

Specific cell–cell contact serves as the developmental signal to deactivate discoidin I gene expression in *Dictyostelium discoideum*

(cell–cell recognition/endogenous lectin/mRNA levels/developmental manipulations/cAMP)

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ABSTRACT Specific cell–cell contact is a major regulatory signal controlling cell differentiation in *Dictyostelium discoideum*, causing dramatic changes in the developmental program of gene expression. In this report, we focus on the relationships between specific cell–cell contact and the activity of the genes for discoidin I, an endogenous lectin that has been implicated in the cell–cell cohesion process. By performing quantitative RNA dot-hybridization assays and RNA gel blot-hybridization analyses, using as a probe a recombinant plasmid containing a discoidin I cDNA insert, we have measured changes in discoidin I mRNA levels during normal development and in response to specific manipulations of the state of cellular aggregation. Our major findings are as follows. (i) During normal development on filters, there is a close temporal correspondence between the establishment of specific cell–cell contacts and the decline in discoidin I mRNA levels. By the tight-aggregate stage, discoidin I mRNA is barely detectable. (ii) When tight aggregates are disaggregated and the cells are maintained in the disaggregated state, there is a dramatic rise in discoidin I mRNA content. (iii) When cells are developed in suspension (conditions that interfere with the establishment of tight cell–cell contacts), discoidin I mRNA accumulates to abnormally high levels, and these persist well after the levels in filter-developed cells have declined. Taken together, these results strongly suggest that cell–cell contact is the normal developmental signal to deactivate discoidin I gene expression; thus, a contact-deactivated gene for which a recombinant DNA probe is available has now been identified. Furthermore, we demonstrate that exogenous cAMP almost completely blocks the disaggregation-induced reactivation of discoidin I gene expression. Possible mechanistic relationships between specific cell–cell contact, intracellular cAMP levels, and developmental gene expression are discussed.

The morphogenetic assembly of cells into functional tissues involves complex interactions mediated by cell–cell contact. These interactions generally display striking specificity for cell type, often leading to the formation of stable intercellular cohesions and the triggering of new cellular programs such as division, motility, and differentiation. The major tasks presently confronting investigators are the identification and characterization of the molecular components of the recognition apparatus and the elucidation of their mode of action in cell-contact regulation of cellular behavior (1).

The cellular slime mold *Dictyostelium discoideum* provides an attractive model system in which to study the molecular events underlying cell–cell interactions (2). When vegetative amoebae are washed free of their food source and deposited on a solid substratum such as a buffer-saturated filter paper disc, they stop dividing and, after a short lag period, begin moving chemo-

tactically together (with cAMP as the chemoattractant). They eventually form “tight aggregates” containing up to 10^5 cells, which then undergo a series of defined morphogenetic transitions, culminating in the formation of a multicellular fruiting body containing two major differentiated cell types: stalk cells and spores. Of central importance for this discussion is that during the aggregation stage, the cells acquire the capacity for mutual cell surface recognition and cohesion (3). As a result, aggregation occurs not by the migration of individual cells but instead by the formation of long multicellular aggregation streams in which cells remain in continuous contact with their neighbors as the streams coalesce to form mounds. In addition to its presumed role in spatially organizing cells during their morphogenetic assembly, specific cell–cell contact has emerged as a pivotal event regulating the pattern of developmental gene expression, causing the activation of some specific genes (4–9) and the deactivation of others (5, 10, 11). Despite the extensive progress during the past decade in identifying putative molecular components of the cohesion apparatus (3, 12, 13), virtually nothing is known about the cell surface or intracellular events responsible for contact-mediated switches in gene expression.

