a large glycosyl 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). Sequence analysis of the 80 kD CMF indicates that it is unrelated to any known polypeptide growth factor (Jain *et al.* 1992). 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 weight (2 kD) and is present in much higher amounts compared to CMF and thus does not appear to be related to CMF (Willey *et al.* 1991). Another protein that has an effect somewhat similar to 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 1990 a,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 signalling.

CMF-L has many similarities to the 80 kD CMF. Both are protease-sensitive, and appear to contain N-linked glycosyl and O-linked glycosyl residues. The 80 kD CMF and some of the CMF-L's appear to have high
mannose oligosaccharides. For both CMF's, some of the oligosaccharides are substituted with sialic acid and contain Galβ(1,3)GalNAc disaccharide as the core. Both impure and SDS-gel-purified 80 kD CMF break down to CMF-L's. Interestingly, we find that the 80 kD CMF needs the activity of a protein that elutes in the void volume of a Sephadex G-50 column to be able to break down to CMF-L. We do not know whether the breakdown of the 80 kD CMF to an active 65 kD form is autocatalytic or due to protease contamination during the elution from an SDS-polyacrylamide gel (Gomer et al. 1991). The processing of CMF from a larger precursor to a much smaller polypeptide is somewhat similar to the processing of epidermal growth factor (EGF), with the exception that the CMF precursor is soluble whereas the EGF precursor is membrane-bound (Gray et al. 1983; Scott et al. 1983; Mroczkowski et al. 1989). Another difference between CMF-L and EGF is that there are at least seven different CMF-L's. Since there was no apparent repeating sequences in the 80 kD CMF cDNA, the diversity of CMF-L was probably due to partially cleaved polypeptides that overlap a single active site. Because we use cells that are recloned every month, the heterogeneity of CMF-Ls is probably not due to allelic variation (Smith et al. 1989). Assuming an average CMF-L size of 3 kD, the 100-fold increase of CMF activity upon breakdown requires that, even if there are tandem CMF-L precursors, CMF-L molecules should have a higher molar specific activity than the 80 kD CMF. Whether the different CMF-Ls have different functions or receptors is unknown. The sensitivity of CMF-L activity to deglycosylation indicates that both the N- and O-linked glycosyl
modifications are important for activity of the molecules and may be part of the active site.

The biological function of the breakdown of 80 kD CMF to a molecule that diffuses faster (because it is smaller) and is more efficacious is unclear. Previous experiments have shown that prespore and prestalk gene expression requires exposure of cells to CMF for only the first six hours of starvation, well before the breakdown of CMF occurs. This suggests that CMF-L does not function to maintain expression of these genes. One possibility is that the breakdown allows cells that have not reached the aggregate by the time it is starting to form a fruiting body to express CMF-sensitive genes allowing them to proceed to later developmental stages. Some of these genes, such as discoidin, do not require additional extracellular factors for expression. Others, such as prespore and prestalk genes, require high continuous levels of cAMP, and it is currently unclear whether the isolated cells would be able to express this class of genes.

From Table 2.4, the rate of CMF production/secrection can be calculated. Assuming that 6.7 µg (6.7 x 10^{-6}g) of 80 kD CMF recovered in the final SDS-gel purification step represents 32% (from recovery of activity) of what was originally produced by (9 x 10^6/ml x 2400 ml) cells, a cell can secrete (6.7 x 10^{-6}/0.32/9 x 10^6/2400) g, or 9.7 x 10^{-16} g (7.3 x 10^3 molecules) of 80 kD CMF over a period of 20 hours. If the rate of production/secrection is constant over this period of time, a cell will release 6 molecules of 80 kD CMF per minute. If however the total activity obtained after SDS-PAGE purification represented that recovered from 6.7
μg of 80 kD CMF, the rate of secretion would be reduced to about 2 molecules secreted per cell per minute. The actual amount secreted is probably higher because we have not accounted for loss due to degradation, oxidation and/or internalization by cells in the calculation. Given the sensitivity for CMF is about 0.3 ng/ml (~ 2.3 x 10^9 molecules/ml), an isolated cell probably will not be able secrete enough CMF to stimulate itself in 24 hours, even if the 100-fold more efficacious CMF-L is produced during this period of time.

We were able to isolate and purify a 80 kD glycoprotein that mimicks the activity in the crude CM in its ability to induce prespore and prestalk gene expression in cells starved at a density too low for differentiation. The majority of CMF activity can be accounted for and one major activity peak was observed in each purification step (Table 2.4). All of our observations point to the function of CMF as a density sensing factor. Cells starved at too low a density fail to initiate a multicellular developmental program, as determined by the expression of preaggregation and aggregation phase genes (e.g. discoidin I) or by the ability to induce prestalk and prespore gene expression, can become differentiation competent when CMF is added even in the absence of a multicellular form. Since CMF is produced/secreted by high density cells and can be sensed by cells, it suggests that CMF acts as a density sensor: high concentration of CMF indicating high number of cells in the surrounding. Cells probably use CMF to determine whether they are far from an aggregation center and thus need to continue expressing aggregation-specific genes, or are at or near an aggregation center and can thus begin expressing cell-type-specific
genes. Similar effects using different assay conditions have also suggested the existence of a molecule such as CMF during development (Grabel and Loomis 1976) and during vegetative growth before starvation (Clarke et al. 1987, 1988).

CMF is made and secreted by some or all cells from starvation through late development, although it is maximally expressed during the preaggregation and aggregation stages (Mehdy and Firtel 1985). Vegetative cells can be induced to express the discoidin I gene, which is normally only found in developing cells. Therefore we believe that CMF acts not only as a cell-density sensor but also induces the onset of development. This hypothesis also predicts an important role for CMF in aggregate formation. With the availability of purified 80 kD CMF protein and its cDNA as tools, we will be able to understand better its biological function and regulation of development.
CHAPTER III

Regulation of Secretion of Conditioned Medium Factor

I. Introduction

In the previous chapter, I have described the bioassay for CMF’s in which cells are starved at low density in a well of a microtiter plate or slide in buffer possibly containing CMF. cAMP is added to the well, and prespore and prestalk antigens, which can be detected by immunofluorescence, are expressed only if CMF’s are present in the buffer. Fractionation of conditioned medium on Sephadex G-50 shows two size classes of CMF activity. One can be purified to an 80 kD glycoprotein and the other is a set of 0.5-6 kD polypeptides; our data indicate that the smaller peptides are breakdown products of the 80 kD CMF (Gomer et al. 1991; Yuen et al. 1991). The CMF activity can be detected in medium conditioned by starving cells throughout development but not by vegetative growing cells (Mehdy and Firtel 1985; Gomer et al. 1991). However, it has also been shown by our laboratory that the 80 kD CMF transcript and protein can be detected in vegetative growing cells. The factor is associated with a crude plasma membrane fraction from vegetative cells, which suggests that it is trapped inside of vesicles or attached to a membrane (Yuen et al. 1991). Low molecular weight CMF can only be detected after 10 hours of development, and only the 80 kD CMF is found in the membrane fraction of vegetative cells. Taken together it seems to indicate that CMF is
synthesized and sequestered in membrane or vesicles associated with plasma membrane of vegetative growing amoebae. It also suggests that starvation triggers the secretion of CMF.

As described in the previous chapter, preliminary characterization of CMF seems to suggest its function as a density sensing factor. From the purification of the 80 kD CMF, we calculated that on average, a Dictyostelium cell starved at high density in the absence of cAMP pulses secretes approximately 6 CMF molecules per minute. Cells at low densities will express certain genes characteristic of high density cells only when treated with extracellular CMF at levels above ~0.3 ng/ml. Since CMF is detected in medium conditioned by starving cells and can be sensed by cells (as shown by our CMF assay), we have hypothesized that the developing cells simultaneously secrete CMF and monitor its extracellular level, with high levels indicating the proximity of a large concentration of other starving Dictyostelium cells. We have also proposed that at this secretion rate, it would take an isolated cell an infinitely long time to secrete enough CMF so that the CMF level in the vicinity of the cell accumulates to ~0.3 ng/ml. This secretion rate was calculated using conditioned medium made by cells in shaking cultures at a high cell density. To further validate our proposal, CMF secretion rate under some of the various physiological conditions was investigated. These included differences in pH, light intensity, cAMP pulses and initial cell density. The interaction of the cell cycle and CMF secretion was also examined. The findings can also be of a general interest in the understanding of the biological functions of CMF since the secretion of CMF was measured by the ability of the CMF-
containing medium to induce prespore antigen expression in cells that are too far apart to enter into multicellular development.

II. Materials and Methods

Cell Culture and Conditioned Medium Assay

Growth of *Dictyostelium discoideum* KAx-3 cells in HL5 medium supplemented with antibiotics was done as previously described in Chapter II. CMF activity was assayed by starving cells in PBM or PBM containing CMF, and then fixing, staining for the prespore-specific antigen SP 70 (beejin) by indirect immunofluorescence, and counting the number of SP 70-positive cells in the well of an 8-well slide (Gomer et al. 1991; Yuen et al. 1991).

