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Genetics of Early *Dictyostelium discoideum* Development

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EARLY DEVELOPMENT OF *DICTYOSTELIUM DISCOIDEUM*

Dictyostelium amoebae feed on bacteria and divide by binary fission as long as their prey is available. Once their food is exhausted or removed, the cells begin development and synthesize new macromolecules that give them the ability to move toward sources of cyclic adenosine 3′,5′-phosphate (cAMP). All of the apparatus required for recognizing cAMP and moving up a concentration gradient, including the cAMP receptor (106, 130, 131), extracellular cyclic nucleotide phosphodiesterase (97, 126, 185), adenylate cyclase (60, 127, 205), and cAMP secretory apparatus, are produced de novo (144). During their migration into centers of aggregation, the cells become adhesive, and once assembled, they unleash a large number of new transcripts, products of which are responsible for further differentiation into spore and stalk cells (8, 21, 38, 138). There is good evidence that chemotaxis toward cAMP, first used during aggregation, is retained to direct the cells during the later stages of development when complex movements lead to the formation of a migrating slug and then to a fruiting body (120, 220). *Dictyostelium* cells have invented the strategem of coordinating the expression of their genes and morphogenesis by connecting a cell surface cAMP receptor to their sensory and motility apparatus and to the second messenger cascades which they use for gene regulation. Extracellular cAMP, therefore, has immediate effects on chemotaxis and motility and more delayed but crucial effects on gene regulation.

The development of the cellular slime molds begins with starvation, but if what we learn about their development is to have heuristic value to other developmental biologists, *Dictyostelium* should be compared with cell lineages in which a proliferating stem cell enters a developmental pathway. The convenient advantages of *Dictyostelium* are that all cells differentiate and those that form spores are viable when the process is finished. Development is, to use Kaiser's word, gratuitous, and can therefore be experimented on (116). Interesting developmental mutants can be saved as frozen amoebae or as spores formed by mixing with wild-type cells, a procedure that coaxes some aggregateless mutants through development (235).

The chemotactic apparatus develops gradually: cells acquire the ability to move toward a source of cAMP, then the capacity to relay, and finally, after 7 to 9 h, to emit cAMP signals autonomously (101). Amoebae that surround the signalling cell respond by chemotaxis toward the source and then secretion of cAMP so that a wave of cAMP moves outward by a relay mechanism from the central pacemaker cells (see references 58, 129, and 163 for detailed reviews of aggregation). Little is known about the mechanism of secretion, although one study based on electron microscopy suggests that release is through vesicle fusion with the cell membrane (154). The central cells signal every 5 to 9 min, and as the cAMP diffuses radially, it impinges on the cAMP receptors of cells as much as 60 μm away (5). The binding characteristics of the cAMP receptor are complex, but they have a K_d of 40 to 50 nM (103, 106, 129, 163). The amplitude of the wave is reduced by diffusion and the actions of membrane-bound and secreted cyclic nucleotide phosphodiesterase (see reference 137 for the history of this enzyme). The successive waves of cAMP, which travel outward at a velocity of about 300 μm/min, have been observed by an elegant isotope dilution technique (242). The reason for the

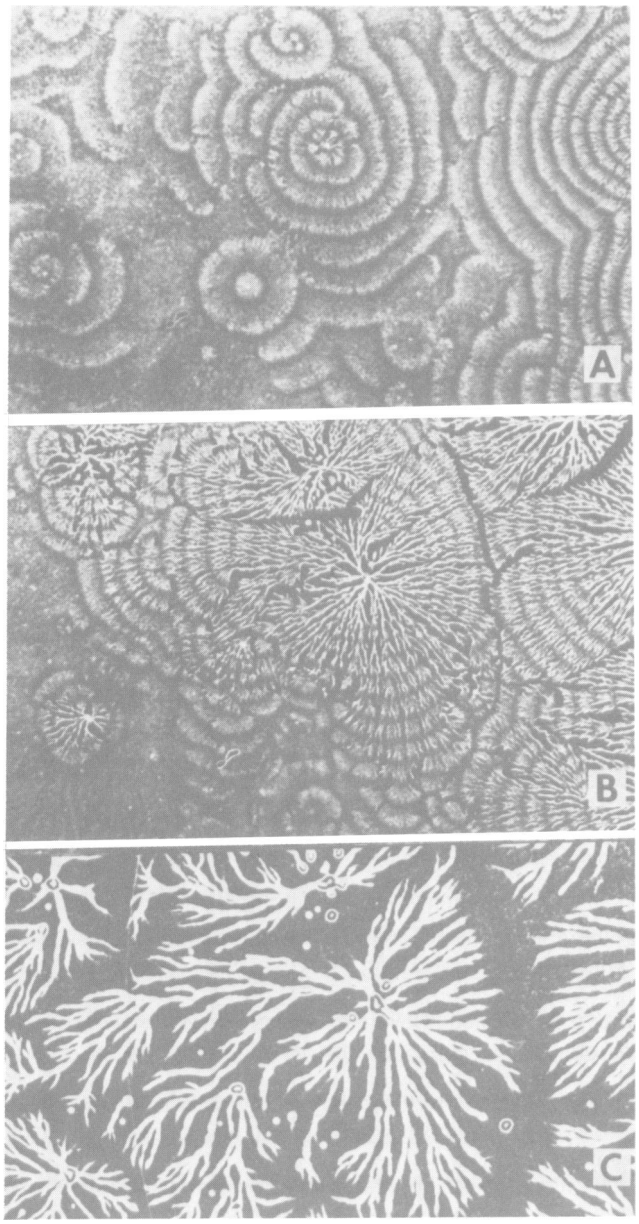

FIG. 1. Dark-field photographs of aggregation territories. *Dictyostelium* amoebae were starved overnight at 6 to 7°C. Under these conditions all of the components necessary for aggregation are made, but aggregation itself cannot occur. When plates containing lawns of amoebae are shifted to room temperature, aggregation occurs within 0.5 h. See the text for further explanation. Aggregation territories are about 1 cm in diameter. Courtesy of Peter C. Newell, University of Oxford.

banded pattern shown in Fig. 1A is that the first cells to receive the signal migrate for only 2 min while the gradient surrounding them is positive and then stop to await another signal that will not come for 5 to 7 min. Meanwhile, cells to their rear are receiving the relayed signal and are still moving. Moving and stationary cells refract light differently; the light bands in Fig. 1A contain moving cells, while the dark bands contain cells that have stopped and are awaiting another wave of cAMP. The changes in shape of individual cells after an increase in cAMP concentration are shown in Fig. 2. Cells that have not yet received cAMP are shown at different magnifications in panels A and C. Cells that have just perceived an increase of cAMP concentration to 1 μM round up in what is called a cringe response, as shown in panels B and E. A few seconds after the jump in cAMP concentration, the cells begin a period of rapid motility as shown in panels C and F. After several minutes the cells become quiescent again. During one round of migration, which covers about 20 μm (5), the cells are deaf to cAMP waves emanating from their rear. This refractory period, as well as the activity of a hydrolytic enzyme on the acrasin, as the signal molecule was called before it was known to be cAMP, were first detected by Shaffer (223, 224).

As the aggregation of the cells progresses, the radial rings of migrating cells break down to form streams in which the elongated cells are attached end to end (Fig. 1B and C) and move more rapidly into the aggregate. The streams remain sources of cAMP and continue to attract straggling cells. Once cells are collected in an aggregate, morphogenesis begins with the formation of a tip, which acts as an embryonic organizer: if the tip is removed, development stops until a new tip is made (22, 153, 199). The tip remains a source of cAMP, and under its influence the mound elongates into a slug which can be several millimeters long. This structure eventually falls over onto the substratum, which in the laboratory is usually agar or moist filter paper. The slug can migrate toward light or up a heat gradient (24, 83), but when presented with overhead light it begins to form a fruiting body by changing its axis to a vertical one again and compacting to form a structure that looks like a Mexican hat. At this stage a tube of cellulose is formed by the central apical cells, and the cells in the peak of the hat (which used to be in the front of the slug) migrate through the cellulose tube, differentiate into stalk cells, and die. Cells in the thick brim of the hat are pulled toward the upper end of the forming stalk, and they in turn differentiate into spores. Under optimal conditions a fruiting body has about 100,000 cells, of which 20% have been sacrificed to form stalk cells. Readers interested in the evolutionary significance of this life cycle should consult reviews by Kaiser (116) and Bonner (23).

GENETIC ANALYSIS

Although aggregation and spore formation are remarkably complex for an organism that contains only about seven times the single-copy deoxyribonucleic acid (DNA) of *Escherichia coli* (80, 238), this is not the only life cycle that *D. discoideum* amoebae can execute. When cells of different mating types are mixed and provided with appropriate conditions, they give rise to structures called macrocysts whose development rivals the more studied pathway in complexity (71, 216–218). The macrocysts are the products of a calcium-dependent cell fusion (161, 245), resulting in the formation of a zygotic giant cell that attracts surrounding amoebae, again by chemotaxis, and devours them. A large multiwalled structure full of pycnotic captured amoebae is formed over a period of days. The macrocyst life cycle can be studied effectively because methods to isolate the early phagocytic giant cells have been developed in the laboratory of Yanagisawa (217).

