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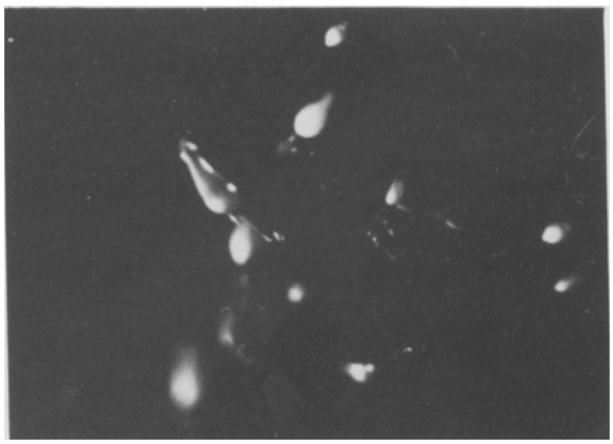
Multi-author Reviews

Molecular biology, growth and development of the cellular slime mold *Dictyostelium discoideum*

The editors wish to thank Dr. R. Mutzel for having coordinated this review.



"It is often said (especially in the writing of research proposals for grants, a process that tends to govern our lives) that cellular slime molds are a good model system. By this one presumably means that one can apply lessons learned using slime molds to our understanding of vertebrate development, and especially human development. To me this way of thinking has always been deeply troublesome. My fascination with the experimental analysis of slime molds is based on the idea that one wants to find out about slime molds! Slime molds are a model system for the study of slime molds."

John Tyler Bonner[3]

Introduction

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Whether or not the authors of the Multi-author Review in this issue agree with Professor Bonner's statement, whether one or another is much more fascinated with understanding how, for example, a human being consisting of more than two hundred differentiated tissues in a proper and reproducible geometry is 'made' from a single fertilized egg, whether one or another is much more fascinated with the idea that this interplay of cells in our bodies is critically dependent, at the end, on whether or not a few copies of some transcription factor are synthesized at some point in time, and how all this was brought about by variability and natural selection, there are good reasons for developmental biologists to work on and think about cellular slime molds.

This Multi-author Review has been assembled to emphasize, once again, the utility of *Dictyostelium* as a model system for addressing the question of why and how eukaryotic organisms become pluricellular. Cellu-

