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Regulation of Discoidin I Gene Expression in Dictyostelium discoideum by Cell-Cell Contact and cAMP

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We have previously presented evidence that cell-cell contact is the normal developmental signal to deactivate discoidin I gene expression in D discoideum [Berger EA, Clark JM: Proc Natl Acad Sci USA 80:4983, 1983]. Here we provide genetic evidence to support this hypothesis by examining gene expression in a cohesion-defective mutant, strain EB-21, which enters the developmental program but is blocked at the loose mound stage. When this strain was developed in suspension, the cells remained almost entirely as single amoebae, unlike the wild type, which formed large multicellular aggregates. In both strains, discoidin I mRNA levels were low in vegetative cells but rose sharply during the first few hours of development. However, the peak level reached at 8 hr in EB-21 exceeded that observed in wild type, and while the level declined markedly over the next few hours in wild type, it remained highly elevated in the mutant. Thus, there was a correlation between the inability of EB-21 to form normal cell-cell contacts and its deficiency in inactivating discoidin I gene expression.

Previous studies from several laboratories, including this one, have demonstrated that exogenously added cAMP can block or reverse the changes in gene expression normally seen upon cell disaggregation. This has led us to propose that cAMP serves as a second messenger regulating the expression of contact-regulated genes. Here we provide additional support for this hypothesis. Intracellular cAMP levels rapidly dropped several-fold when wild type tight cell aggregates were disaggregated and remained low as the cells were cultured in the disaggregated state. Furthermore, overexpression of discoidin I mRNA late in development in EB-21 was corrected by addition of high concentrations of cAMP. These results are consistent with a second messenger function for cAMP in the contact-mediated regulatory response, and they indicate that the cAMP response machinery for discoidin I gene expression is capable of functioning in the cohesion-defective EB-21 strain.

Key words: cell-cell contact, cyclic AMP, Dictyostelium discoideum, gene regulation, discoidin I

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Many types of cells are capable of recognizing and forming specific cohesive associations with other cells, often triggering profound changes in cell growth and differentiation [1]. Thus, like the cellular interactions mediated by secreted chemical signals such as hormones and growth factors, cell-cell recognition and contact is emerging as a major regulatory signal governing cell function. An important challenge is to elucidate the mechanisms by which these cell-cell recognition events, presumably mediated by specific surface-localized molecular components, are translated into new programs of cell behavior.

The cellular slime mold Dictyostelium discoideum is a simple eukaryote whose morphogenetic life cycle involves aggregation of thousands of individual amoebae to form a multicellular organism. During the starvation-induced transition from the growth to the developmental phase, the cells acquire a cell-cell recognition and cohesion system which appears to be mediated by specific surface molecules [2]. Evidence from several laboratories suggests that cell-cell contact serves as a regulatory signal to activate and deactivate specific genes during the developmental program [3,4]. We have been studying the relationship of cell-cell contact to the expression of the genes encoding discoidin I, an endogenous lectin produced during the aggregation stage [5]. Based on the changes in the levels of discoidin I mRNA during normal development as well as in response to specific manipulations of the state of cellular aggregation, we have proposed that cell-cell contact serves as the signal to deactivate discoidin I gene expression during development [6]. Here we provide added support for this hypothesis by examining discoidin I mRNA levels in a cohesion-defective mutant. Furthermore, we present additional evidence that intracellular cAMP functions as a second messenger in the contact-mediated regulatory response.

MATERIALS AND METHODS

Growth and Development of Cells

D discoideum strain NC4 (obtained from W.F. Loomis) and its derivative A3 (obtained from P.N. Devreotes) were the wild type strains used in this study. Strain EB-21 was derived from A3 after mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine and screening individual plaques for aberrant developmental morphologies. Cells of all three strains were grown on SM agar in association with Klebsiella aerogenes [7]. For development, vegetative cells were washed free of bacteria in phosphate buffer (3.2 mM Na_2HPO_4/12.8 mM KH_2PO_4, pH 6.4), then starved either in suspension or on filters (Whatman 50, 4.25 cm) as previously described [6]. Under these conditions, wild type cells on filters form aggregation streams that coalesce into loose mounds by 12 hr and tight aggregates by 16–17 hr. Fruiting body formation is complete by 30 hr. Suspension-developed cells acquire aggregation competence and will fruit if deposited onto filters [8].