There is little doubt that the macrocyst is a sexual structure because of the presence of a synaptonemal complex (183), but recombinant haploid offspring are not usually recovered. In the homothallic species, *Dictyostelium mucoroides*, recombination (though not linkage) has been ob-

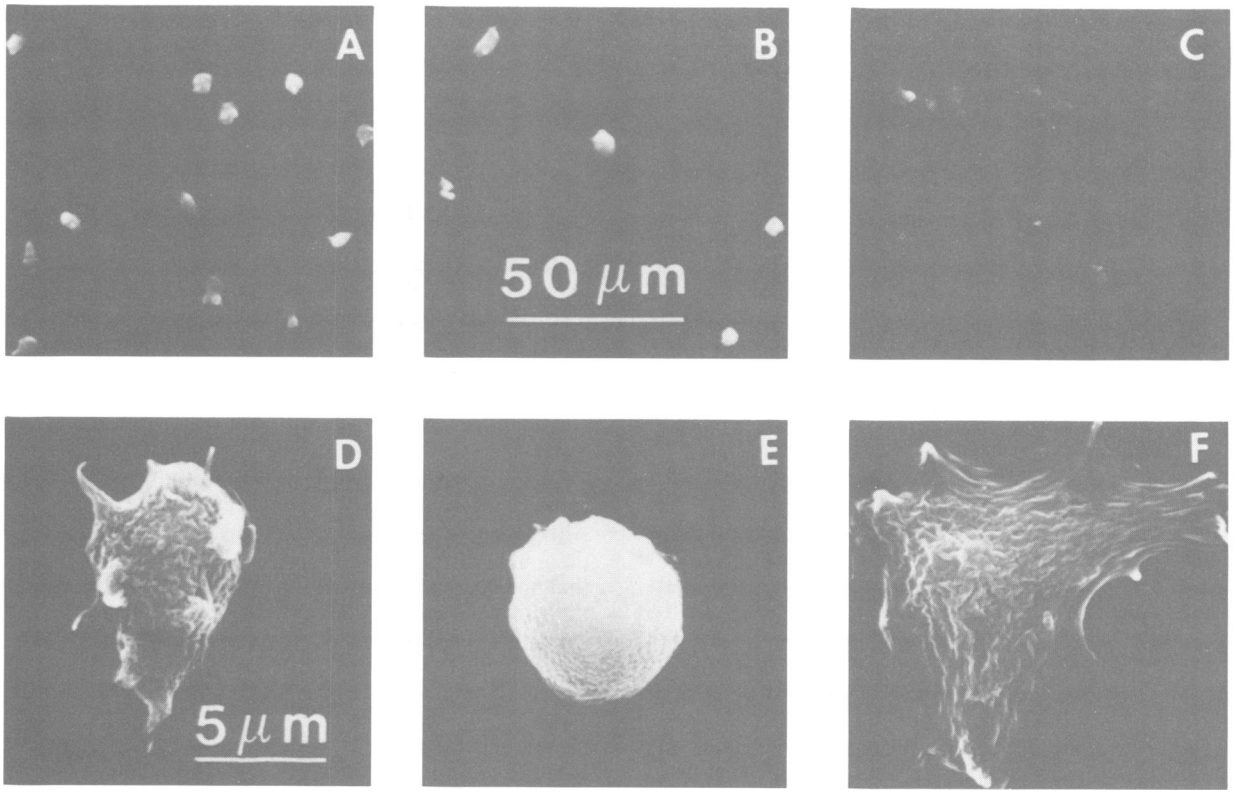

FIG. 2. Developing *Dictyostelium* amoebae before, during, and after a jump in cAMP concentration to 1 μM. See the text for explanation. Courtesy of A. L. Hall, A. Schlein, and J. Condeelis, The Albert Einstein College of Medicine.

served (151), but is difficult to use as a genetic tool. The reasons for the failure of macrocyst germination and the formation of recombinant haploid offspring are obscure, but may result from a mating system for which existing alleles are incompatible. Current evidence supports a one-locus multiple allele mating system (203). Hybrid dysgenesis involving the transposonlike elements known to exist in the genome may operate. Macrocyst formation remains a complicated alternative developmental cycle, not an instrument of genetic analysis.

When studying developmentally regulated genes, one should remember that a cell confronted with starvation does not know which life cycle it will follow and therefore starvation may elicit the activity of genes whose products are synthesized for the path not taken. That the two life cycles share components, notably of the proteins involved in chemotaxis, is clear from studies on phosphodiesterase and phosphodiesterase inhibitor synthesis during macrocyst formation, but these proteins are regulated in ways that do not parallel those used during aggregation (2).

Genetic analysis in *D. discoideum* is carried out by a parasexual system, in which two genetically marked cells (usually temperature-sensitive for growth) fuse at a low frequency and a temperature resistant diploid is selected (117; see references 112, 145, 147, 175, 236 and 261 for reviews). Techniques exist for incorporating unmarked haploids into diploids in which essentially all linkage groups carry genetic markers. Haploidization occurs spontaneously, and its frequency can be increased by treatment with benlate (147). Haploids and homozygous recessive mitotic recombinants are selected with recessive drug resistance markers (147). Mitotic recombination can be used to map markers, and an increasing number of genetic loci have been placed on and within six linkage groups. Markers include the various developmental defects to be described below, drug or metal resistance (147, 261), spore germination, spore color (117), and restriction site polymorphisms (259). *Dictyostelium* amoebae are highly resistant to radiation, and a number of excellent genetic markers that confer sensitivity to ultraviolet or gamma radiation have been isolated (257, 258).

A minimal medium has been developed and auxotrophs have been isolated in axenic strains (84, 85). These include purines, pyrimidines, and vitamins. A thymidylate synthetase mutant has also been recovered (191). Because of the difficulties of working exclusively with axenic media rather than with plaques on lawns of bacteria, the auxotrophic mutations have not been mapped. It would be an advance to grow amoebae clonally on minimal medium agar.

There are seven visible chromosomes, and although they are small, one can detect translocations and aneuploid strains (260). In a recent advance, Welker and Williams have used balanced recessive lethal markers to maintain aneuploid lines (262). While not yet capable of fine-structure analysis, the parasexual genetic system can be used to construct useful strains, to do complementation tests, and to distinguish by mapping between mutants with identical phenotypes.

On a molecular level, *Dictyostelium* amoebae have endogenous plasmids that replicate in the nucleus and themselves contain developmentally regulated genes (165, 181, 184). By fusing a promoter for a *Dictyostelium* actin gene to the Tn*5* gene coding for neomycin resistance, it has been possible to develop a transformation vector. Cells carrying the vector can be selected by the drug G418 (172, 173). By using the replicons of the endogenous plasmids, a nuclear vector that

replicates extrachromosomally has been constructed (81). Introduction and selection of DNA are remarkably similar to the methods used for transfection of mammalian cells. Recent experiments by De Lozanne and Spudich with a cloned myosin gene have detected insertion of the transforming plasmid into the homologous nuclear gene (56). The frequency of this integration event was about 15% of the total transformants. Introduction of a truncated myosin gene into its wild-type homolog led to a phenotype in which karyokinesis but not cytokinesis was normal. The result was a large multinucleate cell still capable of some forms of motility. What remains to be determined is the frequency with which integration by homologous recombination occurs in genes that are not essential for cell division. Transformation with the alpha-actinin gene has also produced a surprising result. Transformants frequently had the endogenous alpha-actinin gene destroyed, without integration of the exogenous DNA (W. Witke, W. Nellen, and A. Noegel, EMBO J., in press). If they prove to be common events, integration by homologous recombination or gene destruction without integration, techniques previously available only to those working on procaryotic organisms or yeasts, would change the dimensions of the genetic questions that could be examined with *Dictyostelium* spp.

The transformation system has led to another useful finding: that antimessenger constructions work well in *D. discoideum*. An antimessenger construction of the myosin gene reduced the production of myosin heavy chains several thousandfold and led to a phenotype exactly like that of the strain carrying an interrupted myosin gene (133). The authors chose an actin promoter that is active when the cells grow in axenic media or are going through development but is inactive when the cells consume bacteria. Therefore, myosin can be eliminated under one set of conditions but the cells can be propagated easily with normal myosin content under other conditions. Other antimessenger constructs have also been successful, notably one that eliminates the synthesis of the *Dictyostelium* lectin discoidin, a molecule involved in cell-to-substrate adhesion (48, 232). The details of the mechanism of antisense inhibition are unclear, but it appears that double-stranded ribonucleic acid (RNA) is formed and degraded (171). Attempts have been made to incorporate antisense constructions of the *ras* gene into the genome, but these have never been recovered, which may signify that *ras* provides a necessary function in growing cells (202).

The amoebae contain transposons of interesting composition that have been characterized in the laboratories of H. Lodish and R. Firtel. Two elements studied by the latter group are called Tdd2 and Tdd3 and have undergone separate rearrangements in two laboratory strains with a common ancestor (195). Neither Tdd2 nor Tdd3 has direct or inverted repeats. Tdd3 causes 9- to 10-base-pair duplications at the point of insertion. The two elements share a 22-base-pair homology near one end, and in two instances Tdd3 has inserted into Tdd2 at this homologous sequence. In addition to these repeated sequences, the two laboratories have independently isolated an element (Tdd1 or DIRS-1) which has inverted repeats at its termini and an internal sequence of 4.1 kilobases (kb) (35, 207). This structure is unusual in that the repeats are 330 base pairs long and contain heat shock promoters which initiate bidirectional transcription. The promoters may be activated by heat shock or when the cells are plated for development at high density. The *Dictyostelium* heat shock promoters are homologous to those present in the *Drosophila melanogaster* heat shock genes and function well in yeasts. These transposons, which are present in 40 to 50 copies per nucleus, have shifted their positions in the laboratory life of the strains, but no method has been found to determine whether the endogenous retrotransposon sequences have mutagenic properties.

MUTANTS ALTERED IN AGGREGATION

Before a discussion of the available mutants, the reader may find it useful to know how many genes are likely to be required for aggregation, into what general categories the mutants fall, and how to select desired classes. The mutations of *D. discoideum* that affect early development include those that block aggregation, those that develop more rapidly than the wild type, and those that aggregate aberrantly or when they should not. Mutations that block aggregation fall into a number of complementation groups, while the rapid development and aberrant mutants are less common. Although aggregation mutants have been studied for more than 30 years (176, 233, 234, 237, 272), it is usually the case that one does not know what is wrong with them, except that they are affected in a critical step. Certain questions can be asked without knowing the nature of the defects.