An important recent breakthrough in the study of this problem was the identification of recombinant plasmids containing *D. discoideum* DNA inserts complementary to specific mRNAs that require continued specific cell–cell contact for their expression (7–9). These probes for contact-activated genes have enabled more direct and reliable analyses of the effects of specific cellular manipulations on gene expression than were previously possible when only the protein products of such genes could be assayed. Clearly, the identification of plasmids containing inserts for contact-deactivated genes would be of great value, though none have been described to date. In this report, we present direct evidence that the genes for discoidin I, an endogenous lectin implicated in the cohesion process (12), are deactivated by specific cell–cell contact. The availability of cloned plasmids containing discoidin I DNA inserts (14–19), coupled with the abundance of discoidin I mRNA in aggregating cells (18), make this the best available marker for contact-deactivated genes. A preliminary version of this work has been presented elsewhere.†

MATERIALS AND METHODS

Growth and Development of Cells. *D. discoideum* strain NC4 and its derivative A3 were grown on nutrient agar plates in association with *Klebsiella aerogenes* (20). To obtain vegetative

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cells; the plates were harvested in phosphate buffer (3.2 mM $\text{Na}_2\text{HPO}_4/12.8$ mM KH_2PO_4 , pH 6.4) upon first detectable clearing of the bacterial lawn, and the bacteria were removed by repeated washing and differential centrifugation in phosphate buffer. For development on filters (20), vegetative cells were washed once in filter development buffer (9.2 mM $\text{K}_2\text{HPO}_4/11.8$ mM $\text{KH}_2\text{PO}_4/20$ mM $\text{KCl}/5.3$ mM $\text{MgCl}_2/0.5$ mg of streptomycin sulfate per ml, pH 6.4), and 10^8 cells were deposited per filter disk (Whatman 50, 4.25 cm). The filters were each supported by a lower absorbent pad (Millipore AP10, 4.7 cm) saturated with filter development buffer. Alternatively, in some experiments 3×10^8 cells were deposited onto larger filters (Whatman 50, 7.0 cm), each supported by two buffer-saturated lower absorbent filters (Whatman 3, 7.0 cm). The filters were incubated at 22°C in a humidified chamber. Under these conditions, the cells 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. When the alternative of suspension development was used, vegetative cells were suspended to 2×10^7 cells per ml in phosphate buffer and incubated in an Ehrlenmeyer flask (5- to 10-fold greater volume) on a gyratory shaker (150 rpm) at 22°C. Suspension-developed cells are fully viable and will aggregate and fruit if deposited onto filters (3).

For disaggregation experiments, cells were first developed to the tight aggregate stage (16–17 hr) on small filters. The aggregates were then rinsed off with filter development buffer and diluted to a volume corresponding to 2.5 ml per filter. Aliquots were removed for initial time points, and the remaining suspension was diluted with an equal volume of filter development buffer supplemented with 20 mM EDTA (titrated back to pH 6.4). Cells were then disaggregated by repeated passage through a 10-ml pipette, and the cell suspension was transferred to a siliconized Ehrlenmeyer flask and incubated at 22°C on a rotary shaker (220 rpm). Cultures maintained this way remain almost entirely as single cells (3, 7).

At the designated time points, cells developing on filters were rinsed off with filter development buffer at 22°C and pelleted by centrifugation. Cells developing in suspension were likewise pelleted by centrifugation. The supernatants were discarded, and the pellets were frozen in liquid nitrogen and stored at -70°C.

Cytoplasmic RNA Isolation and Analysis. Cell pellets were lysed, nuclei were removed by centrifugation, and the post-nuclear supernatants were treated with NaDodSO_4 , all as described by Blumberg and Lodish (21). Total cytoplasmic RNA was then purified by the method of Wieben (22). RNA gel blot-hybridization analysis was performed as described by Thomas (23), except that the gels were 14 cm long and were run vertically for 1.5–2 hr and the dextran sulfate was omitted from the hybridization step. Typically, hybridizations were performed with 0.5–1 μg of labeled DNA in 5–10 ml of hybridization buffer. For quantitative analysis of RNA preparations, we performed RNA dot hybridizations. RNA samples were diluted into 3 M $\text{NaCl}/0.3$ M trisodium citrate and aliquots (200 μl , containing a fixed amount of RNA as indicated) were applied to a nitrocellulose sheet (preequilibrated with the diluent) under gentle suction with a Hybri-Dot Manifold (Bethesda Research Laboratories). Each well was then rinsed with 250 μl of 3 M $\text{NaCl}/0.3$ M trisodium citrate. Conditions for baking, prehybridization, hybridization, washing, and autoradiography were the same as for the gel transfers. Quantitation was performed by cutting out the dots with a cork borer and analyzing them in ACS counting scintillant (Amersham). Blanks (determined by cutting circles from either blank regions of the nitrocellulose or regions spotted with identical quantities of HeLa cell RNA)