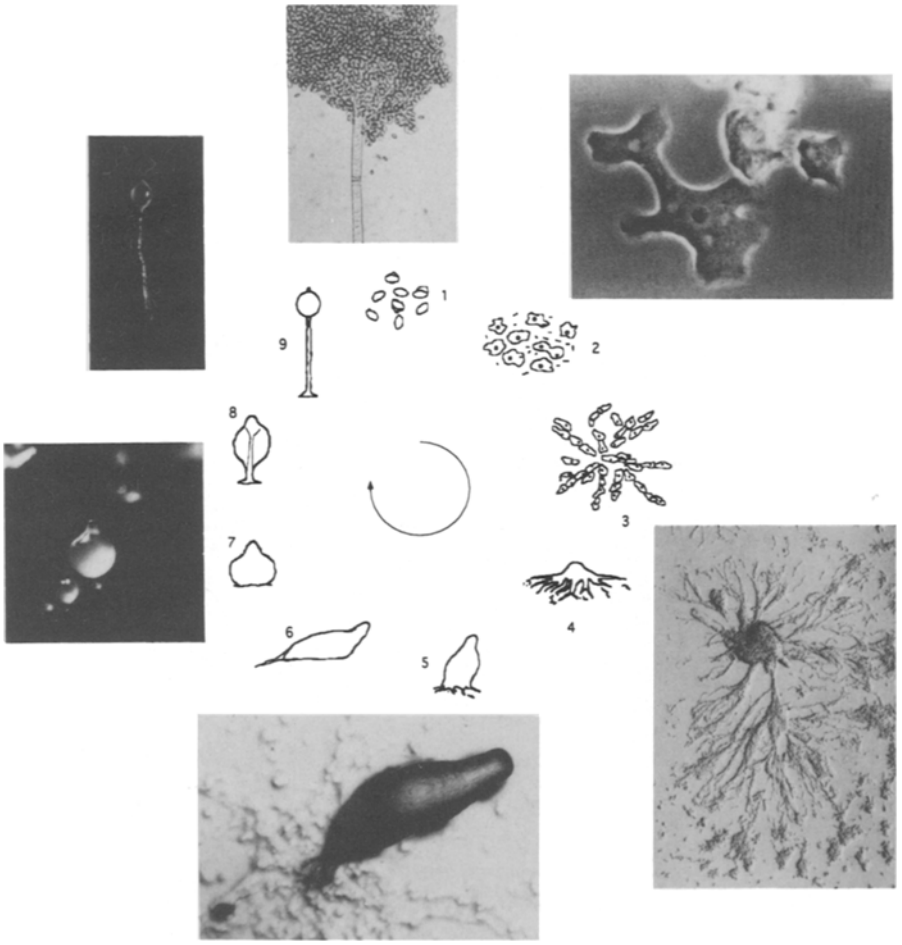

Figure 1. The life cycle of *Dictyostelium discoideum*. Spores germinate (1) and vegetative cells grow (2) until the food source is depleted; after chemotactic aggregation (3) the cell mass of the aggregate (4) elongates to form the 'first finger' (5) and either migrates (6) or directly enters the 'mexican hat' stage (7) to culminate (8) in a fruiting body (9) that consists of the terminally differentiated, dead stalk cells and the spores which can initiate another round of the cycle. Under laboratory conditions development from the onset of starvation to the mature fruiting body can be accomplished in about 24 h. Photographs (taken at various magnifications) show selected stages: top right, an amoeba; bottom right, an aggregate; bottom, a migrating slug; left, a mexican hat just starting culmination; top left, a mature fruiting body; top, detail of a fruiting body with part of the stalk and mature spores. Photographs courtesy of Dr. C. Schlatterer.

lar slime molds, better designated by the French 'amibes sociales', by a number of aesthetic and technical criteria, indeed offer an ideal model for the study of fundamental strategies and mechanisms of cellular communication, the molecular mechanics of cell movement, cell differentiation, and morphogenesis. Moreover, from the phylogenetic point of view, this group of eukaryotic microbes not only represents a model of the current status of these processes, but also of their evolution.

In their natural habitat, the upper soil layer of decaying leaves, free-living amoebae feed on bacteria (probably on anything that is smaller than themselves except for cells of the same or related species), ingesting about 1000 bacteria per cell division (see fig. 1 for a schematic sketch of the life cycle). Growth of the population continues until some critical relationship between the availability of further nutrients and cell density is reached; only when growth has ceased the cells will enter development. Pluricellularity in social amoebae is thus not obligatory, but depends on a defined environmental situation, starvation and population density, and therefore is 'gratuitous'[13].

A first interesting point has to be made about this particular life style: since development proceeds in the absence of an increase in the body mass of the organism (although most of the cells will divide once more[27]) each cell has to rely entirely on its molecular and energetic reserves for the massive biochemical changes that will be required for differentiation and the formation of a fruiting body.

Indeed, right from the onset of starvation, profound changes in the biochemical makeup of the cells become evident: transcription of house-keeping genes (e.g., those for ribosomal proteins, or enzymes of nucleotide metabolism) is shut down, while proteins required for intercellular communication and intracellular signaling,

and first signs of cell-type specific pre-determination appear. Up to 100,000 individual amoebae will now chemotactically aggregate. Cells in the center of the field of developing amoebae start to emit pulses of a chemoattractant, to which surrounding cells respond in a dual manner: first, they migrate towards its source; second, they are also stimulated to synthesize and secrete a pulse of the attractant of their own, thus relaying and amplifying the signal and increasing the size of the 'aggregation territory'. *Dictyostelium* uses cAMP (a well-known ubiquitous intracellular second messenger) as the hormone that coordinates aggregation[14], whereas other species speak quite different chemical languages – from pterin derivatives in the small *D. minutum* and *D. lacteum* species, to a peptide in the more complex *Polysphondylium* species (see ref. 19 for review).

During aggregation the amoebae form tight end-to-end contacts and migrate in streams towards the center. From now on, we have every right to look at this mass of amoebae as if it was a developing embryo. At an air/water interface it forms a tip that will act as an organizer during the further stages; pre-differentiated cells originally scattered in the early aggregate sort out; and cells differentiate according to their position. Depending on its environment, the tipped, elongated aggregate then choses one of two alternative morphogenetic pathways. Under favorable conditions it enters fruiting body formation directly. Otherwise it will start searching these conditions by migrating over the substratum as a 'slug', guided by the chemical composition of its environment, and responding to extremely flat light and temperature gradients. The migrating slug is surrounded by an extracellular matrix that is constantly synthesized and left behind. At this stage two pre-differentiated cell types can be easily distinguished: pre-stalk cells occupy the front of the slug, comprising roughly 20% of the entire population, and the rear end consists of pre-spore cells with some few interspersed 'anterior-like' cells that resemble pre-stalk cells.