Number of Genes

One of the interesting problems that can be addressed by using *D. discoideum* but not most other eucaryotic developmental lineages concerns the number of genes that are not necessary for growth but whose activity is required to pass from a proliferating cell to a defined stage in development. The strict separation of growth and development and the availability of the parasexual system of genetic analysis makes an approach to this question possible. Genetic experiments suggest that the number of genes required for aggregation, including the substantial part played by signal reception and cAMP emission during relay, is limited. Several laboratories have made collections of aggregateless mutants in genetically compatible strains (41, 42, 267) and used complementation testing to estimate the genetic complexity of aggregation. The reasoning was that, if a large number of genes is critical to aggregation, then it would be unlikely to have two independently isolated recessive mutations fall into the same complementation group. Diploid strains bearing two complementing mutations aggregate and sporulate normally, making detection of noncomplementing diploids a simple matter. Two groups (41, 267) found that independently isolated mutations fell into the same complementation groups at a frequency that would be expected if 40 to 50 genes were essential for development to the aggregation stage. Using *Polysphondylium violaceum*, a species with similar aggregation properties, Warren et al. (255, 256) also concluded that aggregation required 40 to 50 genes. By comparing the frequency of mutation in specific genes with that in genes that affect aggregation, Loomis (142) concluded that as many as 100 genes could be involved in aggregation and 300 in all of development.

A genetic regulatory system might exist that confounds standard complementation analysis. Gene or functional redundancy would affect the results, as would mutations that cause lethal phenotypes only during aggregation. Dominant mutations, which cannot be used in complementation tests, are rare. The most important caveat is that genes that are useful but not essential are not scored by complementation analysis. Several examples will be described below. Hybrid-

ization studies have been used to estimate the number of new transcripts made during aggregation and later development. The two types of study are not directly comparable because the endpoints of the analysis were different: geneticists studied cells that have not reached aggregation, while molecular biologists studied messenger RNA (mRNA) from populations that had. When mRNA from cells that had been starving for 6 h but had not yet aggregated was used for hybridization analysis, no increase in the proportion of the genome that was transcribed was detected (20). By this time all of the genes required for aggregation should have been induced. Major changes in transcripts and polypeptides occur after the cells form tightly adhesive aggregates of cells (20, 125).

The genetic estimates of the number of aggregation genes are useful for two reasons. First, one can infer that a specific defect, such as a loss of adenylate cyclase, should be recovered from a reasonable collection of aggregateless or temperature-sensitive aggregateless mutants. The genetic estimates also help to define the magnitude of the problem, which is to unravel the molecular circuitry controlling the steps of *Dictyostelium* development. To determine the interactions of 50 or more genes would be a formidable task, comparable to a complete molecular description of a large bacteriophage. The problem becomes manageable only if development can be divided into short periods during which the functions and relations of a smaller number of genes can be studied. Analysis of development is also simplified if genes can be sorted into groups on the basis of their patterns of regulation.

Selection Procedures

The thorough description of aggregation and the clever application of selection techniques is leading to a situation in which workers can recover mutants defective in specific processes and are not compelled to sort through colonies that are interesting only because they fail to aggregate. For example, it is possible to determine which of the subprocesses that make up aggregation (chemotaxis, cAMP stimulation of effector molecules, cAMP secretion, or adhesion) is affected. A strategy for screening mutants presented in the form of a flow chart has been proposed by Devreotes et al. (57). Selection techniques for specific classes of mutant are also evolving.

One technique takes advantage of the fact that amoebae do not aggregate in the presence of constant high levels of cAMP because the cAMP receptor on the surface of aggregating *D. discoideum* cells adapts and uncouples from at least one of its effector enzymes, adenylate cyclase (60, 70, 129). Adaptation is important because it delays the expression of certain genes until conditions for aggregation are more propitious. Even if there were no adaptation, chemotaxis would be impossible if external cAMP levels were stable and continuously above the K_d of the receptor. Mutants that aggregate in the presence of cAMP, presumably because they evade the effects of adaptation, have been isolated by Gerisch and his colleagues (95, 210, 254) who used cAMPS, an analog of cAMP which binds well to the cell surface receptor but is hydrolyzed slowly by the *D. discoideum* cyclic nucleotide phosphodiesterase (212). Most cAMPS-resistant mutants regain their normal morphology when cAMPS is removed, but one strain makes small fruiting bodies with or without cAMPS. The cAMP relay system is no longer used to collect amoebae from a large area, forcing the cells to find each other by random collision. The mutation *casA1000* in this strain has been mapped to linkage group II (254). Some wild-type strains aggregate normally in the presence of cAMPS (95), as do the rapid development (*rde*) mutants described below. It is a reasonable assumption that what has been altered in the cAMPS-resistant mutants is a fundamental aspect of signal transduction, probably bearing on the mechanism of cAMP receptor adaptation.

Exogenous cAMP acts as a regulator of gene activity both before and after aggregation. There is no evidence that cAMP enters the cells in effective concentrations, but there is good pharmacological data suggesting that cAMP works through its cell surface receptor during aggregation (157, 241) and during postaggregation development (102, 105, 186). Therefore, the gene-inductive effects of cAMP must be mediated by second messenger systems. What the second messenger cascades are and how they act are central questions in examining any cell that develops in response to external cues. Genetic examination of signal transduction pathways requires a method to select mutants that may be defective in signal reception or a subsequent cascade. After aggregation, when the cells have become tightly adherent, many new transcripts and proteins appear, but when the aggregated cells are returned to nutrient medium, or even one with buffered dextrose, they lose their new transcripts (78). The abrupt loss of developmental messengers is called erasure (229). Exogenous cAMP holds the cells in development despite the presence of nutrient medium. cAMP stabilizes the postaggregation transcripts and blocks cell division. Berger and Dulaney (15) isolated mutants that escape the inhibitory effects of cAMP in nutrient media. After several cycles of starvation and dedifferentiation in the presence of cAMP, five mutant phenotypes were found. One of these cannot aggregate; three are blocked at aggregation and one is blocked at later stages. One of the virtues of these mutants is that they are spontaneous and have not been ravaged by nitrosoguanidine, a serious problem in an organism in which no way exists to make isogenic strains. The loss of a component of a second messenger cascade would explain the escape and the failure to complete development in subsequent developmental cycles. No cascade essential for growth could be affected.

Mutants that fail in chemotaxis have been isolated by Barkley and Henderson (9), who developed a method based on the Boyden assay for chemotaxis, in which cells were placed on a series of filters over a reservoir of cAMP. Wild-type cells move into the filters in search of cyclic AMP, while those unable to respond chemotactically to cAMP moved in a random walk pattern and remained on the upper filter. Some mutants were temperature sensitive and may include receptor- or motility-defective strains. One mutant, HB3, is defective in the cAMP receptor. When cAMP binding was analyzed, the mutant membranes appeared to have lost a low-affinity cAMP-binding site and to have gained high-affinity (25 nM) sites. This mutation disturbs but does not block aggregation, which occurs without formation of streams and requires a higher than normal cell density (10).

Segall et al. (222) used an identical selection scheme, but, instead of cAMP for the chemoattractant of developing cells, they used folate, which attracts growing cells (187). After achieving dramatic enrichment for mutants incapable of chemotaxis toward folate, the survivors were examined. One lacked the enzyme folate deaminase, others were defective in motility, and a third interesting class failed to migrate toward folate and could not aggregate. Inability to

aggregate and a defect in a growth specific function might seem contradictory, but it is known that, at an early stage of development, declining numbers of folate receptors coexist on the cell surface with increasing numbers of cAMP receptors (62, 246). At this time, the cyclic nucleotide phosphodiesterase, which is normally induced by cAMP, can be induced by folate. Several authors have postulated a common pathway for folate and cAMP transmembrane signalling, such as a second messenger cascade (17, 252). A defect in such a second messenger system would block chemotactic responses to folate as well as later developmental events and may offer a genetic entry into regulation of early development. The absence of the phosphodiesterase would cause an aggregateless phenotype (see below). These as well as other mutants must undergo cosegregation or reversion studies to demonstrate that the failure to move toward folate and the inability to aggregate result from the same lesion.

For studies of proteins important to motility in *D. discoideum*, Clarke (39) forced mutagenized amoebae to grow at the restrictive temperature (27°C) on bacteria that had been preloaded with bromodeoxyuridine. Those amoebae that were capable of phagocytosis incorporated the bromodeoxyuridine and were killed by irradiation with near-ultraviolet light. Survivors were enriched for temperature-sensitive growth mutants, and a number of these had defects in motility. Reversion to temperature resistance was accompanied by a restoration of motility. Curiously, one of the mutants overproduces discoidin (19), but this does not seem to be connected with reduced motility.

A few mutants express a bizarre phenotype that is unique to *D. discoideum*: they develop too fast. The mutant, FR17, aggregates and develops in such a way that all morphogenetic and biochemical markers are telescoped (1, 119, 231). By taking advantage of the fact that spores are formed 16 h after the onset of starvation rather than the normal 22 to 24 h, it was possible to heat mutagenized populations and kill all cells that were not quick to form spores (119). The mutations recovered fall into several complementation groups (1, 119) and are discussed below.

During development, the pattern of glycosylation of developmentally regulated glycoproteins changes (110). Taking advantage of the fact that a late step in oligosaccharide maturation is sulfation (89, 107), Boose and Henderson (25) developed a 35S suicide selection from which they recovered 99 mutants temperature sensitive for sulfur transport or incorporation. Seventy-four were unable to aggregate at restrictive temperature; of these, 29 were blocked at aggregation and 45 were blocked later. Mutations that affect the processing of lysosomal glycoproteins have also been isolated by the group of Dimond (68) and may overlap those of Boose and Henderson.

The potential mutant selection and screening procedures have not been exhausted. Sussman (239) has proposed using coculture with strains of *E. coli* that are only able to grow in the presence of cAMP produced by aggregating slime mold cells as a way to detect amoebae that are defective in cAMP secretion. Adenine auxotrophs exist (85) and can use cAMP as an adenine source, which requires a cAMP phosphodiesterase. Screening procedures have been attempted in which amoebae capable of growing on 5′-AMP but not cAMP as an adenine source in minimal medium were recovered. Instead of being defective in the phosphodiesterase, which was the expected phenotype, the cells have developed a new and interesting phenotype that makes their growth sensitive to cAMP (J. Franke and R. H. Kessin, unpublished experiments). One explanation may be that the cAMP receptor, normally present only during development, may have been dislodged from the genetic program and now appears during growth. Experiments described above showed that the cAMP receptor may use the same second messenger cascade as the folate receptor, and therefore the effect of cAMP could be transmitted to the nucleus of growing cells carrying an aberrantly expressed receptor.