have been subtracted to give the data shown. Data are expressed either as cpm hybridized or relative mRNA levels. Control experiments with each of the DNA probes used indicated that this method is highly reproducible and that, when a fixed quantity of total RNA is applied per sample, the cpm hybridized is proportional to the content of the complementary specific mRNA (data to be published elsewhere). It should be noted that comparisons of the intensities of bands or spots on autoradiograms and comparisons of cpm hybridized are only meaningful within a given experiment with the same probe because the specific activities of the probes and the durations of autoradiographic exposures vary from one experiment to the next. Our results indicate a very good agreement between the RNA dot hybridization and the RNA gel blot hybridization analyses, confirming that both techniques measure the same specific mRNAs.

Recombinant Plasmid Probes. Plasmid pDd812 is a recombinant of pMB9 containing a cDNA insert of ≈ 500 nucleotides derived entirely from the protein coding region of the discoidin Ia gene (14, 16). By RNA gel blot-hybridization analysis, this probe detects a single cytoplasmic RNA band of ≈ 960 nucleotides (discoidin I mRNA) (ref. 14; also see *Results*). Plasmid PL3 is a recombinant of pBR322 containing a cDNA insert that hybridizes with a single mRNA that is absent from vegetative cells and does not appear until late in development on filters; expression of this mRNA appears to require continued specific cell-cell contact (ref. 9; also see *Results*). Plasmid DNA (1 μg) was labeled with 250 μCi (1 Ci = 3.7×10^{10} Bq) of $[5' \text{-}^{32}\text{P}]\text{dCTP}$ (800 Ci/mmol, New England Nuclear) by a modification of the nick-translation method of Rigby *et al.* (24). Incorporation typically exceeded 65% of the input radioactivity, based on trichloroacetic acid precipitation analysis.

RESULTS

In the experiments described below, we analyzed discoidin I mRNA levels in cells during normal development and in cells whose aggregation states had been experimentally manipulated. For comparison, we also used plasmid PL3 to measure levels of another specific mRNA, one whose expression had been shown to require continued cell-cell contact (9).

Time Course of Discoidin I mRNA Levels During Normal Development. The developmental time course of discoidin I mRNA levels in cells developed on filters is shown in Fig. 1. Discoidin I mRNA was absent from vegetative cells and was detected first after 2 hr. Thereafter, the level increased to a peak around 8 hr and then declined sharply during the next few hours. By 15 hr, discoidin I mRNA was barely detectable and remained so throughout the remainder of development. This pattern of expression is consistent with previous reports for strain NC4 analyzed by *in vitro* translation (25), RNA excess hybridization kinetics (18), and RNA gel blot analysis (15) as well as with the pattern obtained for strain V-12M2 analyzed by quantitative primer extension analysis (26).[‡] The mRNA detected by plasmid PL3 showed a very different regulatory pattern, being absent from vegetative cells and throughout the first 15 hr of development on filters (Fig. 1). Thereafter, the level increased continuously during later development stages. These data are consistent with the earlier report of Mangiarotti *et al.* (9), which showed this mRNA to appear first late in development.[‡]

The timing of the peak in discoidin I mRNA (8 hr) and the subsequent decline (8–12 hr) corresponds to the period when

[‡] When axenically grown cells are examined, changes in mRNA levels occur earlier than for bacterially grown cells (9, 14, 15, 26). This is due to differences in the physiologic states of axenically vs. bacterially grown vegetative cells, not to strain differences.