Conditioned Medium Production

CM was prepared in a shaking culture at a standard density of 5 x 10⁶ cells/ml at 21°C or by starving a submerged, monolayer of cells at a density of 1.8 x 10⁵ cells/cm² at room temperature unless otherwise specified. CM and PBM preparation and CMF purification were done as described in Chapter II. CMF assays were performed at room temperature under constant light. For the pH experiments, the pH was adjusted with KOH to values other than 6.1. CM was normally made under standard fluorescent room light; for light/dark experiments illumination was supplemented with a Sylvania 100 W flood lamp 1 m above the plate and an adjacent plate was covered with aluminum foil. To examine the production
of conditioned medium factor by cells starved at different densities, cells were allowed to develop for 20 hours in monolayer culture submerged in 11.2 ml of PBM in Fisher 15 x 100 mm plastic petri dishes which had been lightly frosted with a scouring pad. Mehdy and Firtel (1985) have reported that all cells in submerged monolayers formed large agglomerates at a density of at 36 x 10^4 cells/cm^2, although CM was routinely made with cells plated at 9 x 10^4 cells/cm^2. Therefore to examine CMF secretion at high cell densities, AX-3 cells were plated at densities of 4.5, 9, 18 and 27 x 10^4 cells/cm^2. For the low density experiment, five plates of cells were also similarly starved at 1.8 x 10^3 cells/cm^2. As a control for the low density experiment, the gel-purified 80 kD CMF was added to cells to a final concentration of 0.03 units/ml (2.24 μl of the factor with an activity of 150 units/ml added to 11.2 ml of cell suspension per plate) in a duplicate set of five plates. The CM made by low density cells (low density CM) and that similarly made but spiked with exogenous 80 kD CMF (low density CM + CMF) were collected and clarified by centrifugation as described in Chapter II. CM from cells simultaneously starved at 1.8 x 10^5 cells/cm^2 (high density CM) was diluted 100 times with PBM. A 45 ml sample of each of low density CM and low density CM + CMF and the diluted high density CM were loaded into centrifrep/10 10 kD cutoff concentrators (Amicon, Beverly, MA) that had previously been washed three times with 0.01% gelatin to reduce nonspecific binding. The concentrators were centrifuged at 2000 x g at 4°C until a total of <450 μl of materials remained. The volumes of retained materials were brought up to 450 μl. These concentrated conditioned media were then assayed for CMF activity
as described above. The density experiment was also repeated with shaking cultures where the high and low density conditioned media contained $5 \times 10^4$ and $5 \times 10^6$ cells/ml, respectively. The two media were prepared and concentrated as described for CM made by submerged monolayers of cells.

For cAMP pulsing, a homemade electronic circuit with Hewlett Packard oscilloscope Model 122AR was used to cause a Wiz peristaltic pump (ISCO, Lincoln, NE) to deliver a pulse of liquid every 6 minutes. The output from the pump (either PBM or cAMP in PBM) was fed to a shaking flask through a 155 cm length of silicone tubing. The tubing was taped to the flask so that the end of the tubing was suspended ~ 5 mm above the cell suspension. The pump output was adjusted so that each pulse would dispense 10 µl of a 4 x $10^{-5}$ M solution of cAMP in PBM into 20 ml of cells to produce a final instantaneous concentration of 20 nM at each pulse. One hour after starvation the pulses were applied to the cells and discontinued 10 hours later. CM was harvested at the end of 20 hours as described. The effect of exogenous pulses of cAMP on the kinetics of CMF production were also examined. CM was made in the presence and the absence of added exogenous pulses of cAMP as described above and collected at 2, 4, 6, 8, 10 and 20 hours after onset of starvation.

**Synchronization of Cells**

For synchronization of cells by nocodazole arrest, mid log phase cells growing in axenic culture were pelleted by centrifugation, resuspended in PBM, repelleted, and resuspended to $1.2 \times 10^6$/ml in 200 ml of HL-5 supplemented with antibiotics and 1% v/v of 10 mg/ml
nocodazole (Sigma) in dimethylsulfoxide. After shaking for twelve hours, the cells were pelleted by centrifugation, washed once in PBM and resuspended in 200 ml of fresh HL-5/antibiotics medium to a final density of $1.1 \times 10^6$ cells/ml; cells were counted initially at half hour intervals after the resuspension in HL-5 and then at hourly intervals. Immediately after counting, 10 to 15 ml of cells were pelleted, washed twice in PBM, and resuspended to a density of $5 \times 10^6$/ml in 3 to 5 ml of PBM and shaken for 20 hours. CM was then prepared as previously described.

Synchronous growth of cells was also induced by temperature shift following the procedures described by Maeda (1986). Exponentially growing cells at 21°C were transferred in a flask to an 11.5°C water bath. The cells were gently agitated by rotating the flask continuously. Temperature of the medium usually was cooled to 11.5°C within 20 minutes. After 20 hours of incubation, temperature of the culture was shifted back to 21°C within 1 minute by first immersing the culture in room temperature water, followed by shaking at 21°C. Cells were counted after an hour at 1 hour intervals for 11 hours. Following each cell count, CM was prepared as described above. Cells were also synchronized by releasing from stationary growth (Gomer and Firtel 1987), and CM was made as described above. CMF activity for all the conditioned media made from synchronously grown cells was assayed as described (Chapter II).
III. RESULTS

CMF Increases Its Own Secretion

CMF secretion by starved Dictyostelium cells may be influenced by a variety of conditions such as initial cell density, extracellular pH or light level. The first parameter investigated was the effect of developing cell density on the secretion of CMF. In mammalian cell culture, both cell-cell interactions (Sugimoto et al. 1991; Baes et al. 1987; Schwartz et al. 1989; Hoefer et al. 1984) and the accumulation of a diffusible effector molecule in the medium (Böhmer et al. 1990) have been shown to modulate hormone secretion. To determine if CMF secretion is influenced by cell-cell contact, cells were starved in submerged monolayer culture at a density (9 x 10^4 cells/cm^2) where the cells are nearly confluent, form aggregates, and secrete sufficient CMF into the buffer to allow prespore and prestalk gene expression. Cells were also starved at twice and three times this density, and additionally at a two fold lower density, at which only roughly 50% of the cells form aggregates (Mehdy and Firtel 1985). CM was prepared from cells starved at these four densities and assayed for CMF activity. As shown in Figure 3.1A, for the highest density of cells (27 x 10^4 cells/cm^2), and as previously observed for cells starved in shaking culture (Gomer et al. 1991), a plot of activity vs dilution shows a narrow peak (Figure 3.1A, opened triangles). As expected, cells starved at lower densities produce CM that is effective at lower dilutions. To find out if the amount of CMF secreted per cell varies with cell density, CMF activities were determined, as described, from Figure 3.1A (an extrapolated value was used for the
lowest density). The activity in units/ml found for each sample was multiplied by the volume of the sample then divided by the number of cells in that sample. As shown in Figure 3.1B, the amount of CMF activity secreted per cell increases with cell density, indicating the augmentative nature of cell density on CMF secretion.