Mutations That Affect Early Functions

Although the period of aggregation is relatively simple compared to the rest of development, it is, if the estimates derived from complementation analysis are accepted, complex enough to require subdivision. One interesting period of slime mold development is the time between starvation initiation and the appearance of the capability to move by chemotaxis toward cAMP. The mechanism by which the cells sense starvation is unknown, except that deprivation of amino acids is required (159). No particular amino acid acts as a trigger. Aside from a number of amino acids and a carbon source, the cells require only a few vitamins (including folate) and some minerals (84). After a few hours of starvation, the cells become capable of chemotaxis toward sources of cAMP. Such cells have acquired the ability to bind cAMP to the cell surface receptors, to transduce a signal leading to directed motility, and to hydrolyze cAMP. One way to screen for mutants that are blocked early in the developmental program is to take advantage of the observation that 95% of aggregateless mutants can be made to form aggregates if they are presented with pulses of cAMP (47, 51, 141). One product that mutants rescued by artificially supplied pulses of cAMP must have is the cyclic nucleotide phosphodiesterase, because in the absence of this enzyme periodic additions of cAMP appear to the cells as stepped increases and not as individual signals. The consequence of stepped increases in cAMP concentration is that adenylate cyclase activation and cAMP relay are extinguished by adaptation and aggregation halts until cAMP no longer saturates the receptors. Cells can resume development after many hours or even days of paralysis by excess cAMP. It is not simply that the excess of cAMP blocks the abilities of the cells to find their way because gradients are undetectable over the high background: constant levels of cAMP suppress the induction of a variety of genes whose products are essential for the progression of development.

The frequency of mutants not rescued by cAMP pulses was 1 of 15 in one study (51). As expected, the strains that could be rescued by cAMP made phosphodiesterase. A larger analysis of 218 mutants (47) produced 11 "frigid" mutants that formed five complementation groups. Mutants from three groups appeared to be blocked at a very early stage of development, because even after hours of starvation they failed to move toward cAMP but retained their ability to move chemotactically toward folate. Strains carrying mutations that blocked development at an early stage but were weakly chemotactic toward cAMP made up two additional complementation groups. The collection of frigid mutants should be enriched for defects in phosphodiesterase induction. Analysis of strains with mutations in the frigid A, C, D, and E complementation groups showed that they make little or no phosphodiesterase mRNA under normal developmental conditions (87).

If 50 genes are required for aggregation and fewer than 1 in 10 of these blocks aggregation at a very early stage, the number of genes required to pass from a growing cell to a state where at least the phosphodiesterase gene can be

activated is small. Under our culture conditions the first appearance of phosphodiesterase mRNA occurs within 30 min of the beginning of starvation. An interval lasting from the time starvation begins to the time when phosphodiesterase mRNA first appears defines a simple developmental sequence involving relatively few genes whose relationships may be unraveled.

There are other indications that the genetic complexity of early aggregation is low. Biochemical efforts to define the number of proteins required for very early development have used two-dimensional gel electrophoresis after labeling of cells with [^{35}S]methionine to ask how many new proteins are made. Before aggregation the number was limited (8, 36, 79, 146), and immediately after the beginning of starvation two labeled proteins (158) were found. There are problems with a two-dimensional gel analysis of proteins newly made during a developmental process. First, the technique may not detect mRNAs or proteins that are not abundant, and second, there does not appear to be a consensus within the *Dictyostelium* studies about which proteins are changing. More important, the technique does not measure those proteins that are made by growing cells but which are dedicated to the early stages of the developmental program. Experience with the development of *Bacillus subtilis* suggests that gene products necessary for early steps in development exist in the growing cell. During the sporulation of *B. subtilis*, *spo0* gene products present in growing bacilli are necessary for the initial steps of endospore formation (see reference 149 for a review). In fact, mutants that lack these proteins have a growth advantage. The presence of transactivation factors necessary for a developmental process, but present before it begins, has also been noted in higher eucaryotic cells (see reference 156 for a review). Development-specific genes activated during growth would be readily detected by genetic means but would be missed by methods based on hybridization or two-dimensional gel analysis.

Another of the early events of *Dictyostelium* development is the regulated biosynthesis of discoidin (227), a lectin that bears some resemblance to fibronectin and has a role in maintaining the adhesion of aggregating cells to the substratum (48, 232). Discoidin is apparently not necessary for aggregation, but because of its induction and the cloning of the several discoidin genes (194), a detailed description of the molecule and its regulation is available. Alexander and colleagues (6, 7) have isolated mutants in two complementation groups, *disA* and *disB*, which prevent the induction of the discoidin gene family. A previous study by Shinnick and Lerner (226) reported a mutation in a discoidin gene that abolished cell adhesion. This is in conflict with later results and with what we now know about discoidin. Since the mutation has been lost, there is no way to rationalize the conflicting results. A suppressor of *disA*, called *drsA*, relieves the inhibition of discoidin expression caused by the absence of the *disA* gene product. An unexpected effect of the *drsA* mutation is to cause the expression of the discoidin genes during growth. Since the appearance of discoidin occurs rapidly after starvation, *disA*, *disB*, and *drsA* must be in genes that act within hours after the beginning of starvation. One can infer that the discoidin mutants do not block the appearance of phosphodiesterase because cells carrying *disA*, *disB*, or *drsA* still aggregate, which they could not do if they lacked phosphodiesterase. Starvation must therefore unleash several parallel inductive pathways. The discoidin studies are among the first to use suppressor mutants to study *Dictyostelium* development.

Mutations That Affect Later Events during Aggregation

Signal transduction and relay are crucial events during *Dictyostelium* aggregation. For the reasons described above, the majority of mutations affect later stages of aggregation when the signal transduction mechanism has become fully established and the lesions probably cause defects in cAMP synthesis and release. Signal transduction can be studied by methods unique to this organism. When amoebae starved for a number of hours are given a pulse of nanomolar cAMP, they respond by changing their shape and light-scattering properties. These changes can be followed in an oxygenated suspension of cells in a spectrophotometer (57, 95, 206) and correlated with a rapid intracellular rise in cyclic guanosine 3′,5′-phosphate (cGMP) (15 s) and a slower but greater increase in intracellular cAMP (60 s). Other rapid responses to cAMP include calcium influx (32, 264), proton export (155), actin polymerization (75, 177), myosin phosphorylation (16), and phosphoinositol accumulation (77).

Wurster and colleagues have analyzed one mutant and found that, while cGMP synthesis rose in response to a pulse of cAMP in the mutant agip 53, the normal cAMP response did not occur (269). Nevertheless, after a series of artificial pulses the mutant cells became adhesive. This finding has been used as evidence that, while cAMP may be an important primary messenger, it is not necessary as a second messenger for aggregation. The same group has found that cells of the mutant agip 43 are incapable of synthesizing cAMP when starving in buffer but nevertheless undergo spontaneous and synchronous oscillation, which indicates a communication system in addition to cAMP (271). In wild-type cells oscillations in extracellular calcium levels have been observed that are coordinated with cAMP changes (32).

One of the problems that has prevented the use of aggregation-defective mutants to study signal transduction is that one of the events in which blocks occur, cAMP-induced synthesis of cAMP, could not be assayed in broken cell preparations. This problem has recently been overcome (240). If guanosine 5′-triphosphate is added within seconds after rupture of the cells, activation of adenylate cyclase by cAMP is stabilized. Several mutants have been analyzed and one, synag 7, has a defect in cAMP-induced cAMP synthesis. Cytosolic proteins from wild-type cells restore cAMP-induced adenylate cyclase activity in the mutant. The important fact is that, without the mutant, the complementing soluble activity could not have been detected. In the purification of the soluble factor it is the mutant cells that will provide the basis of an assay. Because of the accessibility of the receptor-adenylate cyclase complex to mutational study (the cells grow without receptor or adenylate cyclase), *D. discoideum* provides a good model for studies of receptor-mediated adenylate cyclase action.

For the later aggregation functions having to do with cellular adhesion, several interesting mutations exist, including variants isolated by Ishida (109, 110). One mutant of *D. mucoroides* produces cells that adhere to each other normally, except at the restrictive temperature. Even when the cells have reached the aggregation stage, the temperature shift causes dissociation and loss of multicellularity. A second mutation isolated by Ishida in *D. discoideum* can form stalk and spore cells in liquid roller culture, which the wild type cannot do. There has been one study of microscopic development of membranes during development. Using freeze-fracture techniques, Gregg and Yu have studied the membranes of mutants blocked at unspecified stages of aggregation. In some of the mutants, large intramembra-

nous particles that normally accumulate during development do not appear (104).

Mutations That Affect Many Events

Rapid-development mutations. In contrast to mutations that block aggregation are those that accelerate it (1, 119, 231). To my knowledge, the Rde phenotype is unique to *D. discoideum*. A possible analog in a mammalian cell stems from the introduction of the oncogene *myc* into Friend erythroleukemia cells and the subsequent acceleration of their dimethyl sulfoxide-induced differentiation (136). Any model to explain coordinate gene regulation during *Dictyostelium* development must include a mechanism by which a single *rde* mutation can accelerate the expression of many genes without changing the order of their expression (3). The first rapid-development mutant made spores in 16 h rather than the standard 24 h (231). All of the steps of development were telescoped such that marker enzymes that appeared early were made even more rapidly by the mutant FR17. Enzymes (and now transcripts [219]) that appear after aggregation were also induced earlier than in the wild type. The FR17 cells carry the *rdeA* allele and secrete cAMP in an oscillatory manner beginning soon after starvation (46); they aggregate but never form streams. The aggregates make chaotic fruiting structures with multiple tips, are unable to form migrating slugs, and behave as if their cAMP production is wildly abnormal, which it is (4, 46, 119). The disruption of the normal ability to make a slug with a front and a back is consistent with the idea that sensitivity to cAMP, first elaborated during aggregation, plays an important role during later development. In the analysis of FR17, the supposition has been that an event fundamental to all steps of *Dictyostelium* development is deranged. An intracellular cAMP-binding protein other than the one associated with the protein kinase has been detected (244), and while it may be altered in the V12 strain, a wild-type isolate which develops several hours faster than the standard NC4 strain, it appears to be normal in the FR17 strain (243).