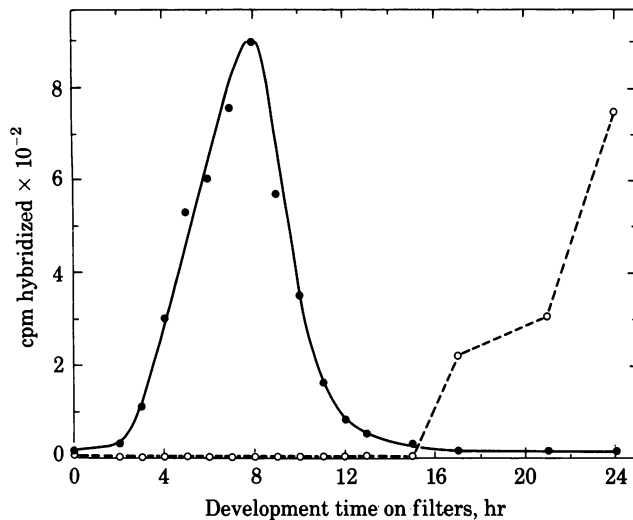


FIG. 1. Developmental time course of mRNA levels during development on filters. A3 cells were developed on filters. Cytoplasmic RNA samples were spotted in duplicate ($5 \mu\text{g}$ per spot) and analyzed with probes pDd812 (—) and PL3 (---).

aggregation streams first form and then coalesce into discrete mounds (2). Cells in such aggregation streams are known to be in intimate contact with one another through the specific cohesion system that is produced during the aggregation stage (3). This suggested to us the possibility that specific cell-cell contact might serve as the signal to deactivate discoidin I gene expression. In the following experiments, we tested this hypothesis by taking advantage of the ease with which the developmental program of *Dictyostelium* can be experimentally perturbed.

Effects of Disaggregation on Discoidin I mRNA Levels. *Dictyostelium* cell aggregates can be readily disaggregated (without the need for hydrolytic enzymes) and maintained in suspension under conditions that prevent reaggregation (3). This manipulation has proved to be extremely useful in the analysis of the role of cell-cell contact in gene regulation, particularly in the identification of recombinant plasmids encoding contact-activated mRNAs (7, 9). Therefore, we examined the effects of disaggregation on discoidin I gene expression, with PL3 serving as a control. Cells were developed on filters to the tight-aggregate stage, by which time discoidin I mRNA had increased and then declined to negligible levels and the expression of PL3 mRNA had begun (Fig. 1). Aggregates on one set of filters were rinsed off and disaggregated, and the cells were maintained in suspension in the disaggregated state. A control set of filters was left unperturbed to continue normal development. The effects of these manipulations on gene expression are shown in Fig. 2. Whereas cells maintained on filters in the aggregated state had the expected low levels of discoidin I mRNA throughout the remaining time course of this experiment, disaggregation induced a dramatic increase, with discoidin I mRNA levels increasing >30-fold within 5.5 hr after disaggregation. By contrast, PL3 mRNA showed the opposite behavior (as expected, see ref. 9), increasing normally when aggregates were maintained on filters but decreasing to negligible levels upon disaggregation. These data suggest that continued cell-cell contact is required to keep discoidin I gene expression in the deactivated state.

Comparison of Discoidin I mRNA Levels in Cells Developed on Filters vs. in Suspension. As a second manipulation to test the role of cell-cell contact in regulating discoidin I gene expression, we compared discoidin I mRNA levels in cells de-