Measurement of mRNA levels

To quantitate the levels of specific mRNA's, we employed either RNA dot hybridization analysis as previously described [6] or a modification of the cytoplasmic dot hybridization procedure of White and Bancroft [9]. In this latter method, frozen cell pellets (typically 5×10^7 cells) were suspended by vortexing in 225 μl Tris-EDTA buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.0). In some experiments, this buffer was supplemented with 20 mM vanadyl ribonucleoside complexes (Bethesda Research Laboratories). Fifty microliters of 5% (v/v) nonidet P40 (in Tris-EDTA buffer) was then added, and the suspensions were incubated 5 min on ice. In cases

where the cell pellets differed from 5×10^7, the volumes were adjusted proportionally. Nuclei were removed by centrifugation for 5 min in an Eppendorf microfuge, and the supernatants were transferred to fresh tubes. Aliquots of the supernatants were treated for RNA analysis by addition of equal volumes of a mixture containing 3 parts blotting buffer (3 M NaCl, 0.3 M trisodium citrate) plus 2 parts 37% formaldehyde. Samples were incubated 15 min at 60°C, then frozen and stored at −70°C. The protein content was determined by the method of Bradford [10], using the protocol and reagents supplied by BioRad with bovine serum albumin as the standard. For subsequent analysis, equal amounts of formaldehyde-treated cytoplasm (based on the protein assay) were spotted onto nitrocellulose sheets. This was achieved by first diluting the appropriate volumes of each sample with blotting buffer such that 150–200 μl aliquots contained the desired amounts of cytoplasm. Aliquots were applied in duplicate to a nitrocellulose sheet under gentle suction using a Hybri-Dot Manifold (Bethesda Research Laboratories). The wells were not rinsed. For both the RNA and the cytoplasmic dot blots, baking, prehybridization, hybridization, washing, and autoradiography were performed as described [6,11], except that in some experiments the prehybridization and hybridization buffers were modified by omitting the salmon sperm DNA and raising the bovine serum albumin concentration to 1 mg/ml and the concentrations of Ficoll and polyvinylpyrrolidone each to 0.5 mg/ml. Quantitation was achieved by cutting out circles of the nitrocellulose with a cork borer and counting in 5 ml ACS counting scintillant (Amersham). Blanks, determined by cutting circles spotted with equivalent samples from either HeLa cells or another slime mold species, Polysphondylium violaceum, have been subtracted to give the data shown. Data are expressed as CPM specifically hybridized. Control experiments (not shown) have indicated that this cytoplasmic dot procedure gives results which are quantitatively very similar to those obtained with the RNA dot procedure previously used by this laboratory [6], and that for each probe the CPM specifically hybridized is proportional to the concentration of the corresponding mRNA.

Recombinant Plasmid Probes

Plasmid pDd812 is a recombinant of pMB9 containing a cDNA insert derived entirely from the protein coding region of the discoidin Ia gene [12,13]. By Northern blot analysis, this probe detects a single cytoplasmic RNA band of 960 nucleotides (discoidin I mRNA) [6,12] which first appears very early in development and whose expression is deactivated by cell-cell contact or high concentrations of exogenous cAMP [6,14]. Plasmid PL3 is a recombinant of pBR322 containing a cDNA insert that detects a single mRNA which first appears late in development on filters, and whose continued expression requires maintenance of cell-cell contact or addition of high concentrations of cAMP [6,15]. Plasmid DNA (1 μg) was labeled with 250 μCi of [5′-^{32}P]dCTP (800 Ci/mmole, New England Nuclear) by a modification of the nick-translation method of Rigby et al [16]. Incorporation typically exceeded 65% of the input radioactivity, based on trichloroacetic acid precipitation analysis.

Measurement of Intracellular cAMP

Intracellular cAMP was measured by radioimmunoassay, using a modification of the protocol of Brenner [17] to obtain cell extracts. For filter-developed cells, duplicate filters at each time point were rinsed on a buchner funnel under gentle