The *rdeA* allele is located on linkage group IV and causes all of development to be telescoped (119). Cell contact is normally necessary for cellular differentiation, but in rapid-development strains this obligation has been alleviated (28). The *rdeA* mutants resemble the cAMPS resistant strains because they aggregate in the presence of cAMPS (210). That they aggregate in the presence of cAMPS means that the adaptation that blocks aggregation when cAMP is in excess in wild-type cells has been evaded in strains carrying the *rdeA* mutation, as it has been in strains carrying the *casA1000* allele (254).

The *rdeC* mutation, located on linkage group III, leads to a defective fruiting structure but appears to speed development only after aggregation (1, 4). In contrast to *rdeA* mutants, little cAMP is produced at any stage. Whether or not the *rdeC* strain can aggregate in the presence of a large amount of cAMP is not known. A strain carrying both mutations has not been made. A mutation in strain HIfm-1 that accelerates an early stage of aggregation has been described (230).

Bypass mutations. The proteins induced during development have a precise order of appearance. Gerisch and his colleagues have used a strain that is resistant to the inhibitory effects of cAMPS and introduced a second mutation that causes premature expression of certain genes, including adenylate cyclase and the contact sites A (csA) glycoprotein, during stationary phase (95). These gene products usually appear only during aggregation. The second mutation does not cause developmentally regulated genes to be expressed in log phase, and therefore the mutant (and presumably wild-type) cells undergo an event critical to the regulation of developmental genes as they pass from mid-log to stationary phase. The mutant HG592 expresses enough of the developmental program that it becomes adhesive, and aggregates form while the cells are still in the spent growth medium. Wild-type cells do not aggregate under these conditions. In addition to expression during stationary phase, the genes are reexpressed at the correct time during aggregation. The second mutation has not yet been genetically separated from the *casA1000* mutation, so it is not clear that the property of premature expression depends on *casA1000* and a second mutation. An inference that can be drawn from these "bypass" mutations is that, if the time of expression of two genes is advanced by a single mutation, then their regulation has something in common. The premature expression caused by the mutation in strain HG592 resembles that produced by the *drsA* allele characterized by Alexander et al. (6).

Mutants Defective in Known Functions

The majority of the mutations that disrupt *Dictyostelium* aggregation can be sorted into groups that affect subprocesses such as chemotaxis, motility, or adhesion. A few have been detected in proteins and glycoproteins with specific functions.

Cyclic nucleotide phosphodiesterase. Brachet, Darmon, Barra, and their colleagues isolated mutants that do not respond to pulses of cAMP and produce very little phosphodiesterase (14, 27, 49, 54). Two of the mutations did not complement. When the ability to hydrolyze extracellular cAMP was restored by addition of phosphodiesterase, aggregation and other events in development were normal until a late stage in fruiting-body construction (49). Whether the failure at this time was due to the absence of phosphodiesterase or to a secondary mutation is not known. These mutants provide the best evidence that the phosphodiesterase is essential to *Dictyostelium* aggregation and that its absence causes an aggregateless phenotype. Recent experiments by M. Faure in our laboratory have restored aggregation to these mutants by transformation with the genomic phosphodiesterase gene.

Cyclic nucleotide phosphodiesterase and the phosphodiesterase inhibitory glycoprotein have been purified and characterized in our laboratory (86, 87, 121, 166, 185, 193, 211, 225). A gene which codes for the inhibitor has been recovered (J. Franke and L. Wu, unpublished experiments), and the cysteine-rich sequence of this molecule should soon be known. The gene that codes for the phosphodiesterase has been cloned and sequenced (137, 192). One phosphodiesterase gene codes for a membrane-bound and an extracellular enzyme. We do not yet know the mechanism by which the membrane-bound phosphodiesterase is attached to the cell surface or how it is released. What is known about the enzyme and its interaction with a specific secreted inhibitory glycoprotein is summarized in Fig. 3. The glycoprotein inhibitor of the phosphodiesterase is a heat-stable molecule with a high cysteine content and is synthesized by cells when no cAMP is detected in the medium. The secreted inhibitor inactivates extracellular phosphodiesterase by binding to it and changing the K_m of the enzyme for cAMP from 5 μM to 5 mM. When RNA extracted from the phosphodiesterase mutants was examined, very little mRNA was detected

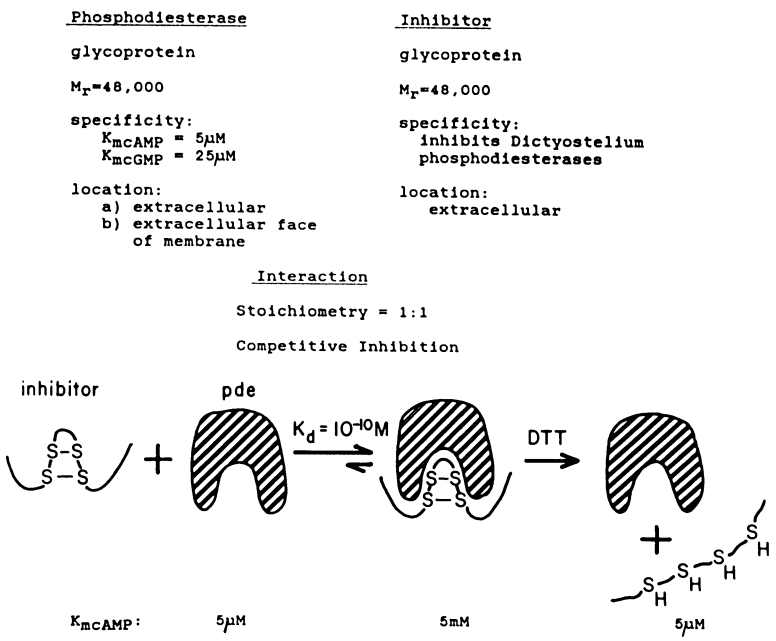

FIG. 3. Biochemical properties of the cyclic nucleotide phosphodiesterase (pde) and its glycoprotein inhibitor. See the text for details. DTT, Dithiothreitol.

during growth or development, but if the cells were pulsed with cAMP to induce the mRNA and enzyme, a very small amount of the developmentally regulated cyclic nucleotide phosphodiesterase mRNA appeared. Although the mRNA was only a fraction as abundant as in the wild type, it had the same size, 2.2 kb, as is present in normally developing cells. mRNA coding for the phosphodiesterase in growing cells has a size of 1.8 kb, is much less abundant, and could not be detected in these mutants (87). These results can be interpreted in terms of a mutation at the phosphodiesterase locus that affects transcription during vegetative growth and development, but does not completely eliminate recognition of the phosphodiesterase gene by a component of a cAMP-induced second messenger system. The behavior of the mutants could also be explained by the absence of a trans-activating factor that affects no other crucial gene. This is made somewhat less likely by the discovery that the phosphodiesterase genomic sequence can be used to cure the aggregateless phenotype of the *pdsA* mutation (M. Faure et al., unpublished experiments). Because of the paucity of mutations that block the appearance of the phosphodiesterase, it is likely that, between the starvation signal which initiates development and the first induction of the phosphodiesterase, the expression of relatively few genes is required. That at least one new protein is necessary for transcription is known from the effects of cycloheximide, which blocks appearance of the 2.2-kb phosphodiesterase mRNA (87). New proteins are likely to include a transcriptional factor because a new promoter is used to synthesize the development specific 2.2-kb mRNA. Whether or not this function is coded by one of the genes identified by the frigid mutations is not yet known.

The structural gene that codes for the phosphodiesterase has been cloned and contains a promoter that directs transcription of a 1.8-kb mRNA as well as 5′ exons which are spliced during development to form a 2.2-kb mRNA (G. J. Podgorski et al., manuscript in preparation). The 2.2-kb mRNA is transcribed from a stronger promoter which is far away from the structural gene and has not yet been cloned. The structural gene and 2.2 kb of 5′ flanking sequence have been inserted into wild-type amoebae by cotransformation with a G418 resistance plasmid (M. Faure et al., manuscript in preparation). The transformants have 150 to 200 additional phosphodiesterase genes and 160 times the normal amount of enzyme. The constitutive 1.8-kb mRNA (but not the 2.2-kb mRNA) is made in excess, indicating that the promoter of the 2.2-kb mRNA is not contained in 2.2 kb of 5′ sequence. One short additional exon is contained in the 2.2 kb of 5′ flanking sequence. This structure makes the phosphodiesterase gene the most complex *Dictyostelium* gene yet studied and the only one with a large developmentally regulated splice.