The increase in secretion by cells developing at higher density observed in Figure 3.1B may be a result of cell-cell interaction or a synergistic effect of CMF on its own secretion or both. To resolve these two possibilities, the amount of CMF activity secreted per cell at extremely low cell densities was determined. Cells were starved as above in submerged culture at 18 x 10^4 cells/cm^2 and also at a 100 x lower density. At this lower density the cells are many cell diameters apart from one another, do not form aggregates and the CMF concentration is too low to allow prespore and prestalk gene expression (Mehdy and Firtel, 1985). A CMF assay of medium from these two samples after adjusting for the difference in density (see Materials and Methods) showed that the high density cells secreted 1.4 x 10^{-5} units of CMF per cell (as observed above) while the low density cells secreted 2.1 x 10^{-6} units/cell. Plotting activity vs. cell densities at 0.18, 4.55, 9, 18, and 27 x 10^4 cells/cm^2 (Figure 3.1C) shows that as the amount of CMF secreted per cell decreases faster as cell density approaches 0. To determine if the decrease in the apparent amount of CMF secreted per cell at low density is an artifactual loss resulting from CMF binding to the culture dish or during the concentrating process, we included as a control CM made by low density cells spiked with 0.03 units/ml of SDS-gel purified 80 kD CMF (the equivalent of 3.4 x 10^{-6}
Figure 3.1. The secretion of CMF is density dependent. (A) *Dictyostelium* cells were starved at densities of 4.55 (50X), 9 (100X), 18 (200X), and 27 (300X) \times 10^4 \text{ cells/cm}^2 as monolayer submerging cultures. The conditioned media were prepared, collected, and assayed at serial dilutions by a factor of 2 as described in the Materials and Methods. The percentage of cells expressing prespore antigen SP70 as induced by CMF was plotted against the dilution factor. (B) Activity (the inverse of the dilution where 50% of maximum response occurs) of each CM was determined from (A) as described (see Materials and Methods in Chapter II) and was converted to units per cell by dividing by the cell density in each medium. The value for activity at 50X density was obtained by extrapolation. A plot of activity per cell versus cell density was constructed. (C) Values for activity per cell for conditioned media made by cells starved at densities of 0.018, 0.45, 0.9, 1.8, and 2.7 \times 10^5 \text{ cells/cm}^2 were obtained as described and a plot similar to the one described in (B) was constructed.
units/cell). After concentrating 100 fold, the conditioned medium from this experiment contained 10.3 units/ml of CMF activity (the equivalent of 1.2 x 10^-5 units/cell). This increase was induced mostly by the 80kD CMF since the concentrating process, which was done by centrifugal ultrafiltration through a 10 kD cutoff membrane, should have effectively removed CMF-L from the concentrated CM (Chapter II; Yuen et al. 1991). Since 3.4 x 10^-6 units of CMF per cell came from exogenous supplemented CMF, the low density cells secreted a net 8.6 x 10^-6 units (subtracting 3.4 x 10^-6 from 1.2 x 10^-5 units/cell), a roughly four-fold increase. Similar effects were also seen with CM made from cells in shaking cultures at densities of 5 x 10^6 and 5 x 10^4 cells/ml (data not shown). Since cells in shaking cultures exist mostly (90%) as single cells (Mehdy et al. 1983), increase in CMF secretion probably arose from a synergistic effect of CMF on its own secretion, independent of cell-cell contact; such effect was especially noticeable when few CMF molecules were present.

**CMF Secretion Is Not Affected by pH or Light**

*Dictyostelium* cells can proceed through aggregation stage normally within a pH range of 5.5 to 8 (Loomis 1982). However, secretion of many molecules in a variety of organisms can be influenced by pH of the environment. Therefore we examined the effect of pH on the secretion of CMF by starved cells. Cells were starved at a standard density in shaking culture in PBM adjusted to pH 5.0, 5.5, 6.0, 6.5 or 7.0. The high molecular weight components of the conditioned media were isolated and
assayed for CMF activity (at pH 6.1). All had approximately the same activity.

Because of possible advantages for aggregation during either day or night and because developmental programs such as macrocyst formation are light-sensitive (Habata et al. 1991), we assayed CM from cells starved as submerging monolayers under bright light or in the dark; no difference in CMF activity was observed (compare 2 with 1.7 units/ml).

**Exogenous Pulses of cAMP Do Not Affect Secretion of CMF at 21 °C**

Another important parameter that may regulate the secretion and/or production of CMF is cAMP pulses. To complete aggregation, developing *Dictyostelium* cells need pulsatile signals of cAMP to achieve chemotaxis. The apparatus that effects pulsatile signals includes the surface receptor, extracellular phosphodiesterase and its inhibitor, and adenylate cyclase (Kessin 1988). The expression of all the components in the apparatus and many other early genes is regulated by pulses of cAMP. CMF was detected in the membrane fraction of vegetative cells (Yuen et al. 1991), and its activity is required for the first 6 hours of development (Gomer and Firtel 1987). It should thus be classified as an early gene product (Chapter II) and may be under the regulation of cAMP pulses. Although the level of CMF mRNA was unaffected by exogenously added pulses of cAMP (Jain, R. unpublished observations), its secretion rate may be under the regulation of pulses of cAMP. CM was made by starving cells in the absence or presence of exogenously added pulses of cAMP for 2, 4, 6, 8, 10, and 20 hours in the shaking culture. The pulsatile cAMP administered this way
Figure 3.2 The kinetics of CMF secretion. Cells were starved in shaking cultures in the presence (opened circles) and the absence (filled circles) of exogenous pulses of cAMP. CM was collected at 2, 4, 6, 8, 10, and 20 hours after starvation and assayed as described. Activity values are an average of three experiments.
has previously been shown to be capable of inducing the expression of cAMP pulse-regulated genes. As shown in Figure 3.2, there is no difference in activity between CM made by cells pulsed (opened circles) or unpulsed (filled circles) with cAMP at any time points examined. The mean amount of CMF initially increased linearly with time. The reason for the high values of standard deviation is unknown but has also been observed by two other researchers in the laboratory.

The results shown on Figure 3.2 were contrary to what was reported previously by our laboratory. A cell developing in a shaking culture that was not pulsed with cAMP secreted about $9.7 \times 10^{-16}$ g of CMF (Chapter II; Gomer et al. 1991) whereas that developing in the presence of cAMP could secrete about $2.2 \times 10^{-15}$ g of CMF over a period of 20 hours (Jain and Gomer, unpublished observation). This level was roughly a two-fold increase in the amount of secreted CMF per cell. Cells which were pulsed with buffer that did not contain cAMP produced a CM that had the same CMF activity as unpulsed cells (data not shown). However these initial experiments were done when the temperature of the culture room was not controlled and stayed above 23°C. We also observed that CMF activity in medium prepared at higher temperatures was about 160 units/cell (Chapter II, Table 2.4) whereas CM prepared similarly at 21°C had a typical activity of 2-10 units/ml, indicating higher temperature induces greater CMF secretion.
Mitotic Cells Secrete Reduced Amount of CMF

One way in which early developing cells differ from one another is the cell cycle phase in which they happened to be at the time of starvation (Weijer et al. 1984; McDonald 1986; Schaap and Wang 1986; Gomer and Firtel 1987; Wang et al. 1988). Cells starved at any particular position of the cell cycle have been shown to exhibit differences in sensitivity to the chemoattractant cAMP, and in EDTA-resistant cohesiveness (Ohmori and Maeda 1987). In addition, starvation that occurs at S or early G2 phase induces cells to preferentially differentiate into prestalk cells whereas cells starved at other phases differentiate into prespore cells (Gomer and Firtel 1987). Some cell cycle-related events would thus determine the initial ratio of prespore and prestalk cells in an aggregate. Later in the development, the levels of extracellular ammonia, adenosine, oxygen, and a set of secreted chlorinated hydrocarbons (DIFs) can affect the composition of prespore and prestalk cells in the migrating slugs (Gross et al. 1983; Schaap and Wanf 1986; Brookman et al. 1987; William et al. 1987; Sternfeld 1988; Kwong and Weeks 1989; Xie et al. 1991). Prestalk-specific genes are usually expressed earlier than prespore-specific genes (Barklis and Lodish 1983; Haberstroh and Firtel 1990; Esch and Firtel 1991). It was observed that cells starved at S or early G2 phase (prestalk cell-inclined) contain higher cAMP binding and higher phosphodiesterase activity as compared to those starved at late G2 phase (prespore cell-inclined). The reason for earlier prestalk-specific gene expression has thus been suggested as being the result of higher levels and earlier expression of chemotactic signals generated and detected by prestalk-inclined cells (Wang...
et al. 1988). We have shown that pulses of cAMP (the chemotactic signal) can increase CMF secretion at 23°C or higher temperature. The higher chemotactic potential exhibited by cells starved at S or early G2 phase may cause much higher secretion of CMF. Since low density cells can be induced to differentiate when enough CMF is present, CMF may contribute to greater chemotactic potential in these cells. The secretion of CMF from cells starved at different cell cycle phases was measured.

To obtain cells at the same cell cycle phase, vegetatively growing Dictyostelium were synchronized in the presence of nocodazole which arrests cells at mitosis (Burke and Gerace 1986). As shown in Figure 3.3A, cells synchronized by release from nocodazole arrest divide within the first hour, and increase in density only slowly until 10 hours after release. The second cell division occurred during the next hour resulting in roughly a 35% increase in cell density. Mitosis usually occurred a half hour before cytokinesis. Cells were taken at various times after release from nocodazole arrest and starved for CM production in shaking cultures; the CM was then assayed for CMF activity (Figure 3.3B). The conditioned media made by cells starved at different cell cycle phases generally contained 5 to 7 units/ml of CMF activity, with two exceptions. Cells starved at six hours after release (roughly mid G2 phase) produced about 9.5 units/ml of CMF activity. And cells starved 1 hour before the second cell division (at 10 hours after release from nocodazole arrest, roughly M phase) produced no measurable CMF activity, although CMF assay of the CM made did show 2-3% of cells expressing prespore-specific antigens (data not shown). This small amount of CMF may be secreted by the 65%
Figure 3.3  CMF secretion profile at different phases of cell cycle: synchronization with nocodazole. (A) Vegetative Dictyostelium cells were arrested at mitosis by incubating with 10 mg/ml nocodazole in HL-5 medium for 12 hours. They were then released into fresh growth medium and cell density determined initially at 0.5-hour intervals then at 1-hour intervals. Each sharp increase in cell density signifies cell division of a great number of cells and mitosis normally occurs a half hour before cytokinesis. (B) CM was made in shaking cultures by cells taken at various time after release from nocodazole arrest. The conditioned media were then assayed and activity measured as described.
of cells that did not undergo mitosis.