suction with 5 ml of filter development buffer at 22°C. Each filter was placed in 5 ml of ice-cold 10% (w/v) trichloroacetic acid previously spiked with 0.3 pmoles of [2,8-^{3}H]cAMP (New England Nuclear, 34.5 Ci/mmol) to monitor recovery. Cells were gently scraped off the filters, vortexed, and incubated on ice for 30 min, and the samples were passed through nitrocellulose membranes to remove insoluble debris. The original filters were rinsed with an additional 1 ml of 10% trichloroacetic acid (not containing [^{3}H]cAMP) which was then combined with the original filtrates. For cells developing in suspension, 4-ml aliquots (in duplicate) were removed at each time point, and the cells were pelleted and extracted with 5 ml of 10% trichloroacetic acid spiked with [^{3}H]cAMP as described for the filter-developed cells. To remove the trichloroacetic acid, samples were extracted four times with 25 ml of water-saturated ether, and after removing residual ether with a gentle stream of nitrogen, the samples were lyophilized. The residues were suspended in 0.6 ml of 50 mM sodium acetate buffer, pH 4.75, transferred to plastic microfuge tubes, frozen, and stored at −70°C for subsequent assay. The cytoplasmic protein content of cells at each time point was determined from either duplicate companion filters or duplicate aliquots of cell suspensions which were processed and assayed for protein as described for the cytoplasmic dot hybridization procedure.

For the cAMP radioimmunoassay, each assay tube (done in duplicate in 1.5 ml plastic microfuge tubes) contained 50 μl of 3×10^{-11}M [^{125}I]-2′-0-succinyl (iodotyrosine methylester)-cAMP (New England Nuclear, 2,200 Ci/mmole, carrier-free), 90 μl of a 1:1,000 dilution of anti-cAMP antiserum (donated by Squibb), the appropriate dilutions of either unlabeled cAMP (for standard curves) or the cell extracts described above, and 5 μl of acetylation mix (triethylamine:acetic anhydride, 2.5:1, prepared fresh for each use), brought to a total volume of 500 μl with 50 mM sodium acetate buffer, pH 4.75. To obtain nonspecific binding, normal rabbit serum was used instead of anti-cAMP. Following overnight incubation at 4°C, 40 μl of a 10% (w/v) suspension of formalin-fixed Staphylococcus aureus (Pansorbin, Calbiochem, washed twice with 50 mM sodium acetate buffer, pH 6.2) was added to each tube. Samples were incubated 1–2 hr at room temperature (during which time total CPM were determined), then centrifuged 12 min in an Eppendorf microfuge. Pellets were washed once with 50 mM sodium acetate buffer, pH 4.75, then counted in a Beckman gamma counter. Percent radioactivity specifically bound was determined for each sample, and the cAMP content of each sample was obtained by comparison with the standard curve, using a computer program based on a linear regression analysis of log dose and a logit response transformation of raw standard curve data [18]. Values were corrected for yield based on the percent recovery of the [^{3}H]-cAMP (which typically exceeded 55%) and then normalized to protein content. Data are expressed as pmoles cAMP per milligram cytoplasmic protein.

RESULTS

Overexpression of Discoidin I mRNA in a Cohesion-Defective Mutant

Cohesion-defective mutants provide an opportunity to study the relationships between cell-cell contact and developmental gene expression. Strain EB-21 was identified during a screening of plaques from a mutagenized cell population and was chosen for further study based on its aberrant developmental morphology. When starved on a solid support such as agar or filters, this strain aggregated with timing

comparable to that of wild type but remained blocked at the loose mound stage and did not fruit (Fig. 1A). Microscopic examination of cultures of suspension-starved cells revealed a dramatic alteration in cell-cell cohesiveness; whereas nearly all cells in the wild type culture entered large multicellular aggregates, the EB-21 culture remained almost entirely as single cells (Fig. 1B).

In view of this cohesion defect in EB-21, it was of interest to study the expression of genes believed to be regulated by cell-cell contact. Table I shows the levels of discoidin I mRNA (detected by plasmid probe pDd812) during development in EB-21 and its parent, A3. In both strains, the levels were very low in vegetative cells but increased dramatically during the first several hours of starvation. In fact, the peak level obtained in EB-21 was considerably higher than that in wild type, and