The effects of the excess phosphodiesterase synthesized by the transformants during growth and development are dramatic. Growth is not blocked but aggregation and development are deranged. In liquid culture, aggregation is more rapid than normal. In strains that carry a large number of copies of the phosphodiesterase gene, no fruiting bodies are produced and the cells form no slugs and are unable to create a tissue with a front and a back, which is consistent with a role for the enzyme in pattern formation during postaggregation development (120, 220). Phosphodiesterase production continues long after aggregation of the transformants. When cells from normal slugs were separated into prestalk and prespore cells, only the prestalk cells contained detectable levels of phosphodiesterase mRNA (87). Rutherford et al. (214, 215) have found the phosphodiesterase activity to be localized in prestalk cells.

csA(gp80). When cells have been starving for 8 to 10 h, they become adhesive in a manner that is resistant to ethylenediaminetetraacetic acid. Fab fragments directed against an 80,000-dalton glycoprotein (gp80) blocked this type of adhesion, which led to the thought that the glycoprotein antigen was a cell adhesion molecule (18, 92, 93, 167). A mutation that blocks the appearance of csA(gp80) has been isolated (180) in a strain carrying the cAMPS-

resistant and bypass mutations described above. It was the expression of the csA antigen on single cells rather than on clumps of adhering cells that made selection of adhesion mutants by a combination of fluorescence-activated cell sorting and colony immunoblotting possible. Using a clone of the gene coding for gp80, Noegel et al. (179) demonstrated that there is only one csA gene and that its transcript is missing in the mutant. As expected, ethylenediaminetetraacetic acid-stable contacts between cells are abolished in the strain carrying the defective csA(gp80) allele. These results are consistent with those of Loomis and colleagues, who studied the effects of the *modB* mutation on gp80. This mutation prevents one variety of glycosylation of gp80 and other glycoproteins without affecting the intracellular targeting of the molecule (100, 148, 169, 263). In strains carrying *modB* or csA mutations, production of gp80 is reduced and ethylenediaminetetraacetic acid-stable contacts are eliminated, but aggregation proceeds. The csA determinant appears as a membrane protein of lower molecular mass in strains carrying *modB* mutations. Knecht et al. have recently identified a 24-kilodalton polypeptide which may be involved in early cell adhesion (132).

One result of the investigations on csA has been to confound the search for a *Dictyostelium* cell adhesion molecule because the cells that lack gp80 and its transcript adhere and aggregate. This finding does not necessarily eliminate the participation of gp80 in the adhesion process: one can postulate a backup system or one which can function without one of its components. Luna and her colleagues have isolated a membrane fraction enriched in cell contact sites and find that gp80 is preferentially associated with these points of membrane contact (108). That the csA glycoprotein does not participate in cellular adhesion in as exclusive a way as originally hoped should not detract from the fact that it is the best-characterized example of a class of genes that is inducible by pulses of cAMP, but whose induction is completely blocked by saturating amounts of the nucleotide (see below).

ras. *Dictyostelium* contains a single copy of a *ras* gene that is homologous to its mammalian and yeast counterparts (200) and like the mammalian gene contains an important glycine residue at position 12 (201). The mRNA disappears during aggregation (200) but the protein remains (200). The gene, which contains three short introns, has been sequenced, and its complementary DNA has been altered in an attempt to examine the phenotype of the cells after transformation. Alteration of a Gly-12 to Thr-12 within *ras* changes the activity of the protein. Transformation with the altered construct slows growth and affects the aggregation and development of the amoebae, producing aggregates with multiple tips (201). Aggregation streams in submerged culture were blocked by the alteration to Thr-12. Overproduction of a *ras* molecule which contains the wild-type amino acid Gly-12 had no effect. No effect of the threonine substitution on adenylate cyclase activation or adaptation could be detected. There appears to be no effect on the activation of guanylate cyclase in response to a cAMP signal, but there is a somewhat stronger desensitization of the guanylate cyclase (248). It is likely that *ras* plays an important role in signal transduction.

cGMP phosphodiesterase. The cytosol of starving *Dictyostelium* amoebae contains a cGMP-specific phosphodiesterase (45, 64) whose role is the degradation of the cGMP produced within seconds after the cell surface cAMP receptor binds cAMP. The laboratory of Newell (208, 209) has characterized mutants called streamers which have long periods of cell movement. It was subsequently discovered that there is little cGMP phosphodiesterase activity in the mutant strains and that cGMP levels remain high after a cAMP stimulus (139, 209, 251). Stimulation of the growing cells with folic acid also leads to prolonged increases in intracellular cGMP levels (251). The *stmF* locus has been mapped to LG II by Coukell and Cameron (43), who have also isolated revertants and suppressors (44). These results indicate that a normal aggregation phenotype can be restored in some revertants without affecting the elevated levels of cGMP and that *stmF* is likely to mark the cGMP phosphodiesterase structural gene. Purification of the enzyme has proved difficult. The cGMP phosphodiesterase gene is an example of a locus that is useful but not essential for aggregation and would not have been scored by complementation analysis.

Other developmentally regulated proteins. *D. discoideum* cells synthesize and secrete a number of glycosidases shortly after the initiation of starvation. These enzymes are present at low levels in cells growing on bacteria but increase dramatically in activity after the beginning of starvation (66). Using a screening procedure based on the clonal growth of single mutagenized amoebae (30), Loomis and colleagues were able to recover mutations in glycosidase genes including *N*-acetylglucosaminidase (65, 143), alpha-mannosidase (88), and beta-glucosidase (69). Strains defective in alkaline phosphatase have also been isolated (152), as has a specific alpha-glucosidase mutation (90). In combination with histochemical techniques, these mutations have provided excellent genetic markers. None of these mutations blocked development. *nagC*, a locus that affects synthesis of *N*-acetylglucosaminidase and no other glycosidase, has been defined by Judelson et al. (115). Second-site mutations that block alpha-mannosidase synthesis and aggregation have been isolated (140). A mutation of the UDPG-pyrophosphorylase gene was recovered by single-cell screening techniques (67). This enzyme is essential for synthesis of the polysaccharides necessary for the construction of a fruiting body, and its absence caused a failure at culmination. The pyrophosphorylase gene has been cloned and studied in the laboratory of Dottin (82).

Dictyostelium is an excellent organism for studies of cellular motility, and as a consequence several genes involved in this process have been identified, mutations affecting them have been isolated, and in some cases the genes that code for them have been cloned. Examples include the actin-associated proteins alpha-actinin (182, 253) and severin (253). The mutants were recovered by immunological screening of large numbers of mutagenized amoebae arranged in arrays on agar plates. The mutants were probed with the cloned gene fragment and shown to lack the relevant transcript. The gross aspects of motility and phagocytosis in the mutants seem to be normal. In addition to contributing to studies of nonmuscle motility, these experiments have introduced extraordinarily useful colony blotting techniques to *Dictyostelium* mutant screening.

GENES EXPRESSED DURING AGGREGATION

Many genes expressed during development have been isolated for which no mutations or function are known. These have been used to analyze the expression of specific gene transcripts during development and, in cases in which the genomic nucleotide sequence has been determined, have provided a picture of the unusual gene structure used by this organism (125). In *D. discoideum* introns are rare and

generally less than 200 nucleotides long. The intergenic DNA contains, on average, 85% A and T residues, many of which are found in homopolymer tracts. The presence of these sequences is striking because of the unusual properties of AT-rich DNA, which include inhibition of nucleosome packaging (198), promotion of sharp bends in the double helix (134), and formation of the Z-DNA configuration (162). We do not know if the mechanisms of gene regulation used by other eucaryotes operate within this unusual sequence context, or if novel means of regulation have evolved to function in the AT-rich sequences.

The intergenic AT-rich sequences are reminiscent of yeast centromeric regions. Lower eucaryotic organisms occasionally have dispersed centromeres and bind the mitotic spindle at multiple points on the chromosome. One of the effects of dispersed centromeres is that they prevent nondisjunction when the chromosome is fragmented. It may be relevant that slime molds are extraordinarily resistant to ionizing radiation. An argument against the idea of dispersed centromeres is that genetic markers can be aligned with respect to a centromere. Perhaps the AT-rich sequences are vestiges of a dispersed centromere or perform a centromeric backup function.

Most of the *Dictyostelium* genes that have been characterized display motifs pointed out by Kimmel and Firtel (125). These include a TATA box at approximately 30 to 40 nucleotides from the start of transcription and a stretch of T residues between the TATA box and cap site. Much of the information about gene structure has been derived from examination of the discoidin and actin gene families: the former contains 4 closely related genes (63, 194) and the latter contains 20 genes (204). There is differential regulation of the actin genes: some are expressed preferentially during development (see references 40, 174, and 204).

The cloned genes of *D. discoideum* have been useful in defining the temporal and spatial patterns of gene expression even though many encode proteins of unknown function (213). Understanding the biological significance of a pattern of gene regulation is easiest when the gene product and its function are known. In addition to the phosphodiesterase (137), cysteine proteinases 1 (265) and 2 (52, 190, 196, 197), discoidin (194), csA (179), UDPG-pyrophosphorylase (82), the regulatory subunit of the cAMP-dependent protein kinase (170), and *ras* have a known or presumed function in development. Sequences that code for the cAMP receptor gene have also been cloned recently (P. Devreotes and A. Kimmel, personal communication). The sequence data predict a molecule with multiple membrane-spanning sites and carboxy-terminal serines and threonines that may be phosphorylation targets. Only one receptor gene has been detected by Southern blotting. Its sequence has an astonishing homology with rhodopsin, to the point that antirhodopsin serum recognizes the *Dictyostelium* cAMP receptor (Devreotes and Kimmel, personal communication).

The advent of transformation made it possible to study promoter structure and function. Firtel and his colleagues have shown that 500 nucleotides of 5′ flanking sequence of the *D. discoideum ras* gene (201), and 1 kb of 5′ flanking sequence of the cathepsinlike proteinase gene (52, 53) contain all of the *cis*-acting elements required to direct normal developmental regulation. The cathepsinlike proteinase gene studied by Firtel and co-workers is the same as the cysteine proteinase 2 gene of Williams and colleagues. The phosphodiesterase gene requires much more sequence for regulated expression during development. Some of the genes that have had their 5′ sequences determined are shown in Table 1, which was compiled with Jeffrey Williams. This is a consensus sequence TGGGGG/T about 200 nucleotides 5′ to the initiation codon of each gene. Pears and Williams (190) and Datta and Firtel (52) have mutagenized the TGGGG/T element which is in proximity to the proteinase 2 gene and suggest that it is useful for efficient induction of the contiguous gene and affects the amount of transcription rather than the timing. EB4 and D11 are genes which function late in development and under some circumstances produce more transcript when cells are presented with cAMP (11–13). The astute reader will notice that the sequence is present in the 5′ sequences of genes whose expression times do not overlap completely. Therefore, TGGGG/T is not a sequence that is fully responsible for the cAMP-inducible phenotype. The phosphodiesterase gene has the TGGGG/T sequence in one promoter region that does not appear to be regulated by cAMP. The cAMP-sensitive promoter used during development to make a large transcript is distant from the structural gene and has not yet been found.