Synchronization can also be achieved by a rapid shift of temperatures (Maeda 1986). The temperature of vegetative cultures was shifted from 21°C to 11°C and back to 21°C 12 hours later in succession. Our results showed that first cell division took place three hours after shifting temperature from 11 to 21°C (density increased roughly 50%) (Figure 3.4A). A second division probably occurred at 10 hours after temperature shift (an approximately 20% increase in cell density) to give a 7-hour cell cycle as reported (Maeda et al. 1989). CM made by cells starved at different cell cycle phases showed no measurable activity for cells starved at approximately M phase (2 and 9 hours after temperature shift), and roughly 3 to 7 units/ml of activity for cells starved at other phases (Figure 3.4B). Again, a very low percentage of cells (a lot lower than 50% of maximum response) was induced to produce prespore antigen in the presence of CM made by M phase cells. A slightly greater amount of CMF secretion from cells starved in late G2 was also observed. CMF activity was also greatly reduced in CM made by M phase cells that were synchronized by release from stationary phase (data not shown). Assuming the ability of CM to induce cell differentiation comes from CMF and the activity reflects amount of protein, secretion of CMF by cells fated to prestalk or prespore cells was roughly the same except for those undergoing mitosis. Given the standard deviations for CMF activity in CM were high (as indicated in Figure 3.2), the increases in CMF activity observed in cells starved in late G2 were probably in significant. However, the reduction of CMF activity in CM produced by mitotic cells was
Figure 3.4  CMF secretion profile at different phases of cell cycle: synchronization by temperature shift. (A) Vegetative cells growing at 21°C were incubated at 11°C for 20 hours with constant agitation and the temperature was immediately raised back to 21°C. Cell density was measured hourly. At least one complete cell cycle was observed. (B) CM was prepared from cells synchronized by temperature shift at the time indicated and CMF activity determined as described in Materials and Methods.
observed in cells synchronized by three different methods.

III. Discussion

We do not know how and whether the production of CMF in vegetative cells is regulated except that cell density does not affect CMF mRNA levels. Although CMF activity is not detected in the medium conditioned by vegetative cells but is found 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 (Jain et al. 1992). It has been calculated that there are roughly $2.3 \times 10^5$ 80 kD CMF molecules sequestered in a vegetative cell (Yuen et al. 1991) but only $7.3 \times 10^3$ 80 kD CMF molecules secreted per cell over a period of 20 hours (Chapter II). Taken together, these data suggest that the activity detected in the medium conditioned by developing cells probably arises from secretion of the existing CMF pool found in the vegetative cells rather than de novo synthesis during development. The results also indicate that regulation of CMF activity is at the level of secretion. Since we did not have an easy way to measure exactly the amount of secreted CMF protein, activity provided a good estimate of the amount of CMF secreted. This measurement seems to be a valid assumption because most of the CMF activity in crude CM can be purified to the 80 kD CMF.

We have investigated various physiological parameters that may influence the CMF secretion rate. Of all the conditions Dictyostelium cells
may encounter at the onset of development, including differences in pH, light, cell cycle phase and cell density, CMF secretion is only severely affected in cells that are starved when they enter into M phase. Secretion by cells at other cell cycle phases indicates that both prespore and prestalk cells secrete CMF. Reduction in the secretion of proteins by mitotic cells may be a characteristic property of M phase cells since such findings have been reported in vertebrates (Kanki and Newport 1991).

cAMP pulses which induce expression of many early genes can increase the amount of CMF secretion by two fold but only at temperatures above 23°C. The potentiation of CMF secretion by cAMP pulses is absent if the cAMP signal transduction is hindered by caffeine, which blocks the activation of adenylate cyclase by the cAMP receptor (Brenner and Thoms 1984; Mann and Firtel 1987), or by the absence of surface cAMP receptors (Ammann and Gomer, personal communication). These observations suggest that secretion of CMF is dependent on cAMP receptor-mediated signal transduction, more specifically the signal transduction pathway that requires the activation of adenylate cyclase. From the activity calculated for CM made at 21°C or at 23°C and higher temperature, the secretion is also enhanced by temperature.

CMF secretion was found to be cell density dependent: we observed a 9-fold increase in secretion per cell over a 150 fold range of cell density. Such density dependency was most pronounced at very low cell density where cells did not form aggregates. However cell-cell contact does not seem to be important for this density-dependent increase in secretion based on the following two reasons. At higher cell densities where all of the cells
are in aggregates, increase in CMF secretion was still observed. In addition, when cell-cell contact was prevented by shaking, secretion of CMF by cells starved at low density was also lower. The increase in secretion appears to be due to a positive feedback effect of CMF itself. This synergistic effect of CMF on its own secretion is further supported since adding small amount of purified CMF (at a concentration too low to induce cell differentiation) to cells starved at low density enhanced CMF secretion 4 fold. Interestingly, the amount of CMF secreted per cell by high density cells in submerged monolayer conditions (Figure 3.1B) is similar to that of cells starved in shaking culture, which is 160 units/9 x 10^6 cells or 1.7 x 10^{-5} units/cell (Chapter II, Table 2.4). The similarity in the CM secretion per cell observed under these two conditions may be partly due to a temperature effect: the reported values were derived from CM made at 23°C or room temperature.

Based on results reported in this chapter, I propose a model for CMF secretion: at onset of starvation, secretion of CMF is triggered; as more cells aggregate chemotactically towards a few signaling centers in response to pulses of cAMP, the total amount of CMF around a cell would increase as a result of an increase in cell number; at temperatures above 21°C, CMF secretion is also enhanced by pulses of cAMP. The increase in CMF further stimulates its own secretion (positive feedback) that may speed up the process of aggregation; once enough cells have aggregated and CMF accumulates to the threshold levels of 0.3 ng/ml, differentiation can be induced. Such a model predicts a role for CMF in directing aggregate formation and is the subject of investigation for the following chapter.
CHAPTER IV

Elucidation of the Function of CMF by Antisense Transformation

I. Introduction

To determine whether CMF activity resides solely in the purified 80 kD protein, it is necessary to show that no CMF activity occurs when CMF-H is not present in cells. To confirm this hypothesis, the approach used was to repress the expression of the 80 kD CMF protein in Dictyostelium cells. The “conditioned medium” made from such cells should not have CMF activity if the 80 kD band is the sole factor responsible for the ability of conditioned medium to induce differentiation. Studying the behavior and the phenotypical consequences of cells lacking CMF will also help us to understand the biological functions of CMF. There are vectors available in Dictyostelium to block gene expression by antisense transformation. Many groups have successfully demonstrated the viability of antisense transformation in Dictyostelium discoideum as a means of generating a mutation in a specific gene. Notable examples include the antisense construct that eliminates the synthesis of discoidin (Crowley et al. 1985) and the myosin antisense construction that leads to several thousandfold reduction in the production of myosin heavy chain (Knecht and Loomis 1987).
The transformation vectors used to generate these antisense mutations in *Dictyostelium* are based on a modified *E. coli* vector pBR322 containing a *Dictyostelium* actin 6 promoter fused in frame with the bacterial neomycin resistant gene, which encodes a phosphotransferase (APHIII) (Nellen *et al.* 1984a,b). This phosphotransferase deactivates aminoglycoside drugs such as neomycin of Tn5, kanamycin, and G418. Actin 6 is expressed at moderate to high levels during vegetative growth but its expression becomes substantially reduced during late development (McKeown and Firtel 1981; Romans and Firtel 1985). Cells that are transformed can be selected by gradually increasing levels of G418 up to 50 μg/ml. Untransformed vegetative cells are sensitive to G418 at a dose as low as 1-5 μg/ml. In cells that are resistant to 50 μg/ml of G418 the average vector copy number is about 150-200/cell (Nellen and Firtel 1985; Knecht *et al.* 1986; Witke *et al.* 1987). In transformed cells this type of vector is integrated into the *Dictyostelium* genome as a large tandem array. This integration is not a result of homologous recombination since the transformants expressed the actin 6 gene in a way that is indistinguishable from untransformed cells (Nellen *et al.* 1984b).

Antisense constructs can be generated by ligating a cloned gene in reverse orientation downstream from a promoter, so that transcription from the promoter will produce antisense RNA. In high copy number transformants, the antisense message is present in great excess over the sense message transcribed from the single-copy endogenous gene. The sense and antisense messages are thought to hybridize to form a double-stranded RNA. Unlike other eukaryotic systems transformed with
antisense constructs, where sense-antisense hybrids accumulate in the nuclei because of an inhibition of the transport process, the double-stranded RNA is not detected in *Dictyostelium* cells and is thought to be rapidly degraded (Alexander *et al.* 1983; Springer *et al.* 1984; Crowley *et al.* 1985).