Culmination then proceeds in a manner that is reminiscent of gastrulation in higher embryos. The slug arrests and the pre-stalk zone moves into an upright position. A small population of 'rear guard' cells at the base differentiates into the stalk cells forming the basal disk. Pre-stalk cells from the tip migrate through the mass of prospores down to the base, thereby terminally differentiating into mature stalk cells. As more and more stalk cells become stuck to each other during this morphogenetic movement, the mass of differentiating spores is lifted off, giving the structure its final body form: an elongated stalk of dead, vacuolized, plant cell-like stalk cells surrounded by a rigid cell wall which support a small sphere of resistant spores that can be dispersed and eventually germinate to initiate another asexual cycle.

The goal of the present collection of reviews is to walk along this developmental cycle and to highlight some of the features of *Dictyostelium* that makes the organism such a unique model for the study of development. Before doing so, we will be introduced to the systematics of cellular slime molds and their place in evolution, and to the molecular techniques that were a prerequisite to much of the progress that has been made in the last decade.

As mentioned above we can hope to learn about the evolution of pluricellularity by studying cellular slime molds. For this, their point of divergence from the main eukaryotic branch must be defined as properly as possible: Are the ancestors of these microbes the most ancient eukaryotes that found their way out of the 'hole of unicellularity', defining a prototype which natural selection kept preferring over hundreds of millions of years? Or have they found their own way of becoming pluricellular after they diverged from a unicellular ancestor? 'Classical' molecular phylogeny – 5.8 S and 18 S rRNA comparison – places *Dictyostelium* at a very low branch of eukaryotic descent[17]. Loomis and Smith, in our first review, call for a revision of this point of view; and if rRNA phylogeny has become covered with some patina, they definitely scratch it. Summarizing morphological and biochemical properties, and the organization of the genome, they show that *Dictyostelium* cells resemble much more higher cells (e.g., mammalian macrophages) than ciliates or true slime molds, not to speak of yeast. Making the point that the *Dictyostelium* genome is extremely biased towards a high A + T content, they argue that this mutational pressure should also result in a bias in the A + T content of rRNAs, yielding a misleading topology of the resulting rRNA tree. If, indeed, *Dictyostelium* rRNA sequences to a significant extent reflect an adaptive answer to a metabolic constraint, mutational pressure, and not only their phylogeny, then we have to look at different molecular clocks. In fact, the protein trees that Loomis and Smith presented originally in 1990[15], and which they now underpin with additional comparisons, suggest that the branch leading to *Dictyostelium* diverged much later than suggested by rRNA comparison, namely after the yeasts, but before the plant/animal boundary. A propos: while *Dictyostelium* amoebae resemble macrophages, mature stalk cells are much more like plant cells... A very reassuring fact in their protein analyses is that these appear to be quite robust: although branch lengths vary with the protein analyzed, the topology does not change with protein family or the method of phylogenetic analysis.

What should we expect from a simple microbial model for eukaryotic development? Development should be rapid, synchronous, and easy to observe and control; we should be able to cultivate large numbers of genetically identical cells for biochemical analysis; there should be

convenient ways to isolate and characterize mutants showing abnormal development; a small genome should facilitate the molecular analysis of the genes involved in development; and we should be able to use all the tools and tricks of classic genetics. With the exception of the last, *Dictyostelium* offers all of these advantages. Unfortunately, there is no easy way to perform genetic crosses between different strains. Although under certain conditions haploid amoebae of strains with different mating types can convert into gametes, mate and form a zygote which pseudo-cannibalizes surrounding cells and forms a macrocyste in which meiosis occurs[7], the efficiency with which these macrocysts germinate is extremely low. On the other hand, diploids can be made and haploidized after recombination, but the procedure is quite laborious and time consuming[18].