An excellent way to recover *Dictyostelium* genes has been developed by the group of Jacquet, which has used *Dictyostelium* sequences to complement yeast auxotrophic mutations. The efforts have concentrated on the pyrimidine pathway, but the wealth of mutations in yeasts do not preclude selection of genes such as the *Dictyostelium* adenylate cyclase gene or genes that control the cell division cycle. To date, the genes coding for six activities have been cloned. The dihydroorotate dehydrogenase gene contains a single activity, but carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase, the first three steps in the pathway, are coded into a multidomain protein that is the product of a single gene (113, 114; M. Faure and M. Jacquet, personal communication). This association of genes parallels that found in *Drosophila* and mammalian cells. The dihydroorotate dehydrogenase gene and the gene coding for the other three activities are linked in the genome with 1 kb of sequence between them and are transcribed in opposite directions. The last two steps in the pathway, orotate phosphoribosyl transferase and orotidine 5′-phosphate decarboxylase, have also been recovered by complementation (26) and are coded by a single transcript. One of the prerequisites of these selections is the construction of highly transformable yeast strains so that libraries are screened directly in yeast cells without passing through *E. coli*, where genetic rearrangements of AT-rich sequences are common. Most attempts to recover *Dictyostelium* genes by

TABLE 1. Genes for which 5′ sequences have been determined

Gene	Approx nucleotides from cap	Strand	Sequence	Regulation by cAMP
Phosphodiesterase (constitutive promoter)	−180	Not transcribed	TGTGGG	None
Cysteine proteinase 2	−210	Transcribed	AGGGGT	+
EB4	−150	Transcribed	TGGGTT	+
D11	−170	Transcribed	TGGGGG	+
Consensus			TGGGGG/T	

complementation have succeeded. None of the 5′ genomic regions of the pyrimidine genes has been sequenced far enough to be compared with certainty to the genes listed in Table 1 except to say that they retain the AT richness found among the actins, discoidins, and developmental genes. The pyrimidine biosynthetic genes are interesting because they will provide a counterpoint with which to compare developmentally regulated genes and because they can be compared with the pyrimidine pathways of other organisms. The mRNAs of the pyrimidine genes disappear quickly after starvation and reappear at between 12 and 18 h of development. The pyrimidine biosynthetic genes are useful genetic resources. They will provide promoters that can be fused to other genes to alter the times of their transcription. If integration by homologous recombination proves to be a frequent occurrence, introduction of the pyrimidine genes by transformation should produce pyrimidine auxotrophs. These are among the few genes known which would produce a specific phenotype after integration by homologous recombination. The phosphodiesterase gene is another. Growth-specific genes that are inactivated during development have also been recovered by Singleton et al. (228) and Kopachik et al. (135).

CONTROL OF AGGREGATION

Without biochemical underpinning, discussion of mutant defects and the multiple effects of exogenous cAMP on gene regulation becomes a compendium of confusing results. What follows is an attempt to combine information about cAMP control of gene regulation and morphogenesis with genetic results into a framework with predictive value and is based on several reasonable assumptions. (i) cAMP works through a receptor, and therefore signal transduction inducing rapid events, such as chemotaxis and motility, or slower events, such as gene regulation, must be the result of activation of second messenger systems. (ii) Mutations stimulating or eliminating these cascades should have profound effects on development. (iii) A limited number of genes is essential for aggregation; as a result, among aggregateless mutants defects in plasma membrane signal transduction and second messenger systems should be frequent. The important questions are: What are the second messenger cascades? Do mutations that affect them exist? Given the selection and screening possibilities offered by *Dictyostelium* species, can we isolate informative mutants?

Second Messenger Cascades

The presence of cAMP outside the cell is transduced by second messengers, but it has been difficult to assign a particular second messenger to the induction of any cytoplasmic or nuclear response. Most of the second messenger systems studied in other organisms exist in *Dictyostelium* (see references 114a and 163). Intracellular cGMP and cAMP are synthesized seconds after a pulse of cAMP, and there is good evidence that second messenger cascades exist for these nucleotides. *Dictyostelium* has a cAMP-dependent protein kinase that is regulated during development and appears maximally after aggregation (55). The kinase appears to be absent during exponential growth. The regulatory subunit gene has been cloned and is homologous to cAMP-binding protein in other organisms (170). There are cGMP-binding proteins in the cytoplasm in addition to the cGMP phosphodiesterase (188), and one has a molecular weight of 25,000 (221). Work on purification has recently progressed (B. Wurster, personal communication). cGMP seems to have a role in the adaptation of the movement response to a cAMP signal, a conclusion drawn from analysis of streamer mutants described above.

There is evidence of phosphoinositide turnover during aggregation. The laboratory of Newell, using digitonin-solubilized cells, has shown that after a cAMP stimulus there is a rapid increase in inositol trisphosphate and a subsequent mobilization of calcium (76). An increase of inositol trisphosphate in permeabilized cells can be induced by guanosine 5′-triphosphate, implicating the intercession of a G protein. Calcium enters the cytoplasm from several directions: the extracellular space and the rough endoplasmic reticulum and mitochondria. An increase of intracellular calcium has been correlated with a rise in cGMP and actin polymerization (74, 164). If all compartments are depleted of calcium, aggregation is inhibited (72). Bumann et al. (34) and Europe-Finner and Newell (73) have shown that entry of calcium from the extracellular space occurs and, unlike other responses to continuous cAMP, the influx of calcium does not adapt in the continuous presence of cAMP as adenylate and guanylate cyclases do. Milne and Coukell (personal communication) have purified a plasma membrane-associated calcium pump which requires adenosine 5′-triphosphate, is magnesium dependent, and is inhibited by vanadate.

The *Dictyostelium ras* gene is almost certainly a participant in the first steps of a second messenger cascade. The effect of the *ras* mutation on phosphodiesterase or other cAMP-inducible genes has not yet been analyzed. That there is a G protein involved in adenylate cyclase stimulation and that it does not appear to be *ras* indicate that there is a family of proteins with the ability to bind guanosine 5′-triphosphate Evidence for members of a G-protein family other than *ras* rested until recently on pharmacological data: no protein has been purified (122, 240, 247, 250) or identified convincingly by using adenosine 5′-diphosphate ribosylation by cholera or pertussis toxin. However, two G protein genes with high homology to their counterparts in higher organisms have recently been cloned by a collaborative effort of the Firtel and Devreotes laboratories (P. Devreotes, personal communication). These sequences detect multiple genes, providing the potential for a large family of proteins involved in signal transduction.

While *Dictyostelium* amoebae have genetic and biochemical advantages for dissecting signal transduction and second messenger systems, certain pharmacological experiments that are used to study second messenger systems in higher organisms cannot be used. Neither forskolin nor phorbol esters seem to be effective, nor has protein kinase C or diacylglycerol been demonstrated. Calcium channel blockers and ionophores have not been used extensively, although this situation is changing. Several calcium channel blockers used in mammalian cells have been shown to suppress induction of the phosphodiesterase by cAMP (M. B. Coukell, personal communication). A number of good agonists and antagonists of the cAMP receptor have been developed (61, 249). Caffeine, for reasons that are not clear, inhibits activation of the *Dictyostelium* adenylate cyclase by cAMP and has been used to define steps that are dependent on intracellular cAMP synthesis (29, 123).

Adaptive and Nonadaptive Gene Regulation

In the presence of constant concentrations of extracellular cAMP, proteins associated with late events in aggregation

never appear, but when cells are given pulses of cAMP these proteins appear earlier than normal. Two such proteins are the csA(gp80) glycoprotein (94, 273) and the cAMP receptor (37, 273), which in this form of regulation resemble a number of less-studied genes and gene products (157). The converse form of regulation has been discovered by Kimmel and Carlisle (123, 124), who showed that the M4-1 gene, which is transcribed in growing cells and inactivated during development, is repressed during development by pulses but is retained in the presence of constant amounts of cAMP. The K5 gene transcript is synthesized very early in development and then repressed, but only by pulses of cAMP (157). While certain genes respond only to pulses, others do not discriminate between two methods of cAMP application. The extracellular cyclic nucleotide phosphodiesterase is induced by steady amounts or by pulses of cAMP (121, 137, 273) and thus the response of the gene is nonadaptive. The phosphodiesterase, inhibitory glycoprotein (121; unpublished data) is repressed by cAMP, whether pulsatile or constant. Thus, there are four possible results of cAMP application (Table 2): induction and repression without adaptation and induction and repression with adaptation. In the cases of M4-1 and K5, we postulate that constant cAMP maintains rather than induces transcription of the gene (123). It is important to note that gene regulation by cAMP after aggregation does not appear to be adaptive; constant application of cAMP maintains synthesis of numerous proteins. Thus, the csA(gp80) and M4-1/K5 genes which respond only to pulses are the exception. The distinction between adaptive behavior when the application of constant amounts leads to suppression and nonadaptive behavior when constant cAMP leads to induction has been pointed out by Gerisch et al. (99). Wurster and Butz (270) have also demonstrated that the organism has two types of response to cAMP, one adaptive and the other nonadaptive. The distinction is crucial to understanding the development of this organism.

What is the molecular basis of adaptation? Current evidence suggests that adaptation occurs at the level of the cAMP receptor. Biochemical purification of the receptor has been accomplished by the groups of Devreotes (58, 130) and Klein (128, 150), who, as yet, have detected only one receptor molecule. More than one receptor type may exist, but if so the cryptic cAMP receptors must fail to bind labeled 8-azido-cAMP. The major form of the receptor is hyperphosphorylated in response to cAMP. One result of phosphorylation is to change the molecular weight of the receptor from 41,000 to 43,000 (59, 131) or, in the hands of another group, from 45,000 to 47,000 (150). When continuous cAMP is applied, the receptor remains phosphorylated and at the higher molecular weight while, simultaneously, a normal response of cAMP stimulation (cAMP synthesis and secretion) is extinguished (129). When cAMP is removed from the cells, the receptor is dephosphorylated and adaptation is relieved; i.e., the cells become responsive to extracellular cAMP again. In addition to engaging adenylate cyclase, the receptor must affect the activity of guanylate cyclase, and perhaps an as yet undemonstrated PIP2 phosphodiesterase and a variety of ion channels (168).