CMF antisense transformants were generated in this study which failed to aggregate even long after control transformed and wild type cells formed fruiting bodies. Addition of purified CMF restored these cells to a wild-type development, indicating that a CMF concentration of above roughly 0.3 ng/ml is required not only for the expression of prespore and prestalk genes but also for the earlier process of aggregation.

**II. 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 previously described (Chapter II; Gomer *et al.* 1991) with some modifications: the concentration of glucose was decreased to 15.4 g/l and that of phosphate was 0.5 g/l Na$_2$HPO$_4$ and 0.48 g/l KH$_2$PO$_4$. CM was made from cells starved in PBM for 20 hours and CMF activity was assayed as previously described (Chapter II; 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 diameter black Millipore filters cut into quarter sections and covered with 2.5 x 10^7 cells. Filters were transferred to new pads soaked with either PBM, 2 ng/ml hydroxylapatite-purified or 1 ng/ml SDS-gel-purified CMF (Gomer et al. 1991) at 0, 2, 3, 4 and 6 hours after starvation. 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 x 10^4/ml and counting until cells reached saturation. Vegetative cell morphology was examined using phase contrast and a 40 x objective on a Nikon microphot Fx microscope.

*Dictyostelium Transformation*

The cloning and sequencing of the CMF gene has been described in detail (Jain et al. 1992). To generate an antisense construct for transformation, the 1.4 kb EcoRI cDNA fragment isolated from the lambda gt 11 library was cut with Pst I and the two fragments separately subcloned into EcoRI and Pst I digested pBluescript KS (+). The two fragments thus had opposite orientations in the vector (see Figure 4.1). The two cDNA fragments and portions of the polylinkers were excised by digesting with SalI and BamHI and again separately ligated 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
Figure 4.1  The transformation constructs for making sense and antisense CMF transcripts. (A) is a graphic representation of the 80 kD CMF cDNA. Digestion with EcoRI and PstI produced 0.52 kb and 0.9 kb fragments. When subcloned into a pBluescript KS(+) vector digested with EcoRI and PstI, the two fragments will be in the opposite orientation. (B) To construct transformation vectors, 5ΔP (pBluescript KS(+) with the 502 bp Eco RI/PstI CMF cDNA insert) and 8ΔP (the same vector with the 905 bp PstI/EcoRI CMF cDNA insert) were digested with SalI and BamHI and subcloned into the vector pA6NPTII which was previously linearized with the same enzymes. Arrows indicate the direction of transcription. The vector containing 5ΔP insert is designated pA6N5ΔP and will give a transcript containing partial sequence that complements to CMF mRNA whereas the vector with 8ΔP, designated pA6N8ΔP, will give rise to a transcript containing partial sense CMF sequence. However since this sequence was not fused in frame with the neomycin resistant gene, no functional CMF can be synthesized.
orientation) and pA6N5ΔP (containing the 502 bp PstI-EcoRI cDNA fragment in an orientation appropriate for antisense repression of the CMF gene). The locations of the SalI and BamHI sites in pA6NPTII cause the insert to be expressed as a portion of the neomycin phosphotransferase transcript located downstream from the SV40 stop codon. The CMF fragment in pA6N8ΔP was not expected to be expressed in transformed cells due to frame shift. After subcloning, constructs having the CMF cDNA in an antisense orientation were identified by digestion with Pst I.

*Dictyostelium* cells were transformed using the calcium phosphate method (Nellen *et al.* 1984) as described by Knecht *et al.* (1990) and selected for clonal transformants by plating in soft agar (Knecht *et al.* 1990) containing 3 µg/ml G418 (Sigma). Colonies that appeared were first transferred 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 G418 and grown axenically. The concentration of G418 was gradually increased in an increment of 10 to 50 µg/ml every time the growth rate for transformants stabilized. Transformants were maintained in HL-5 containing 50 µg/ml G418. Developmental morphology and the production of CMF were monitored occasionally to ensure the stability of the transformants.

**RNA Purification and 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). This involved extracting RNA from cells lysed on ice in PBM containing
1% diethylpyrocarbonate (Sigma) and 1% SDS (Sigma), followed by extraction with 1:1 phenol-chloroform solution four times. A one-tenth volume of 3M sodium acetate, pH 7.4, was added prior to the last two extractions. RNA was then precipitated once with ethanol, once with LiCl (Sigma) added to a final concentration of 4M, and twice more with ethanol. Size fractionation of RNA was done on 1.2% formaldehyde gels 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 of the RNA to Duralon-UV (Stratagene, La Jolla, CA) was done following the manufacturers directions. Labelling of DNA probes was done with α-32P dCTP using a random hexamer primer procedure (Boehringer Mannheim, Indianapolis, IN). The probe for the D2 gene was the 0.83 kb PstI cDNA fragment (Mann and Firtel 1987), the generous gift of Dr. R. A. Firtel. The M4-4 gene was probed with the 0.9 kb PstI cDNA fragment (Kimmel and Carlisle 1986), a gift from Dr. A. R. Kimmel, and the DdrasG gene probed with the 0.8 kb EcoRI cDNA fragment (Robbins et al. 1989), a gift from Dr. G. Weeks. One of the cAMP-receptor genes, CAR1, was detected with the 1.3 kb EcoRI cDNA fragment, gift from Dr. A. R. Kimmel. Blots were prehybridized at 42°C for 4 hours with a change of hybridization buffer (recipe described by Stratagene) and hybridized at the same temperature for an additional 20-24 hours. Blots were washed three times with 2 x SSC, 0.1% SDS at room temperature for 15 minutes each and then three times for 15 minutes each with 0.1 x SSC, 0.1% SDS at 55°C.
Autoradiography on prefished Kodak X-omat AR5 film was done at -70°C for one to three weeks. The blot was then stained with methylene blue (Monroy, 1988) to verify that transfer of RNA had occurred for all samples.

III. Results

**Antisense Transformants Do Not Secrete CMF**

We began to examine the function of the CMF gene by using antisense constructs to transform *Dictyostelium* cells. In this technique, transcripts from backward oriented gene fragments apparently hybridize to the endogenous transcripts, followed by rapid degradation of the double-stranded hybrids (Alexander *et al.* 1983; Springer *et al.* 1984; Crowley *et al.* 1985). Although antisense transformation does not always repress gene expression well in higher eukaryotes, antisense transformation is known to repress expression of selected genes 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 promotors in the *Dictyostelium* transformation vectors pA6NPTII (Knecht *et al.* 1986). 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 promotors 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 hours of development and at lower levels thereafter.

Since transformants were grown in the presence of antibiotic, they grew much slower than their parental AX-4 cells. However the growth rate of the vegetative growing antisense transformants was indistinguishable from that of cells transformed with either vector or sense construct. There were also no morphological differences among transformants under light microscopy. All transformed cells developed on filter pads or starved in plaques on bacterial lawns formed normal fruiting bodies. Since actin6 promoter activity reaches maximum between 2-6 hours of development and decreases several fold after 10 hours (Nellen et al. 1984b; Knecht et al. 1986), antisense transformants eventually form fruiting bodies between one to two days later.

To choose an antisense transformation isolate several criteria were applied. First, transformed cells needed to be resistant to G418 during growth. The concentration of the antibiotic was gradually increased to 50 μg/ml to select for cells that contain high number of transformation vectors. The expression of endogenous CMF gene was also examined at the transcriptional level. RNA from vegetative growing parental AX-4 cells, cells transformed with pA6NPTII vector (vector-alone), and those transformed with the antisense construct pA6N5ΔP were isolated, subjected to Northern blotting and probed with a portion of the CMF gene. It was observed that both parental AX-4 and vector-alone transformed cells contain about equal amounts of CMF message; however, RNA from the
antisense transformants did not hybridize to the CMF probe, indicating that the CMF gene was not expressed in these cells (Jain et al. 1992).