Dictyostelium researchers therefore started quite early to rely on pseudo-genetic analyses, and the second review, by Kuspa, Dingermann and Nellen summarizes the progress that has been made during the last decade. A major breakthrough has been a transformation protocol set up in 1984 by Nellen and his co-workers in the laboratory of R. Firtel[20], opening the way to overexpression of proteins and antisense technology, gene disruption and targeting by homologous recombination, tagging of genes with easily detectable antigenic and biochemical markers, and recently, restriction enzyme-mediated plasmid integration (REMI) which promises a further boost to the pace at which genes in *Dictyostelium* can be analyzed. To illustrate the importance of these techniques with just one example, it was the possiblity of analyzing the expression of marker genes like CAT and *E. coli* β-galactosidase under the control of cell-type specific promoters that led to the demonstration that pre-stalk cells comprise several sub-populations in the slug and culminant[11], and more recently, by using labile reporter contructs, that there is a constant re-differentiation of predetermined cells in the developing organism[6]. On the other hand, physical mapping of the *Dictyostelium* genome is well under way, and it is probably only a matter of years until we can have its entire sequence (which will probably not be the Holy Grail for the slime mold community, but doubtless a fairly useful tool).

In our third contribution, now entering the developmental cycle, Clarke and Gomer ask when and how a population of growing cells realizes that the time has come to begin their 'aventure sociale'. Their work on protein factors that regulate the onset of development and control the size of an aggregate indeed suggests that pluricellular behavior is established well before the onset of starvation. A first, 'prestarvation factor' (PSF) is constantly synthesized and secreted by vegetative amoebae and can induce the expression of certain genes, the products of which are required for development. Interestingly, the presence of food bacteria inhibits its effects (PSF probably being bound by the bacteria), thereby enabling the amoebae to measure the relationship between their density and the availability of bacteria. Consequently, development is initiated when the food source has been optimally used, i.e. when a critical relation between the available food source and the yield of vegetative amoebae is reached. Starving cells then secrete a second, 'conditioned medium factor' (CMF) which appears to be important at least until aggregation since CMF antisense mRNA mutants can no longer aggregate. The exciting point about CMF is that its concentration appears to define the number of cells in an aggregate, providing a model for the regulation of tissue size in higher eukaryotes.

The cytoskeleton is one of the few true distinctive features of eukaryotic cells. For example, it enables macrophages and cellular slime molds to 'catch' their prey and engulf it. Noegel and Luna review the state of the art with *Dictyostelium*. Knowledge of the myriad of cytoskeletal components and the complexity of their interactions that ultimately allow coordinated cell movement, phagocytosis, intracellular vesicle trafficking, or the cleavage of daughter cells, has profited in a paradigmatic manner from molecular techniques and their combination with biochemical, immunological, and cell biological approaches. Happily, we are looking inside a 'simple' machine, constructed by a 'lower' eukaryote. Simple? There are about twenty active actin genes, for example, whose protein products can be tyrosine-phosphorylated and acylated, and more than ten myosin genes. During cell movement actin filaments undergo rapid rearrangements that are finely tuned by a vast community of capping, severing, bundling, crosslinking, and sequestering activities. Actin-binding proteins that determine the localization of filaments have been identified; some of them, like ponticulin, hisactophilin, coronin, coactosin, or p30b still await the identification of their counterparts in 'higher' organisms, while others share structural and functional homology with cognate proteins from vertebrates. The list of these proteins may be far from completion since new activties are constantly being identified and characterized; moreover, analysis of mutants often reveals only minor phenotypic changes, suggesting the presence of additional, complementing components.

The following three chapters deal with communication and the transduction of cellular signals during multicellular development. Van Haastert summarizes the facts from reception of the extracellular signal, cAMP or folate (and possibly other compounds), down to the formation, release and action of intracellular messengers. Readdressing the question of whether *Dictyostelium* cells respond to temporal, spatial, or both temporal and spatial changes in the extracellular concentration of chemoattractants, Van Haastert presents a number of most entertaining calculations, concluding

that the difference in receptor occupancy between the front and rear ends of a chemotaxing cell at limit attractant concentration is minute. It is clearly the signal transduction and amplification cascades that are activated upon these small changes in receptor occupancy which allow the cells to make sense of these alterations 'at the verge of stochastic and thermal fluctuations'. Again, there appears to exist a remarkable conservation of signal transduction mechanisms (down to the MAP kinase kinase kinase pathway) from *Dictyostelium* to higher organisms, starting with the molecular architecture of cell-surface receptors and their coupling to G proteins and to the intracellular messengers that are liberated upon receptor activation; and again, the system is readily amenable to analysis and experimental manipulation due to its relative simplicity, allowing, for example, the elucidation of the functions of each of the four distinct cAMP receptors, or of the different G protein subunits, to be analyzed in great detail. However, the system also can behave quite surprisingly, for instance, when deletion of the single phospholipase C gene fails to produce any apparent phenotypic alterations at the level of chemotaxis and development, although there is a host of biochemical and cell biological data that implicate the enzyme in chemotaxis and development.