TABLE 2. Results of cAMP application

Method of application	Gene			
	Nonadaptive		Adaptive	
	Phosphodiesterase	Inhibitor	csA, cAMP receptor	M4-1/*K5*
Constant	Induces	Represses	Represses	Induces
Pulsatile	Induces	Represses	Induces	Represses

A simplified diagram of cAMP receptor behavior is shown in Fig. 4A. See reference 129 and a recent review on signal transduction by Janssens and van Haastert (114a) for more detailed accounts. The receptor may exist in three states: one in which there is no cAMP and the receptor is disengaged from its effector enzyme(s), a second in which cAMP is present and an effector molecule such as adenylate cyclase is stimulated through the action of a G protein, and a third in which cAMP concentrations in the external milieu do not change but, because of phosphorylation, the receptor ceases to stimulate its effector enzyme. In the last instance the system has adapted. If we imagine that as a second messenger system emanates from the receptor and effector and try to select conditions to stimulate it maximally, it is obvious that the absence of cAMP and an excess of cAMP are two conditions to be avoided. If the postulated second messenger cascade has a half-life measured in seconds or even minutes and is necessary for induction of the csA and cAMP receptor genes, then in the absence of cAMP or in the presence of constant cAMP, the csA, cAMP receptor, and other proteins would not appear. This is what is observed. Periodic application of cAMP, with cAMP destroyed by phosphodiesterase between pulses, would stimulate the second messenger cascade by avoiding lengthy periods of adaptation or nonstimulation and lead to premature induction. It has been known for a long time that pulses of cAMP advance the synthesis of csA and of the cAMP receptor in starving cells (94, 273). The events brought forward by pulses of cAMP include one that is rate limiting because the developmental state in general is advanced by pulses of cAMP (50, 51, 99). Note that, although cAMP is the second messenger used as an example in Fig. 4A, there is no evidence that it is responsible for induction of csA or cAMP receptors. The M4-1 transcript may depend on increases in intracellular cAMP pools for its repression (123).

To explain the regulation of the phosphodiesterase and inhibitor by cAMP, we postulate another second messenger system that emanates from the cAMP receptor but is not subject to adaptation in the presence of extracellular cAMP (Fig. 4B). In this case phosphorylation of the receptor would have no effect on an effector molecule producing another second messenger and the cascade would continue, resulting in sustained synthesis of the phosphodiesterase (and blockade of inhibitor and discoidin synthesis). Phosphorylation of the receptor acts as a clutch which shifts development into neutral by disengaging effector molecules like adenylate cyclase but leaving others to operate. This has an advantage to the aggregating cells because no late functions will be induced before formation of the aggregate is complete, which it cannot be as long as cAMP levels are very high and interfere with chemotaxis. By maintaining the cascade that induces phosphodiesterase, the cells work to restore the conditions under which the cascade leading to csA and receptor induction can proceed coordinately with chemotaxis.

Can the scheme shown in Fig. 4 help to explain how certain mutants such as the *casA1000* or the rapid development mutations evade the paralytic effects of high exogenous cAMP concentrations? One mechanism may be that the receptor is itself mutated to a form in which binding of the ligand and the induction of the second messenger cascade are normal, but the mechanism of adaptation (phosphorylation) is defective, so that signals are transduced and stimu-

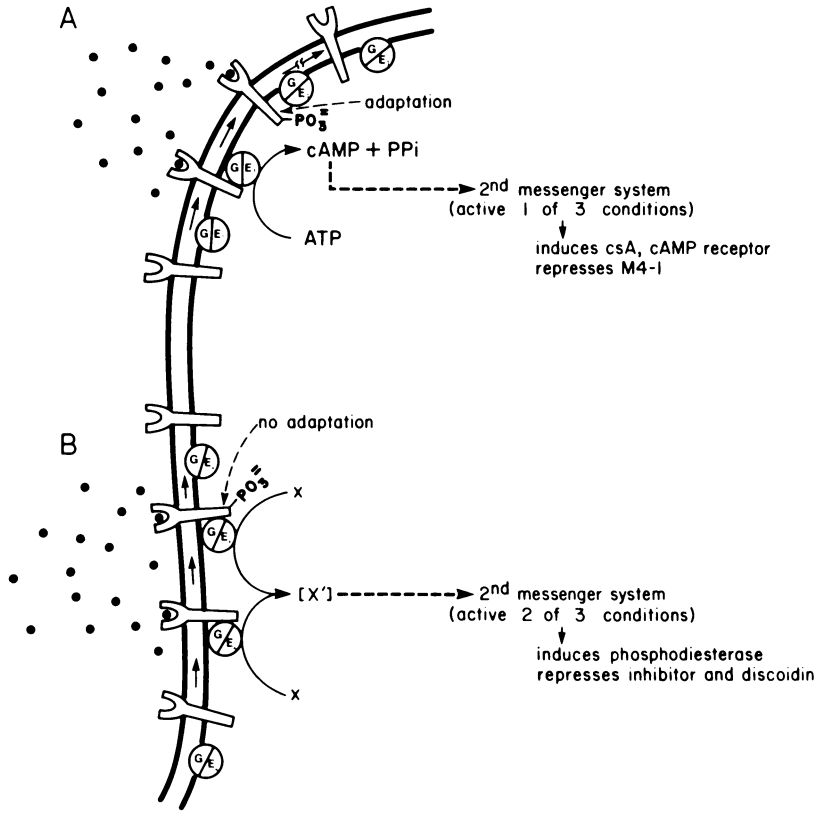

FIG. 4. Correlation of receptor behavior and modes of gene regulation by cAMP. (A) Receptor and adenylate cyclase coupling before, during, and after a cAMP stimulus. The half-time of deadaptation is 2 to 3 min. Pulses given at 2-min intervals do not advance the developmental state (268) even if degradation of cAMP takes place in seconds. Note that adaptation will continue as long as extracellular cAMP concentrations are not reduced or a higher concentration of cAMP is not added. Panel B shows a hypothetical situation with another effector molecule and perhaps another G protein, in which phosphorylation of receptor does not impede generation of a second messenger. The model does not require that a single second messenger system induce and repress, but is shown that way in deference to Occam's rule. ATP, Adenosine 5'-triphosphate; PP_i, inorganic pyrophosphate.

late responses in the cytosol and nucleus of the cell, even in the presence of continuous cAMP. The first cascade may continue to function in mutants (*casA1000* and *rdeA*) that evade the inhibitory effects of high levels of cAMP. This would result in the induction of the class of proteins represented by csA and the cAMP receptor in the example above, and aggregation could continue, although not normally. Both mutations cause defective aggregation. A gratuitously active second messenger cascade is reminiscent of the actions of oncogenes such as *erbB* (91, 178).

When cells make contact at aggregation, a milestone has been reached and proteins required to that point are repressed. Cell contact functions to stimulate the synthesis of a new class of proteins and transcripts (8, 20, 21, 38, 125, 138). If this class is induced by a gratuitously active cascade which is normally dependent on cell contact, one might explain why strains carrying the *rde* mutations can form spores without cell contact.

If phosphorylation of the cAMP receptor is the mechanism of adaptation, this model predicts that among the mutants that aggregate in the presence of cAMP there should be some in which phosphorylation is abnormal. Candidates for receptor adaptation defectives among existing mutants include those that carry the *casA1000* allele and a number of less characterized cAMPS-resistant mutants (95, 254). The rapid development mutants should also be defective in the adaptation process.

If second messenger cascades are vulnerable to mutagenesis, and if a second messenger system is responsible for the induction of many genes during development, then in addition to mutations which continue to produce the second messenger, some should be found that act more distally and remove the inhibition built into second messenger systems. For example, if cAMP acts as a second messenger, elimination of the regulatory subunit of the cAMP-dependent protein kinase by mutation or antimessenger techniques could lead to a constitutive kinase that should have specific effects on development.

The selection procedures described above are capable of yielding mutants that could test these ideas. Among the most likely to involve second messenger systems are those produced by Berger and Dulaney (15). These defects may block a second messenger cascade that is essential for the maintenance of differentiation. One would predict that, under normal conditions, the cascade functions to maintain the expression of the many proteins made after the cells have aggregated. An interruption of this cascade by mutation would allow the cells to escape the differentiated state, but as a consequence the cells would become defective in a function necessary for a subsequent developmental cycle. Some of the escape mutants fail at the stage of aggregation. A second interesting collection which should be examined for inductive defects is the one produced by Segall et al. (222) which contains mutants that fail to migrate toward

folate. Strains which neither migrate toward folate nor aggregate should be tested for cAMP inducibility of phosphodiesterase by folate and by cAMP. Because folate and cAMP induction of phosphodiesterase share components, elements of a common second messenger cascade may be defective in some of these mutants. Some of the folate-unresponsive mutants that do not aggregate should be incapable of phosphodiesterase induction by cAMP.

As more is learned about the biochemistry of second messenger systems, the availability of mutants, including those in the *ras* gene, should help us to discriminate which second messenger cascades control important chemotactic and gene-inductive events. For the problem of signal transduction, the ability to measure cAMP synthesis in response to cAMP stimulation should allow rapid screening of mutants for defects which can be complemented by wild-type extracts. Hesitation stemming from the thought that unworkably large numbers of genes will be involved in any subprocess of aggregation is unjustified. Advances in parasexual and reverse genetic techniques combined with the characterization of genes known to be essential for aggregation should permit us to unravel the regulatory circuits that coordinate events during early development of *D. discoideum*.

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