The most important criterion for successful antisense transformation requires the absence of the specific gene product. Because the gene cloned by Jain et al. (1992) could be involved in CMF secretion or other aspects of CMF gene regulation, determination of whether the antisense transformants produced CMF would serve to verify that the cloned gene encoded CMF gene. Wild-type, vector-alone transformed, sense and antisense cells were starved in buffer and the resulting conditioned medium assayed for the presence of CMF. Figure 4.2 shows a sample of CM activity produced by sense, vector-alone, and antisense transformed cells. Both sense and vector-alone transformants secrete an activity that allows cells starved at low density to express a prespore specific antigen. As shown by the CMF activity assay, a clone of cells transformed with the pA6N5ΔP antisense construct secretes much less of this activity. CMF activity of the transformants was also compared to the parental AX-4 cells (Table 4.1). At least in the two dilutions examined (no dilution and 1:3 dilution of CM made by monolayers of submerged cells), AX-4 and vector-alone transformed cells produced conditioned media that had equal ability to induce cells starved at low density to differentiate. Antisense transformants again secreted barely detectable CMF activity. Roughly one in three clones of cells transformed with antisense construct secreted barely detectable amounts of CMF activity. The very low levels of CMF activity secreted by the antisense transformants, and the existence of CMF activity secreted by control transformed cells, was observed for cells starved in a variety of
Figure 4.2 CMF antisense transformants secrete very little CMF. Cells transformed with the pA6NPTII vector alone (circles), the sense pA6N8ΔP vector (triangles), or the antisense pA6N5ΔP vector (rectangles) were starved at $1.8 \times 10^5$ cells/cm$^2$ as submerging monolayers for 20 hours. The conditioned media produced were assayed at 4 dilutions for the ability to induce prespore antigen expression in cells starved at low density.
The diagram shows the number of positive cells against dilution factor. The x-axis represents the dilution factor ranging from 1 to 1000, and the y-axis shows the number of positive cells ranging from 0 to 200. Three lines are plotted:

- **Vector alone**
- **Sense**
- **Antisense**

The vector alone line (open circles) shows a higher number of positive cells compared to the sense (closed triangles) and antisense (closed squares) lines at all dilution factors.
Table 4.1  Transformation vector pA6NPTII does not affect production of CMF.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No Dilution</th>
<th>1:3</th>
</tr>
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<tr>
<td>AX-4</td>
<td>300</td>
<td>74</td>
</tr>
<tr>
<td>6-1 (vector-alone transformants)</td>
<td>392</td>
<td>63</td>
</tr>
<tr>
<td>5-5 (antisense transformants)</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

Conditioned media were made by starving parental AX-4 cells, vector-alone and antisense transformants at density of $1.8 \times 10^5$ cells/cm$^2$ as submerging monolayers for 20 hours. CMF activity was determined for undiluted and 1:3 dilution of conditioned media as the ability to induce prespore antigen expression by cells starved at low density.
conditions including unshaken submerged monolayers, shaking suspension, or cAMP-pulsed shaking suspension. Antisense transformants that met the criteria described were utilized for subsequent studies.

*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 then were starved and allowed to develop on filter pads (Sussman 1987). Aggregate morphology was examined throughout the developmental cycle. We consistently observed that the first three types of cells showed normal development. The antisense transformants just as consistently did not aggregate for at least two and as many as four days after starvation (Figure 4.3B). However, when grown and allowed to starve on a bacterial lawn, the antisense transformants formed normal fruiting bodies with a delay of two to four days (data not shown). This result was expected because the actin promoters used for the antisense constructs do not induce expression during growth on a bacterial lawn until after starvation (Cohen *et al.* 1986; Knecht *et al.* 1986). This also indicated that the absence of aggregation by the axenically grown antisense transformants was not due to the insertion of the vector into the genome or some other mutation.
Figure 4.3  CMF is required for aggregation. Wild-type (A,C)- and 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 can aggregate when starved in buffer containing purified CMF (D). Bar, 2 mm.
Purified 80 kD CMF Can Restore Normal Development in CMF Antisense Transformants

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 4.3 A and C, wild type cells aggregate in the presence or absence of CMF; the antisense transformants do not aggregate in buffer alone (Figure 4.3B) but do aggregate in the presence of CMF (Figure 4.3D). The developmental morphology and timing of the antisense transformants starved in the presence of purified CMF had no consistent difference from that of wild type cells. This, together with the result that vegetative antisense transformed cells did not contain detectable levels of CMF transcript, indicated that the absence of aggregation in the antisense transformants is due to a lack of production of CMF, and not due to a lack of secretion from a sequestered pool of CMF or some secondary effect of the expression of the antisense gene.

IV. Discussion

The goal of this project was to understand a phenomenon first described by Mehdy and Firtel (Mehdy et al. 1983; Mehdy and Firtel 1985): unlike cells starved at high density, when cells are starved at a density where no cell-cell contact is observed, they do not express prespore
or prestalk genes in response to high levels of cAMP; however, these low density cells become responsive to cAMP when supplemented with a medium previously conditioned by high density cells. This observation was interpreted as an indication that prespore and prestalk gene expression is cell density dependent and this dependency is mediated through a factor (CMF) secreted by cells developed at high density. It was also suggested that the failure of low density cells to express late genes (including both prespore and prestalk genes are classified) is due to the inability of these cells to express some early genes.

Using the phenomenon first described by Mehdy and Firtel (1985) as a criterion for assaying CM activity, we were able to identify a glycosylated protein factor with a molecular weight of 80 kD that is responsible for inducing differentiation in low density cells. The CMF gene was then identified using a degenerate nucleotide derived from the amino acid sequence of CMF (Jain et al. 1992). Sequence analysis of CMF cDNA predicts an acidic (pI= 4.87) polypeptide of molecular mass of 62.6 kD with three possible N-glycosylation sites (Jain et al. 1992). These predictions are in agreement with our observations that the 80 kD CMF contains N-linked glycosylation and binds strongly to the anion exchanger DEAE at pH 6.1 (Chapter II; Gomer et al. 1991). The sequence analysis also shows an internal signal sequence indicative of a secreted protein, like the 80 kD CMF.

We were able to utilize a fragment of the CMF gene to generate an antisense construct and, subsequently, antisense transformants. 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 result indicates that the purified 80 kD CMF is the original activity detected in the crude CM and that the isolated cDNA encodes CMF. These results demonstrate 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. It was reported that in addition to the absence of CMF mRNA, the membrane-associated CMF in the vegetative CMF antisense transformed cells contained no CMF activity (Jain et al. 1992). This observation, together with the fact that only a single band was observed in the genomic Southern, suggests that the sequestered CMF activity found in the vegetative cells is the product of the same gene as the secreted CMF isolated by Jain et al. (1992) utilizing the purification protocol described in Chapter II. It also suggests that the cDNA used for antisense transformation encodes CMF rather than a protein necessary for its secretion.

CMF is not necessary for vegetative growth in axenic shaking culture since the viability of the antisense transformed cells was not affected. 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 if CMF is necessary for growth on bacterial cells. However, the normal fruiting bodies formed by antisense transformed cells grown and allowed to starve in plaques on bacterial lawns indicates that the vegetative CMF is sufficient for aggregate formation, since 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 transformants now allow the functions of CMF and high continuous cAMP to be separated. If a high CMF level were only a signal to cells that they have successfully completed aggregation, 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 confirms the prediction in Chapter III that CMF is important in regulating events leading towards aggregate formation. It also indicates that expression of some early genes (probably regulated by CMF) is obligatory for prespore and prestalk differentiation, as suggested.

The Biological Function of CMF

The CMF gene is transcribed in vegetative cells and during early development (Jain et al. 1992) and its activity can be detected in the membrane-associated fractions (Yuen et al. 1991). However, CMF activity is only released by developing cells throughout development at an estimated rate of 6 molecules/minute/cell (Mehdy and Firtel 1985; Gomer et al. 1991; Yuen et al. 1991). This low excretion suggests that initially the secreted CMF may come from the sequestered pool of CMF protein in vegetative
cells. Why CMF is sequestered in vegetative cells is not known, although preliminary studies with antisense transformants imply it may have a role in repression of transcription of early genes in vegetative cells (see Chapter V). Since CMF activity can be detected throughout development, the secreted protein may be synthesized de novo during early development although it may also arise from the sequestered material in the vegetative cell membrane. There are examples of other proteins that are sequestered in vegetative cells and then are secreted during Dictyostelium development (Cardelli et al. 1990).

Dictyotelim cells do not initiate the multicellular developmental program when plated at a low cell density, as determined by the ability to induce prestalk and prespore gene expression (data presented in this thesis) and by the expression of preaggregation and aggregation phase genes (Mehdy and Firtel 1985). The cells will express early genes such as discoidin I or the serine esterase D2 (see Chapter V) and become competent to differentiate into prespore and prestalk cells, if, and only if, they are exposed to sufficiently high levels of a factor (CMF). That is, when cells are developed at low densities, an insufficient concentration of CMF is secreted for the cells to initiate the multicellular developmental program. When starved cells are at a higher density, the concentration of CMF in the medium surrounding the cells is then sufficient to induce cells to differentiate. Presumably during development a cell needs to sense whether the local cell density is sufficient to permit aggregation to occur. It is proposed that during development, Dictyostelium cells use CMF to monitor cell density in order to determine whether they are far from an
aggregation center and thus need to continue expressing aggregation-specific genes, or are at or near an aggregation center and can thus begin expressing cell-type-specific genes. Similar effects using different assay conditions have also suggested the existence of a molecule such as CMF during development (Grabel and Loomis 1976) and during vegetative growth before starvation (Clarke et al. 1987, 1988).