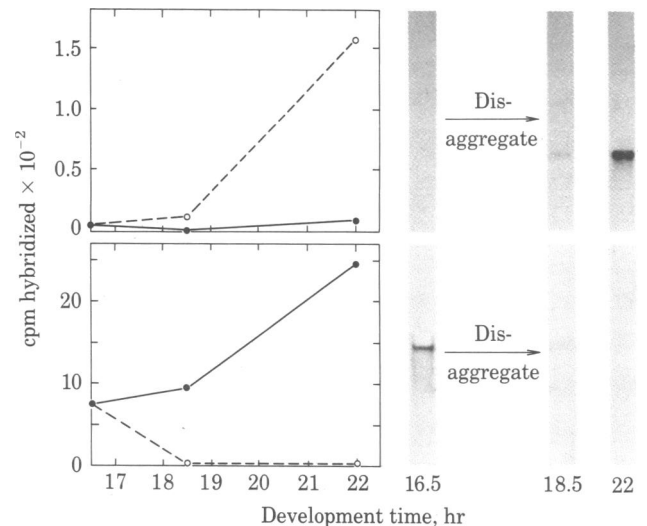


FIG. 2. Effect of disaggregation on mRNA levels. NC4 cells were developed on filters for 16.5 hr. One set of filters was allowed to continue development unperturbed, while cells on the remaining filters were disaggregated and maintained in suspension as described. (Left) RNA dot-hybridization analysis. Cytoplasmic RNA samples ($2.5 \mu\text{g}$) from cells either left unperturbed on filters (—) or disaggregated (---) were analyzed with probes pDd812 (Upper) and PL3 (Lower) as described. (Right) RNA gel blot-hybridization analysis. The indicated cytoplasmic RNA samples ($8 \mu\text{g}$ per lane) were analyzed with probes pDd812 (Upper) and PL3 (Lower), as described.

veloped in suspension vs. on filters. Suspension-developed cells are known to synthesize many of the proteins that normally appear early in filter-developed cells, including the discoidins (27); however, they do not activate genes whose expression requires cell-cell contact (6, 7, 28), presumably because the conditions of suspension development do not allow functional contacts to form efficiently. Thus, it seemed reasonable to predict that during suspension development, discoidin I gene expression might not be deactivated normally, resulting in overexpression of discoidin I mRNA. The experiment shown in Table 1 supports this notion: discoidin I mRNA first appeared at the normal time (around 2 hr) in suspension-developed cells and accumulated during the next few hours. By the time the peak level was observed on filters (8 hr), the suspension-developed cells had accumulated even higher quantities of discoidin I mRNA. Moreover, these high levels persisted much longer in suspension so that by 11 hr, when filter-developed cells had lost >80% of their

Table 1. Comparison of mRNA levels in cells developed on filters vs. in suspension

Plasmid probe	Development time, hr	Relative mRNA levels		Suspension/filters ratio
		Filters	Suspension	
pDd812	2	4	7	1.8
	5	58	71	1.2
	8	100*	184	1.8
	10	39	183	4.7
	11	19	142	7.5
	13	6	75	12.5
	15	3	47	15.6
PL3	0-15	<2 [†]	<2 [†]	—
	24	100*	<2 [†]	<0.02 [†]

A3 cells were developed on filters or in suspension, and the cytoplasmic RNA ($5 \mu\text{g}$ in duplicate) was analyzed by dot hybridization as described.

* Data were normalized so that these values are arbitrarily set to 100.

[†] Below detectable limits.

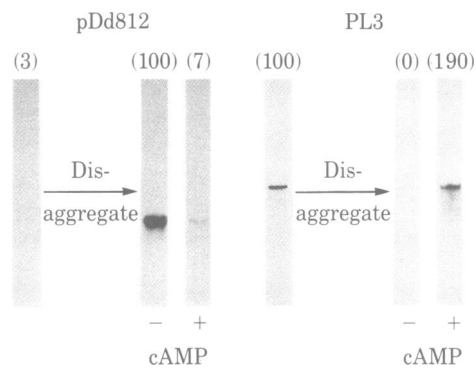


FIG. 3. Effect of exogenous cAMP on disaggregation-induced changes in mRNA levels. NC4 cells were developed on filters for 16.5 hr, then disaggregated, and suspended as described. An aliquot was removed for the initial time point, and the remaining suspension was divided in two for subsequent shaker incubation. One portion received no additions, while the other received additions of cAMP in increments corresponding to 1 mM (final concentration) at 0.25, 1, 2, 3, 4, and 5 hr after disaggregation. Cells were collected at 5.25 hr after disaggregation, and cytoplasmic RNA was prepared and analyzed by RNA gel blot-hybridization analysis (8 μ g per lane) and RNA dot-hybridization analysis (4 μ g per spot, in duplicate) with probes pDd812 (*Left*) and PL3 (*Right*). The numbers shown in parentheses above each gel represent the relative amounts of each mRNA (arbitrary units) as determined by RNA dot-hybridization analysis.

peak discoidin I mRNA, the suspension-developed cells still contained significantly more than was ever attained by their counterparts on filters. Over the next few hours, when discoidin I mRNA dropped to negligible levels in filter-developed cells, the decline in suspension was much more gradual. As expected, PL3 mRNA remained below detectable limits in suspension-developed cells, similar to what has been reported for other contact-dependent mRNAs (7).