The contribution by Newell, Malchow and Gross is the first comprehensive account of our knowledge of the role of calcium ions in chemotaxis, aggregation and cell differentiation in *Dictyostelium*. As for any eukaryotic cell, calcium is extremely important for *Dictyostelium*, regulating processes as diverse as cell motility, chemotactic orientation and locomotion, and gene expression. However, due to its tendency to form complexes with phosphates readily, its free cytosolic concentration must be accurately maintained at a low level. For a fibroblast in our body, this is no great problem, because its extracellular calcium concentration is kept constant. In the soil, a drop of rain could change the free extracellular concentration by several orders of magnitude. Accordingly, *Dictyostelium* amoebae have an enormous capacity to sequester the ion. Thus, while chemotactic stimulation of aggregating amoebae with cAMP leads to changes in the extracellular calcium concentration that can be easily recorded with ion-sensitive electrodes, and free-running oscillations of the extracellular calcium in cell suspensions were first correlated with oscillations in cell shape, oscillations of extracellular pH and $K+$ concentration, oscillations in extracellular cAMP and intracellular cAMP and cGMP as well as with changes in the cytoskeleton, no global cytosolic changes can be seen at the single cell level with the fluorescent indicator dye, fura-2. Discussing these observations with respect to calcium-sequestering organelles and their regulation, Newell et al. then switch to the role of calcium for chemotaxis and cytoskeletal rearrangements, with special emphasis on the interaction of myosin with the cytoskeleton and the possible involvement of cGMP (it is a puzzling fact that, although massive increases in intracellular cGMP after chemotactic stimulation and oscillations of intracellular cGMP in developing cell suspensions were first shown almost 20 years ago, we do not yet know exactly what cGMP does). Finally, data on the regulation of cell differentiation, gamete fusion and spore germination by calcium and calmodulin-binding proteins are summarized.

It has for long been known that cAMP – besides its role in coordinating morphogenetic movement – also acts on the expression of genes during development; a possible link between calcium and cAMP comes from recent experiments by Schlatterer et al.[21] showing that stimulation of aggregative amoebae with the high doses of cAMP required to induce expression of certain genes indeed leads to global increases in the free cytosolic calcium concentration, and that calmidazolium, a calmodulin antagonist and inhibitor of *Dictyostelium* calcineurin (S. Hellstern & R.M., unpubl. obs.), can similarly increase cytosolic calcium (C. Schlatterer, pers. commun.).

Is *Dictyostelium discoideum* really a good model to study the effects of intracellular cAMP, if the cyclic nucleotide is also used as a hormone that coordinates aggregation? Reymond, Schaap, Williams and Véron try to settle a long-lasting dispute on the question of whether *all* effects of cAMP on cell differentiation are brought about by receptor activation, or whether they are *all* mediated by cAMP-dependent protein kinase. Reviewing pharmacological, biochemical, and molecular data on the cAMP receptor/phosphodiesterase system and a host of experiments aimed at altering the cell type-specific expression, properties, and regulation of the kinase, they show that cAMP can clearly act both from the outside (possibly by a signaling pathway including calcium as a second messenger [see above]) and as a 'classical' intracellular messenger via the activation of PKA (note that we still have no clearcut idea of the free cytosolic cAMP concentration and by which enzyme it is synthesized; certainly, synthesis of cAMP that is secreted for signal relay and synthesis of intracellular cAMP must be properly kept apart). It is worthwhile noting that virtually everything that we know about the role of cAMP-dependent protein kinase in preaggregative development, the choice of cell fate, and culmination has only been learnt by the molecular techniques allowing the expression of mutant forms of the protein under the control of defined, cell type-specific promoters.