As a density sensor, CMF can also solve many problems developing Dictyostelium cells may face initially. The soil amoebae eat bacteria that are almost their size and starve asynchronously. The fact that the cells lacking CMF do not aggregate suggests that CMF may function to coordinate the decision of cells to aggregate. In a field of cells, those that have consumed the last of their food source would start to secrete low extracellular CMF levels. Studies on the secretion rate of CMF (Chapter II) indicate that an isolated cell do not secrete enough CMF under many physiological conditions examined to induce differentiation. Thus low levels of CMF would prevent these cells from aggregating until many more of the cells in the aggregation field starve, improving the chances of successfully relaying the chemotactic cAMP signal and also allowing the formation of a larger fruiting body. Another possiblity is that CMF levels allow cells to sense their local density just before they begin to aggregate. This detection 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).

The way CMF secretion is regulated also complements its role in controlling the aggregation processes. When few cells are secreting CMF, the secretion rate is at least 7 fold lower than the estimated 2-6 molecules/minute/cell (Chapter III). At this low level, cells are unlikely to become aggregation competent and thus aggregates of small clumps of cells may be prevented. But as the local number of starved cells starts to rise, the levels of secreted CMF is also elevated, which would further stimulate its own secretion. Higher levels of CMF may enable cells to increase the production of chemotactic and aggregation machinery (a subject that is presently under investigation and some preliminary results are presented in the next chapter) which further stimulates CMF secretion (cAMP pulses increase CMF production at temperature above 23°C; Chapter III). These positive feedback effects by cell density and cAMP enable cells that are further away from the initial signalling center to have a better chance of becoming sensitized for chemotaxis and signal relaying. Once cells reach an aggregation center, CMF becomes less essential and the synergistic effect on its own secretion also become less pronounced.

Many of the genes expressed during the preaggregation and aggregation stages encode gene products used by the cells during aggregation. These include the serine esterase D2, contact site A (a cell adhesion molecule), a cAMP receptor, and a G\(\alpha\) protein (G\(\alpha\)2) subunit that couples the cAMP receptor with phospholipase C (Gerisch et al. 1975; Mann et al. 1987, 1988; Klein et al. 1988; Rubino et al. 1989; Kumagai et
al. 1989; Firtel et al. 1989). The genes are maximally induced by secreted pulses of cAMP that interact with the cAMP cell surface receptor and activate G protein-mediated signal transduction processes. Recent studies (Mann and Firtel 1989) have shown that a basal level of expression, which is variable for the four genes described above, is induced upon starvation. Since CMF is sequestered in the fraction associated with vegetative cell membrane (Yuen et al. 1991), it is secreted as early as 2 hours after development, and its action is necessary only for the first 6 hours of development (Gomer and Firtel 1987), CMF may be responsible for this basal level of expression of these early genes. The receptor and Gα2 are part of the signal transduction system required for chemotaxis and pulse-induced gene expression, and, thus, basal expression of these genes is essential for establishing the initial phases of the signaling system. CMF would allow this initial phase to proceed when the cells were at a sufficient density to aggregate and proceed through multicellular development. Once this level is achieved, CMF does not appear to be absolutely required for later development, although it is still expressed at the slug stage (Mehdy and Firtel 1985; Gomer et al. 1985). The secretion of the 80 kD CMF reaches plateau around 10 hours after onset of development when low molecular weight CMFs start to appear. It may implicate a yet undefined role of CMF during celluar differentiation.

It is at first puzzling why cAMP could not be the factor that allows Dicystostelium cells to sense whether they are near a sufficient concentration of other cells to aggregate. However, the cAMP pulse relay mechanism requires that cAMP is rapidly degraded in the vicinity of the
cell to prevent saturating the cAMP receptor; this degradation would presumably make cAMP unsuitable for use as a factor that mediates the mass sensing.

Of broader biological interest are the parallels between CMF and a 17 kD secreted polypeptide designated C-factor encoded by the bacterium *Myxococcus xanthus* (Kim and Kaiser 1990a,b). Like CMF, C-factor appears to be used by the developing *M. xanthus* cells to sense cell density at the onset of development and is required for initiation of multicellular development. Moreover, like CMF, C-factor is not active undiluted and is only active over a narrow concentration range (4-fold for C-factor and 10-fold for CMF). Because C-factor is membrane-bound and its transmission requires orientation between cells that would achieve tightest packing, it may function as a developmental timer for triggering sporulation only when multicellular aggregates have reached the highest density possible. This is partially analogous to CMF which would synchronize cells to the developmental program only when enough cells are present.
CHAPTER V

Future Directions

Preliminary Results: CMF May Affect Expression of Some Early Genes

CMF antisense transformants have enabled us to establish the role of CMF in aggregation. Precisely how it regulates the aggregation processes remains unknown. With the antisense transformed cells, we were able to study how expression of some of the early genes is modulated in the absence of CMF. Thus developmental expression of some early genes was investigated. Experimental procedures are incorporated in the Materials and Methods in the previous chapter.

Several classes of preaggregation and aggregation specific genes that are not expressed in vegetative growing wild-type NC-4 cells have been identified. One gene, D2, has been studied extensively for its developmental regulation by pulses of cAMP (Mann et al. 1988; Mann and Firtel 1987). The gene product of D2 shares 25% amino acid identity with the acetylcholinesterase from the electric ray fish Torpedo californica (Mann and Firtel 1987) and is important for aggregation ((Rubino et al. 1989). D2 gene begins to be transcribed 1-2 hours after starvation, and its mRNA levels reach a peak between 6 and 8 hours and fall thereafter (Rowekamp and Firtel 1980; Mann et al. 1988). Maximum induction of D2 gene expression requires pulses of cAMP (Mann et al. 1988). Since CMF was suggested to be responsible for inducing the initial low levels of D2 mRNA during the first 2 hours of development, we examined the pattern of D2 gene expression in antisense transformants. Parental AX-4
Figure 5.1  D2 gene is not transcribed during development in the absence of CMF. Total RNA was isolated from vegetative (V) and filter pad-developed AX-4 (parental) and CMF antisense transformed cells collected at the indicated hours after starvation. Approximately 5 μg of RNA was loaded in each lane, separated in 1.2% formaldehyde gel by electrophoresis and blotted. The blot was probed with random primer-labeled 0.83 kb PstI fragment of D2 cDNA and washed at high stringency (for details in the procedures, please see Materials and Methods in Chapter IV). The arrow indicate the position of the 1.9 kb D2 transcript.
and CMF antisense transformed *Dictyostelium* cells were plated on filter pads for development, and RNA was isolated from parental AX-4 and antisense transformed cells at vegetative amoebae stage and at 2-hour intervals for 12 hours after the onset of starvation. Northern blots of RNA from the two cell types and from cells at different developmental times were hybridized with D2-specific probe. The 1.9 kb D2 message can be detected in the vegetative AX-4 cells, contrary to what has been reported for the wild-type NC-4 cells. The maximum expression occurred between 4-6 hours after starvation on filter pads and the levels of D2 mRNA gradually fell after 6 hours (Figure 5.1, parental). The pattern of gene expression for AX-4 cells, an axenic strain derived from NC-4, seemed to be shifted 2 hours earlier as compared to its parental NC-4 cells. The reason for this may be that vegetative AX-4 cells behave like the stationary-phase NC-4 cells in which some of the early development genes can be detected (Mann, personal communication). In the absence of CMF, the expression of D2 gene during development was reduced to undetectable levels (Figure 5.1, CMF antisense). Interestingly, the D2 transcript was detected in vegetative CMF antisense transformants, and the level was elevated to about the same as that of parental cells at 2 hours after development.

Another early gene we examined was M4. The M4 region of the *Dictyostelium* genome consists of two developmentally regulated transcription units. The M4-1 transcription unit is comprised of a single-copy DNA encoding a 0.9-kb mRNA. The message level in vegetative cells is about 0.1% of total poly(A)$^+$ RNA and decreases shortly after starvation.
Its gene expression is repressed by pulses of cAMP (Kimmel and Carlisle 1986). The repeat and single-copy M4-4 gene, however, encodes a 1-kb transcript whose level is about 0.01% of total poly(A)^+ RNA in vegetative cells. As development proceeds, M4-4 gene expression increases about 5 fold (Kimmel and Firtel 1985). The pattern of expression in wild type cells for M4-4 gene is fairly similar to that of the D2 gene, which led us to investigate the developmental regulation of M4-4 in the absence of CMF. As shown in Figure 5.2 (parental), the developmental pattern for AX-4 cells was similar to what was reported, i.e., low but detectable in vegetative cells, maximally expressed about 4 hours after starvation, then declining to the levels found in vegetative cells. Unlike D2 transcripts, the pattern of expression for M4-4 gene in antisense transformants appeared similar to that for untransformed AX-4 cells except that the message levels at all time points examined were elevated about 2-3 fold that found in the absence of CMF (Figure 5.2, CMF antisense). Thus the D2 and M4-4 genes appear to be regulated differently during development.