Effect of cAMP on the Disaggregation-Induced Reactivation of Discoidin I Gene Expression. In several instances, exogenous cAMP has been shown to block or reverse the changes in gene expression normally induced by disaggregation, suggesting the hypothesis that this nucleotide might serve as a critical mediator in the regulatory mechanism (7, 28–31). The experiment shown in Fig. 3 supports this notion for discoidin I gene expression. Addition of exogenous cAMP to disaggregated cells maintained in suspension nearly completely blocked the disaggregation-induced increase in discoidin I mRNA. Similarly, exogenous cAMP blocked the disaggregation-induced loss of PL3 mRNA [consistent with a previous report (9)], and, in fact, allowed the levels to increase comparably to what was seen with cells maintained on filters (compare Figs. 2 and 3). Thus, exogenous cAMP has an effect on discoidin I gene expression similar to what has been shown for other contact-regulated genes, i.e., it blocks the disaggregation-induced changes in mRNA levels. It should be noted that an inhibitory effect of exogenous cAMP on the early starvation-induced accumulation of discoidin I mRNA has been reported (15).

DISCUSSION

The changes in the levels of discoidin I mRNA during normal development and in response to specific manipulations of the state of cellular aggregation strongly support the conclusion that specific cell–cell contact is the normal developmental signal to deactivate discoidin I gene expression. The three lines of evidence are summarized as follows. (i) During normal development on filters, there is a temporal correspondence between the period when specific cell–cell contacts are established and discoidin I gene expression is deactivated. Thus, aggregation

streams begin to assemble at around 8 hr and coalesce into discrete mounds during the next few hours, with the cells continually maintaining intimate contact with their neighbors (2, 3); concomitant with this, discoidin I mRNA peaks around 8 hr and declines precipitously between 8 and 12 hr (Fig. 1). (ii) When tight cell aggregates are disrupted and the cells are maintained in the disaggregated state, discoidin I gene expression is reactivated (Figs. 2 and 3). It should be emphasized that the disaggregation-induced changes are quite dramatic, with discoidin I mRNA levels increasing over 30-fold within 5.5 hr and the ratio of discoidin I mRNA to PL3 mRNA increasing more than 1,500-fold compared to cells that are left unperturbed on filters (Fig. 2). When direct comparisons were made by performing the RNA dot-hybridization analyses side by side, the discoidin I mRNA levels observed 5.5 hr after disaggregation were found to equal or exceed the maximum level achieved during normal filter development (data not shown). (iii) When cells are developed in suspension rather than on filters, discoidin I mRNA first appears at the normal time but accumulates to a level almost double that observed on filters. Furthermore, relatively high levels persist much longer in suspension so that at 13–15 hr, when tight aggregates are forming on filters, the suspension-developed cells contain over 10-fold more discoidin I mRNA than do their filter-developed counterparts (Table 1).