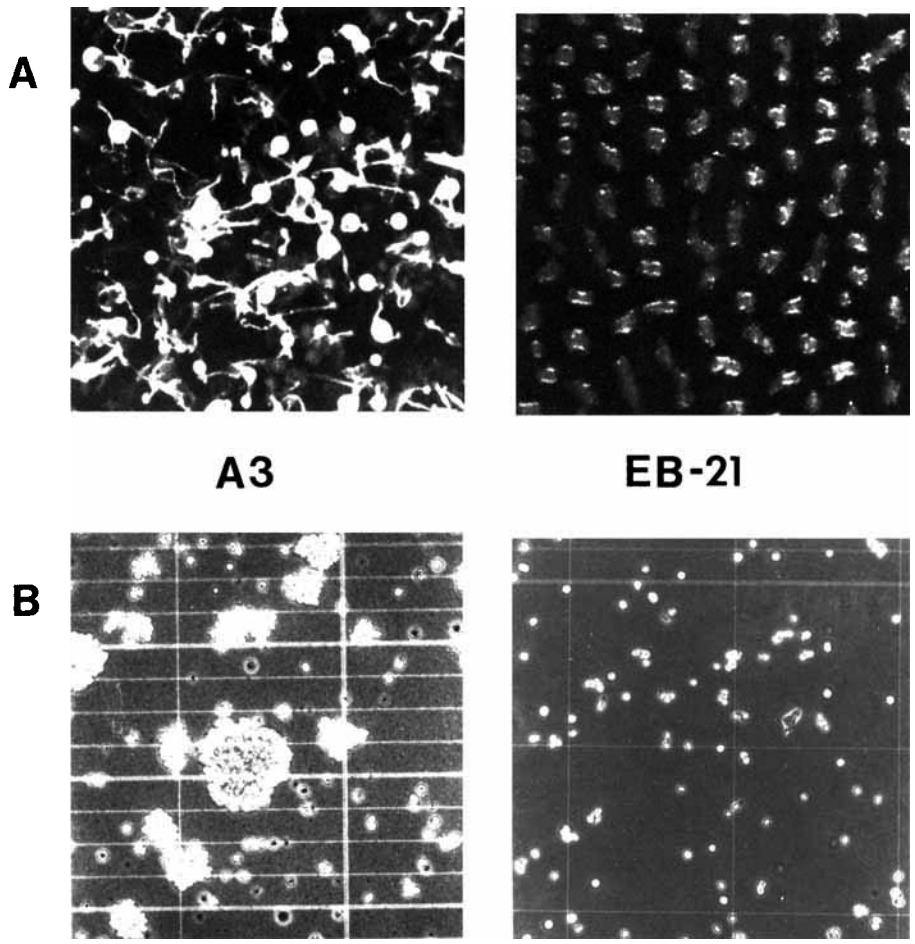

Fig. 1. Morphological properties of wild type and mutant strains. A) Cells developed on solid substrate. Cells were grown on nutrient agar plates, and the terminal developmental morphologies after clearing of the bacterial lawn are shown. Similar results were obtained with cells developed on filters. B) Cells developed in suspension. Cells were starved 18 hr. For photography, the EB-21 culture was diluted approximately fivefold to enable visualization of single cells.

during the next 6 hr, when the wild type level declined markedly, the EB-21 level remained highly elevated. These results provide additional support for the hypothesis that cell-cell contact is responsible for the normal developmental decline in discoidin I mRNA. Consistent with this, PL3 mRNA, whose expression has previously been shown to be dependent on continued cell-cell contact [6,15], was not expressed in EB-21 (Table I).

cAMP as a Second Messenger

Previous work from this and other laboratories using wild-type cells has indicated that exogenously added cAMP can prevent or reverse the changes in gene expression normally observed upon disaggregation. Thus, the disaggregation-induced rise of discoidin I mRNA [6] and decline of contact-dependent mRNA's such as PL3 [3,6,15] are blocked or reversed by addition of high levels of cAMP to the incubation. Based on this, we have postulated [6] that cell-cell contact functions to elevate intracellular cAMP which in turn serves as a second messenger to activate some genes and deactivate others. According to this hypothesis, we would predict that disaggregation should cause a drop in intracellular cAMP and that the level should remain low as the cells are maintained in the disaggregated state. Figure 2 confirms this prediction: At the earliest time examined (30 min) after disaggregation of filter-developed cells, cAMP levels dropped more than four-fold compared to unperturbed cells and remained greatly reduced throughout the remainder of the incubation. It should be noted that the intracellular cAMP levels shown here, including the decline between 14 hr and 19 hr of development on filters, are in excellent agreement with those reported independently by two other laboratories [17,19].

If cAMP is indeed a second messenger for cell contact-mediated gene control, then it might be predicted that the alterations in gene expression in a cohesion-defective mutant would be correctable by cAMP. This is the case for discoidin I gene expression in EB-21 (Fig. 3). In this experiment, discoidin I mRNA levels were again highly elevated late in suspension development in this strain. However, addition of cAMP caused a sharp drop compared to the untreated control. We have performed

TABLE I. Relative mRNA Levels in Wild Type vs. Mutant Cells

Plasmid probe	Hours of Development	CPM specifically hybridized A3	CPM specifically hybridized EB-21	Ratio, EB21/A3
pDd812[a]	0	149	103	0.6
	8	1,415	4,217	3.0
	14	133	2,451	19.0
PL3[b]	0–15	< 20	< 10	—
	17	225	—	—
	18	—	5	—
	24	750	5	0.01

[a]Cells were developed in suspension for the indicated times. mRNA levels were determined by the cytoplasmic dot hybridization procedure (5 μg protein per dot for each strain). The background obtained with Polysphondylium violaceum cytoplasm was 181 CPM, and has been subtracted to give the data shown.