One of the early genes that may be important in the signal transduction pathway for signal relay and chemotaxis is DdrasG gene. ras gene products resemble G-protein in that they bind guanine nucleotides (Scolnick et al. 1979) and contain GTPase activity (McGrath et al. 1984), and thus may be important in mediating signal transduction. Two closely related ras genes have been identified in Dictyostelium discoideum. The Ddras gene is transcribed in pseudoplasmodial stage into 0.9 and 1.2-kb mRNA species, which are enriched in the prestalk cell population, and maximum levels are only observed after formation of the multicellular
Figure 5.2  The expression of M4-4 gene is elevated in the CMF antisense transformants. The isolation of RNA and Northern blotting were done as described in the Figure 5.1 legend. "Parental" indicates RNA isolated from AX-4 cells whereas V designates RNA from vegetative cells. The hybridization and washing conditions were as described. The probe was the 0.9 kb PstI M4-4 cDNA fragment. The arrow indicates the position of the 0.9-kb of M4-4 transcripts.
aggregate (Reymond et al. 1984; Robbins et al. 1989). The Dd rasG gene is early development (Robbins et al. 1989). This 1.2-kb transcript increases about 2 fold during the first 2-3 hours of development and falls to negligible levels by the aggregation stage (Khosla et al. 1990). This decline in the amount of mRNA is effected by a developmentally regulated gene product and pulses of cAMP (Robbins et al. 1991). There was basically no difference in levels and pattern of expression for Dd rasG gene with or without the presence of CMF (Figure 5.3). The level of transcript in undifferentiated vegetative cells may be slightly higher in the CMF antisense transformants. In addition, cells transformed with the CMF antisense construct contained an extra 0.9-kb transcript in vegetative growth and 4 hours after the onset of development. The reason for the presence of this extra mRNA species is not known. It was not an artifact of the blot since hybridization with other probes showed a normal expression pattern. It may represent a new and novel class of ras gene.

Of the three genes we investigated, there were three types of transcription regulation by CMF. Since both D2 and Dd rasG gene expression are modulated by pulses of cAMP, the next candidate for investigation is the surface cAMP receptors which are involved in signal relay, chemotaxis, and cAMP-induced gene regulation (Devreotes 1982; Janssens and van Haastert 1987). Based on cAMP binding kinetics, sizes of cAMP binding proteins, responses to divalent cations and guanine nucleotide analogs, and rate of desensitization and phosphorylation, several forms of cAMP receptors during early development have been proposed (Snaar-Jagalska et al. 1988) and subsequently three classes of the receptor
Figure 5.3 An extra $DdrazG$ transcript is detected in cells lacking CMF. The isolation and detection of $DdrazG$ mRNA were as described using a random prime-labeled 0.8-kb EcoRI $DdrazG$ cDNA fragment. The designations are the same as in Figure 5.1, V being the RNA from vegetative cells, numbers being the hours after starvation when RNA samples were isolated, and parental being AX-4 cells. The upper arrow points to the reported $DdrazG$ transcript while the lower arrow indicates the position of the extra 0.9-kb mRNA band observed in the CMF antisense transformants.
<table>
<thead>
<tr>
<th>CMF Antisense</th>
<th>Parental</th>
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<tr>
<td>12 10 8 6 4 2 V</td>
<td>12 10 8 6 4 2 V</td>
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genes have been discovered (Saxe et al. 1991). It has been proposed that the \textit{CARI} gene encodes the surface receptors responsible for the activation of adenylate cyclase. The expression of the \textit{CARI} gene coincides with the establishment of a signal relay system in early development. Its mRNA is undetectable during vegetative growth, accumulates substantially 5 hours into development, and remains at high levels through aggregation (10-12 hours) when significant heterogeneity in the message size is observed. The multiple size forms of \textit{CARI} mRNA probably result from differences in their 5'-untranslated regions (Saxe et al. 1991). As the \textit{CARI} mRNA level decline, the \textit{CAR2} gene (which encodes a single sized RNA) becomes expressed during postaggregative development. \textit{CAR3} gene, which encodes one size class of transcripts, is expressed at 10\% of \textit{CARI} mRNA levels during early development. The maximum expression occurs during tight aggregate formation and the message levels decline thereafter. Regulation of \textit{CARI} gene expression by CMF was investigated using CMF antisense transformants. As shown in Figure 5.4 (parental) and as reported, no transcript was found in vegetative cells; maximum expression of the \textit{CARI} gene occurred around 4 hours after onset of development and a larger transcript began to appear between 8-10 hours into development. CMF antisense transformants showed approximately the same pattern and levels of expression for the smaller transcript (\(\sim 1.9\) kb) as compared to the parental AX-4 cells with two exceptions. These cells did not express the larger 2.1 kb transcripts during aggregative stage but transcribe the 1.9 kb mRNA species in the vegetative cells in addition to two other mRNAs (3 and 8.9 kb).
Figure 5.4  The 2.1-kb transcript of CAR1 gene encoding the cell surface cAMP receptor is missing in the absence of CMF. The mRNA for CAR1 gene was detected as described using a 1.3-kb EcoRI CAR1 cDNA fragment. V stands for vegetative cells whereas number indicates the hours after starvation. Parental represents AX-4 cells whereas CMF antisense are the cells transformed with the antisense construct pA6N5ΔP. Upper and lower bars, respectively, indicate the positions of the 4.1- and 1.9-kb rRNA bands.
Expression of a cAMP receptor gene

Parental
V  2  4  6  8  10  12

CMF Antisense
V  2  4  6  8  10  12
Expression patterns during development for cells transformed with vector alone were not included in all the experiments described in this section, therefore all the changes observed for CMF antisense transformants may be artifacts irrespective of CMF regulation, possibly due to the presence of 50-200 copies of actin 6 promoter. We are currently repeating these experiments with the further controls. However, given the factors that developmental patterns and morphology, CMF mRNA levels, and CMF activity for vector-alone transformed cells were indistinguishable compared to the AX-4 cells, we would for now assume that results observed for AX-4 were representative for both types of cells. Consequently, all the data so far suggest that CMF regulates the expression of many genes at the transcriptional level through a variety of mechanisms during both vegetative growth and early development.

One prediction of CMF as the density sensing factor necessary for the completion of aggregation is that it plays a role in the regulation of the expression of early genes. The expression of the four early genes examined were all modulated by CMF consistent with a role during early development. Some genes require CMF for the induction of expression (for example, D2 gene is not transcribed in the absence of CMF) whereas others need CMF to maintain proper levels of expression (the example is M4-4 gene whose transcripts accumulate to higher levels in the CMF antisense transformants). CMF may be necessary for either initiation at the correct promoter or for proper processing of transcripts. The patterns and levels of expression of CAR1 gene and DdrasG gene were unaffected by CMF. However, without CMF, an mRNA species is missing (as in the case
of cAMP receptor gene) or an extra mRNA species appears (as in the case of Dd rasG gene). These results imply that CMF may have some indirect action on differential initiation or splicing processes to generate transcripts of alternative sizes. The observation that amount of mRNA becomes elevated (M4-4 and Dd rasG genes) or is raised to detectable levels (CARl and D2 genes) in vegetative growing CMF antisense transformants implies a role of CMF in undifferentiated vegetative growing cells, contrary to what was previously believed. This is a consistent with the finding that CMF message and gene product are found in vegetative cells.

Other Possible Experiments

Preliminary data not presented have shown that CMF had an opposite effect on the expression of phosphodiesterase and its inhibitor, i.e., phosphodiesterase transcript levels are lower and phosphodiesterase inhibitor transcript levels are higher in the absence of CMF. Since the expression of the 1.9-kb transcripts is normal in the CMF antisense transformed cells, low levels of phosphodiesterase and high levels of its inhibitor may disrupt cAMP pulse production. Thus, an obvious experiment would be to examine whether the normal oscillatory rise and fall of cAMP levels every 6 minutes can be observed.

Morphological comparisons will be examined in the cells developed in the presence and the absence of CMF at electron microscopic levels with scanning electron microscope (SEM). The absence of CMF is mimicked either by the CMF antisense transformants or wild-type cells developed at very low density. High density wild type and vector-alone transformed
cells and low density wild type cells in the presence of exogenous CMF should provide the "normal" morphology. Preliminary results seem to indicate the number of membrane protrusions may be affected by CMF.

The development of CMF specific antibody and the identification of CMF receptors are also under intensive investigation by members of our laboratory.
REFERENCES


Kimmel, A.R. and Firtel, R.A. (1985) Sequence organization and developmental expression of an interspersed, repetitive element and


accumulation of inositol 1,4,5-triphosphate in Dictyostelium cells that are labelled with $^3\text{H}$:inositol by electroporation. Biochem. J. 258: 577-586.