In reaching our conclusions, we felt it necessary to apply each of the above experimental protocols because of our concern about possible side effects of the conditions used in the standard disaggregation protocol and our limited understanding of the minimal requirements for functional cell–cell contacts. Demonstration of the temporal correspondence between contact formation and discoidin I gene deactivation during normal filter development avoids the use of disruptive treatments with possible side effects but by itself represents only a correlation. The disaggregation protocol used is indeed very effective in disrupting tight cell–cell contacts and allows the culture to be maintained primarily as single cells, but the requirement for EDTA raises questions about possible side effects on gene expression not related to disruption of contacts. Finally, while it is generally assumed that cell–cell contacts are formed less efficiently in suspension and that the aggregates produced in suspension are not as tight as those obtained on filters (7, 28), it must be acknowledged that aggregates in fact do form in suspension and that these display all of the properties of the specific cohesion system [i.e., species specificity, developmental specificity, and preference for end-to-end cohesion (3)]. Therefore, it is not surprising that discoidin I mRNA levels do decline eventually in suspension, albeit more slowly than on filters. As yet, we have been unable to prevent aggregate formation in suspension in the absence of EDTA, even at high shaker speeds (data not shown). Thus, while each of the experimental protocols used has its limitations, taken collectively they strongly suggest that specific cell–cell contact is the common denominator in the deactivation of discoidin I gene expression. However, at this point we cannot rigorously exclude the possibility that the requirement is for close proximity rather than for actual contact. We presently are attempting to develop other protocols to block or disrupt specific cell–cell contact with the goal of dissecting in detail the requirements for functional contacts and elucidating the functional relationships between contacts formed on filters vs. in suspension.

With these findings, discoidin I now represents a contact-deactivated gene for which a recombinant DNA probe is available. Previous reports suggesting a suppressive role for cell–cell contact in the expression of certain genes focused on components of the cAMP chemotaxis system such as cAMP phosphodiesterase and surface cAMP receptors (5, 10, 11). These

studies relied on activity assays for these proteins in cells subjected to disaggregation and suspension development and, although informative, were subject to the limitations inherent in using measurements of functional protein products to assess the activities of the corresponding genes. In this regard, it should be noted that analysis of the effects of such treatments on discoidin I protein levels would not have revealed the regulatory behavior occurring at the gene level because the lectin itself is quite stable and persists at high levels late in development, long after discoidin I mRNA has declined (12). This highlights the advantage of using cloned DNAs as probes to measure specific mRNA levels directly, particularly when studying gene deactivation. It is also interesting to note that the contact-deactivated genes identified thus far all encode components of the chemotaxis and cohesion systems. This suggests a feedback regulatory mechanism of obvious rationale because further synthesis of these components would seem to be unnecessary once cells have aggregated and established functional contacts with one another.

It is interesting to note the temporal relationships between the appearance and disappearance of the contact-regulated mRNAs. During filter development, discoidin I mRNA begins to decline at 8 hr, but PL3 mRNA does not appear until after 15 hr (Fig. 1). Although this time delay might at first seem to suggest that the two processes are induced by different initial events, this need not be the case because a considerable delay is seen also with the mRNA changes induced by disaggregation: PL3 mRNA completely disappears within 1 hr (and possibly earlier), whereas discoidin I mRNA just begins to appear at 2 hr (Fig. 2). Thus, the delays between mRNA disappearance and appearance may reflect, at least in part, differences in the times required for the cells to mobilize the machineries for specific mRNA degradation vs. synthesis and not necessarily differences in the initial events triggering these changes.

A final point of discussion concerns the role of cAMP in the expression of contact-regulated genes. Previous work from other laboratories has demonstrated that exogenous cAMP accelerates the normally occurring changes in gene expression, and that cAMP addition to cells that have been disaggregated blocks or reverses the disaggregation-induced changes (7, 28–32). It is generally assumed that such effects are due to the increase of intracellular cAMP by passive diffusion into the cells because high concentrations (typically 1–5 mM) are required and the levels of surface cAMP receptors are low in cells that have reached the tight aggregate stage (10). Thus, cAMP may function as an intracellular “second messenger” in the contact-mediated regulatory response, analogous to what has been proposed for surface receptor-mediated functions in a variety of biological systems (33). Our results support this notion because added cAMP suppresses the reactivation of discoidin I gene expression normally produced by disaggregation (Fig. 3). Thus, one important focus for future mechanistic studies should be to test directly whether cell–cell contact does indeed serve to elevate intracellular cAMP levels. With recombinant probes now available for both contact-activated and contact-deactivated genes, studies of the cell surface and intracellular mechanisms involved in contact regulation of gene expression should proceed at a greater pace.

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