[b]Cells were developed on filters for the indicated times. Filter-developed cells were used because prespore mRNA's such as PL3 are not expressed in cells developed in suspension [3,6]. mRNA levels were determined by the RNA dot hybridization procedure (5 μg RNA per dot for A3, 10 for EB-21).

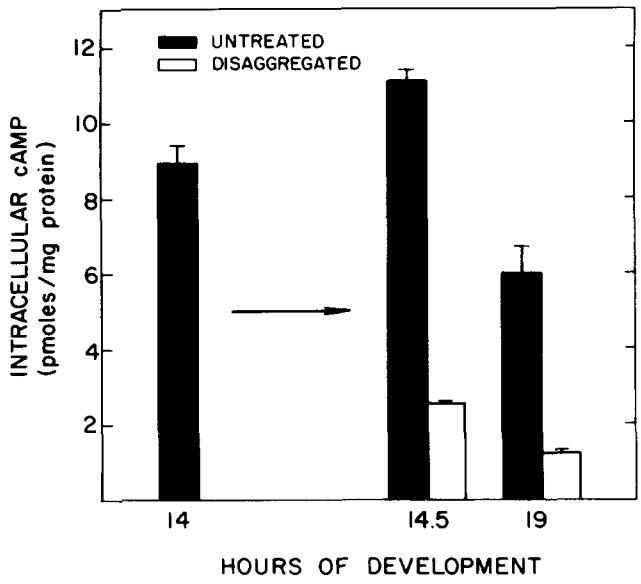

Fig. 2. Effect of disaggregation on intracellular cAMP levels. NC4 cells were developed on filters (10^8 cells per filter) for 14 hr. One set of filters was allowed to continue development unperturbed. Cells from a second set of filters were removed and disaggregated by vortexing in filter development buffer supplemented with 10 mM EDTA. The cell suspension was diluted to 5 ml per original filter (corresponding to 2×10^7 cells per ml based on the original cell count), transferred to a siliconized Ehrlenmeyer flask, and incubated at 22°C on a rotary shaker at 220 rpm. Values represent the mean cAMP contents of duplicate samples of filter developed (closed bars) or disaggregated (open bars) cells, with the error bars indicating the values obtained for individual samples.

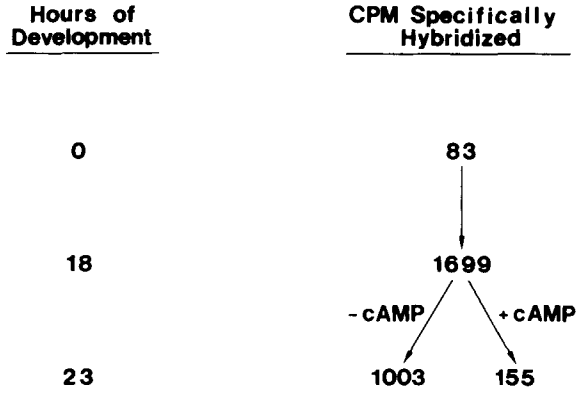

Fig. 3. Effects of exogenous cAMP on discoidin I mRNA levels in EB-21 cells. EB-21 cells were developed in suspension for 18 hr. The culture was then divided into two portions, with one receiving no additions and the other receiving cAMP in increments corresponding to 1 mM (final concentration) at 18 hr and hourly thereafter. Discoidin I mRNA was determined by the cytoplasmic dot hybridization procedure (5 μg per dot). The background obtained with Polysphondilium violaceum cytoplasm was 44 CPM and has been subtracted to give the data shown.

experiments similar to this with EB-21 cells prestarved for much shorter periods (11–16 hrs) with qualitatively identical results. This suggests that, at least for discoidin I gene expression, the machinery for the response to cAMP is capable of functioning in EB-21.