Specific adhesion of cells in a developing embryo or in a tissue has been on biologists' minds for a long time. As Bozzaro and Ponte note in their review on adhesion in *Dictyostelium*, research in this field (up to the CAMs and cadherins so fashionable today) has very much

profited from studies on the cellular slime mold, pioneered by G. Gerisch and his co-workers over a period of more than 30 years. Summarizing comprehensively the data on contact molecules that are important for aggregation and post-aggregative development (in particular the contact site A glycoprotein, one of the best-understood examples of a cell-adhesion molecule), Bozzaro and Ponte also draw our attention to cell-substratum adhesion, the binding of bacteria to the cell surface which is required for phagocytosis, and the role of contacts in intra- and interspecific sorting out of cells. How critical specific adhesion can be for the organism is impressively illustrated with the example of *Dictyostelium caveatum*[24], the predatory slime mold which feeds on related species, and in which a single mutation can cause break-down of self-nonself recognition, making the predator a cannibal (ref. 25; for discussion see also ref. 19).

Our last chapter, by Wilkins and Williams, deals with what caused Brefeld to call social amoebae *Schleimpilze*[4], the 'slime sheath' of the migrating slug, and which, of course, is an extracellular matrix (ECM). After treating the molecular constituents of the ECM (comprising cellulose, like plant cells, but also proteins that have their homologues in the animal world), and their synthesis by pre-stalk cells and an epithelial layer of cells along the slug, the authors consider functional aspects of the ECM and its protein components. Molecular techniques have again contributed much to the picture that begins to emerge, and defined mutants are available. Examples are mutations in *modB*-dependent O-glycosylation which make the slug stall during migration, or the *ecmA* null-mutant which displays a rather complex phenotype: delayed posterior maturation that causes the slugs to elongate, and a delayed transition from migration to culmination. The most puzzling observation with *ecmA* null-slugs is however, that they become 'right handed' approaching a light source preferentially from the right, whereas wild-type slugs approach from both sides.

My favorite perspective

Cellular slime molds have proven a most suitable developmental model in the past, and we trust they will do so for quite some time in the future. There are at least two important model aspects of *Dictyostelium* development that are not covered by the present collection of reviews. The first is regulation of cell-type specific differentiation and proportioning by diffusible signals, in particular differentiation-inducing factor, DIF. This topic has been covered by R. Kay elsewhere (ref. 12; see also ref. 9). The second is programmed cell death. Although the question of whether or not the subpopulation of pre-stalk cells in developing *Dictyostelium* aggregates is doomed was affirmatively answered by Whittingham and Raper 35 years ago[26], it has been re-addressed only very recently at the molecular level[5]. Current research on what is now called apoptosis focusses very much on the 'hows', yet we could probably learn about the 'whys' by studying social amoebae (see ref. 2). Remember there are about 60 species known today, from the most primitive *Protostelium* (not yet very social) where a single vegetative cell differentiates into a spore sitting on a small stalk, to *Acytostelium* with all cells in the aggregate making a common effort to synthesize the acellular stalk, to *Dictyostelium discoideum*, sacrifying about one quarter of the cells for stalk construction, to the complex *Polysphondylium* species in which up to 90% of the cells will construct the stalk (cf. ref. 1).

Ciliates, another deep and very fruitful branch of the eukaryotic tree[8], appear to have driven the exploration of unicellularity to its very limits, bringing about single cells with many forms of specialized 'organs'; have cellular slime molds to a similar extent explored a particular form of metazoan life? Clearly, if we want to pursue the argument, there is a need to understand the phylogeny of the whole group in its molecular details. Coming back to the place of *Dictyostelium* in evolution, whether we trust the rRNA tree or the protein tree, *D. discoideum* sits at the end of a very long branch; what will this branch look like once we can decorate it with molecular data from a number of different species of cellular slime molds? Similarly, as Bozzaro and Ponte point out, comparative studies on cell adhesion in more or less closely related species would be helpful. Likewise, it might be worth testing our models of the multiple roles of cAMP in a species that uses an extracellular signal other than cAMP for aggregation.

After a long time in which almost all of cellular slime mold research focussed on *Dictyostelium discoideum* as 'a model for the model', there are a number of new starting points for comparative molecular and biochemical studies. For example, transformation of *Polysphondylium* has become feasible[23], and cDNA libraries from *D. minutum*[22] and *P. pallidum*[16] have been described that allowed the characterization of the counterparts of the ecmA and contact sites A mRNAs from these species. Moreover, *Acytostelium* and *Protostelium* have been re-discovered for biochemical and molecular studies[10]. A slime mold aficionado's favorite perspective is therefore, to speak with John Bonner: that *Dictyostelium* shall prove a good model for the study of slime molds.

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