DISCUSSION

Based on the sharp decline in discoidin I mRNA levels during the late aggregation stage of D discoideum filter development, and on the reappearance of this mRNA upon EDTA-induced disaggregation, we have previously proposed that cell-cell contact serves as the normal developmental signal to deactivate discoidin I gene expression [6]. Recognizing that the EDTA used in the disaggregation protocol might have side effects on gene expression unrelated to the disruption of cell-cell contacts, we indicated that it was important to examine discoidin I gene expression under conditions where contact formation is prevented without the use of EDTA. As one approach, we examined discoidin I gene expression in cells developed in suspension. Under these conditions where contact formation is presumed to occur less efficiently than on filters, discoidin I mRNA levels decline much more gradually [6]. However, with these wild type cells, the formation of aggregates occurs even in suspension [8] (also Fig. 1B) and discoidin I mRNA levels do indeed decline [6] (also Table I). In this report, we took advantage of a mutant strain, EB-21, which enters the developmental program normally but is defective in cell-cell cohesion (Fig. 1B). The findings (Table I) that discoidin I mRNA levels in EB-21 rise sharply upon starvation in suspension and remain highly elevated during later development provide added support for the hypothesis that cell-cell contact is indeed the signal for discoidin I gene deactivation. However, it must be noted that we have yet to define the precise molecular defect in EB-21 and specifically have not yet learned whether the mutation is in a gene encoding a primary component of the cohesion system vs. a gene required for its proper expression or function. Thus, the possibility exists that the cohesion defect and the failure to inactivate discoidin I gene expression are independent consequences of an indirect mutation, rather than effects which are causally related to each other. The same argument applies to experiments from others workers showing that cohesion-defective mutants fail to express late developmentally regulated genes [20]. Furthermore, even if cell cohesion is required for the normal regulatory behavior, the results to date do not distinguish whether contact per se is the primary signal or whether it simply serves to bring the cells into sufficiently close proximity to facilitate the transmission of another signal. A major challenge is thus to learn whether or not the initial trigger for contact-regulated gene control is the actual molecular interaction between (some of) the components of the specific cohesion system, analogous to hormone/receptor interactions.

The decline in intracellular cAMP caused by disaggregation (Fig. 2) coupled with the ability of exogenous cAMP to deactivate discoidin I gene expression in EB-21 cells (Fig. 3) as well as in disaggregated wild type cells [6] are consistent with the hypothesis that cAMP serves as a second messenger in the contact-mediated regulation. The responsiveness of discoidin I gene expression in EB-21 to high levels of exogenous cAMP suggests that what is defective in this strain is the ability to generate sufficient concentrations of intracellular cAMP for normal gene regulation. Preliminary observations (not shown) do indeed demonstrate that intracellular cAMP levels are substantially lower in suspension-developed EB-21 cells than in the parental strain.

Also of interest are reports from other laboratories showing that during normal development on filters (for cells grown on bacteria as in our studies), intracellular cAMP levels rise sharply beginning around 7–8 hr (which corresponds well to the time when discoidin I mRNA levels begin to decline), and remain relatively high throughout subsequent development [17,19]. Furthermore, Finney et al [21] have also shown that the cAMP levels drop upon disaggregation, but that they rise sharply again as the cells are cultured under conditions which permit reaggregation. These results are consistent with the notion that cell-cell contact serves to elevate intracellular cAMP for use as a second messenger for gene regulation. However, interpretation is complicated by the other known function of cAMP in this organism, namely as a chemoattractant. It is well known that during aggregation, chemotaxis occurs via a cAMP signal relay system in which cells synthesize and secrete cAMP in an oscillatory manner in response to the binding of extracellular cAMP to surface receptors [22]. The cAMP synthesized in this process accumulates to considerably high levels during each oscillation but persists only transiently within the cell before being secreted. It is thus not clear whether this cAMP is sustained at an elevated level for a sufficiently long time, or even whether it is in the appropriate intracellular location, to serve as a second messenger for gene regulation. Indeed, there has been considerable controversy over whether the oscillatory increases in intracellular cAMP produced during the chemotactic response play any direct role in developmental gene regulation [22–33]. Clearly, the possible existence of multiple cAMP pools with different cellular functions complicates interpretation of any measurements of total intracellular cAMP, both during development as well as in response to various manipulations. Resolution of these questions is critical to any further progress in elucidating mechanistic relationships between cell surface events, cAMP metabolism, and gene regulation. Mutants such as EB-21 which are apparently capable of chemotaxis but which have defective cohesion, as well as other strains which have faulty signal relay but can be induced to acquire specific cohesion [31], should play prominent roles in unraveling these complex regulatory events.

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