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A localized differentiation-inducing-factor sink in the front of the *Dictyostelium* slug

(gradient/morphogen/cell sorting/differentiation-inducing factor 1 dechlorinase/DIF-1)

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ABSTRACT **Differentiation-inducing factor 1 [DIF-1; 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-hexan-1-one] induces stalk cell differentiation during *Dictyostelium* development. It is present as a gradient in the multicellular slug, its lowest concentration being in the anterior. Here we demonstrate the existence of a localized sink for DIF-1, also in the anterior of the slug, which could be responsible for generating the DIF-1 gradient. DIF-1 is metabolized extensively by developing cells, initially by a mono-dechlorination. We used an enzyme assay for DIF-1 dechlorinase to examine its distribution in the slug. DIF-1 dechlorinase activity is 30-fold higher in prestalk cells (largely anterior) compared with prespore cells (posterior) when these are separated from each other on Percoll density gradients. Dissection experiments showed that DIF-1 dechlorinase is 25-fold enriched in the anterior 13% of the slug compared with the rest. These experiments also showed that DIF-1 dechlorinase is more anterior-enriched than the standard prestalk markers, the ecmA and ecmB mRNAs. When cut from a slug, both prestalk and prespore fragments regulate to restore the missing cell type. Prespore fragments rapidly regain (by 30 min) a DIF-1 sink in their anteriors, and prestalk fragments restore a posterior zone with low DIF-1 dechlorinase by 4 hr after cutting. The reappearance of the DIF-1 sink in the anterior of prespore fragments is accomplished without detectable cell sorting and may, therefore, be in response to positional signals. Finally, a localized sink may provide a general way of producing a gradient of a signal substance in a developing embryo.**

Much of embryonic development is believed to be organized by gradients of morphogens in the embryo (1–3). The local morphogen concentration is measured by the developing cells and then used to direct them to different fates at different points on the gradient. In principle, morphogen gradients could be produced in an embryo by diffusion of the morphogen from a localized source or by destruction of the morphogen in a localized sink. The bicoid protein gradient in *Drosophila* is an example of a gradient produced by diffusion from a localized source (4), and the special organizing centers, discovered in various embryos, could also be sources of morphogens (5–7). However, to our knowledge, there is not yet an example of a gradient being produced by destruction of a morphogen in a localized sink. The *Dictyostelium* slug contains a gradient of differentiation-inducing factor 1 [DIF-1; 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-hexan-1-one] (8), the inducing signal for stalk cell differentiation (9); its low point is in the anterior. We are interested in how this gradient is generated, even though there is no proof yet that the gradient has an informative role in development.

The *Dictyostelium* slug is sausage-shaped and up to a few mm long. The anterior one-third consists of prestalk cells, and the remainder consists of prespore cells with a scattering of prestalk cells [called anterior-like (AL) cells (10)] among them. At culmination, the prestalk and prespore cells form the stalk and spores, respectively, of the mature fruiting body, and the AL cells form ancillary tissue, such as the basal disc (11, 12). The very front of the slug, the tip, has special organizing properties, indicating that it is a center for morphogenetic signaling (5, 13, 14).

The slug is highly regulative. When either the prestalk or the prespore zone is cut off, the remaining tissue can reorganize, restore the missing cell type, and eventually form a normally proportioned fruiting body. Repatterning of a prestalk isolate seems to use positional cues (presumably given by an underlying gradient) to direct the new prespore zone to form in the posterior of the isolate (15). The mechanism of repatterning of a prespore isolate is controversial; in one view, it could depend on sorting of AL cells (10, 16) or, in another view, it could depend again on a positional mechanism (17, 18).

DIF-1 is a substance (19) that is released by developing cells and appears to be the central regulator of prestalk and stalk cell differentiation. DIF-1 directs cells developing in culture to differentiate into stalk cells instead of spores (20) and induces cells to express prestalk-specific genes while repressing the expression of prespore-specific genes (21–23). Treatment of migrating slugs with DIF-1 alters their pattern, causing a near doubling in the proportion of prestalk tissue (24, 25). Finally, mutants that make only low levels of DIF-1 are blocked in development as mounds and do not express prestalk markers unless DIF-1 is provided (26).

Dictyostelium cells metabolize DIF-1 extensively to produce at least 12 metabolites (27). The first metabolite, initially called DM1, has recently been identified as DIF-3, the monochloro derivative of DIF-1 (see ref. 28). The first step in DIF-1 metabolism is, therefore, a reductive mono-dechlorination. This step is catalyzed by a cytoplasmic enzyme with glutathione as its cofactor (28). In this paper we report on the localization of DIF-1 dechlorinase in slugs and its regulation as prespore and prestalk isolates restore their normal patterning.

MATERIALS AND METHODS

Growth and Development of Cells. All procedures with live cells were at 22°C. V12M2 cells were grown on nutrient agar plates in association with *Klebsiella aerogenes* (29) and freed of bacteria by four centrifugal washes in K/K_2 (20 mM K/K_2 phosphate/2 mM $MgSO_4$, pH 6.1) and a final wash in 10% (vol/vol) NS (NS is 10 mM KCl/10 mM NaCl/1 mM $CaCl_2$) before being plated as streaks of $\approx 10^7$ cells on 1.8% Oxoid

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Abbreviations: DIF-1, differentiation-inducing factor 1 [1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-hexan-1-one]; AL, anterior-like.

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L28 agar/10% NS. The plates were placed in unilateral light, and the resulting slugs were generally used at 18–24 hr of development. In some cases, cells were stained with neutral red at 50 μg/ml (30) before plating.

Cell Separations. Prestalk and prespore cells were separated on Percoll density gradients, as described (31). Slugs were cut into prestalk and prespore fragments with a microknife; each plate of slugs was open for no more than 10 min during cutting. Samples of ≈200 slugs, or slug fragments, were accumulated in ice-cold K/K_2 in a microcentrifuge tube. Cells or slug fragments were briefly centrifuged, and the pellets were frozen at −20°C for subsequent assay.

To determine the proportion of prespore cells in the separated cell populations, fixed cells were stained with an antibody raised against *Dictyostelium mucoroides* spores (32), followed by the appropriate fluorescent antibody, and viewed by fluorescent microscopy.

DIF-1 Dechlorinase Assay and RNA Analysis. Frozen cell pellets were resuspended in lysis buffer [50 mM Hepes/50 mM KCl/1 mM EDTA/1 mM dithiothreitol/10% (vol/vol) glycerol/0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM 7-amino-1-chloro-3-tosylamido-2-heptane (TLCK)/0.02% NaN_3, pH 8.2] at 0°C by a combination of trituration and mixing, then centrifuged at 350,000 × *g* for 30 min (Beckman TL-100). Enzyme activity was assayed in the supernatants, as described (28) with minor modifications. Ten to fifty micrograms of lysate protein was incubated in 50 μl of lysis buffer with 100 nM ^{3}H-labeled DIF-1 (custom synthesis by Amersham, 0.1 μCi per assay; 1 Ci = 37 GBq) and 5 mM glutathione at 25°C for 60 min. At the end of incubation, the radioactive compounds were extracted with 1 ml of ethyl acetate, containing 200 μg of butylated hydroxytoluene. The organic phase was dried down, taken up in 20 μl of methanol/chloroform, 1:1, containing tocopherol at 5 mg/ml, and applied to a Whatman LK6D TLC plate; DIF-1 was then separated from the reaction product DIF-3 by chromatography in hexane/ethyl acetate/acetic acid, 60:40:2. Plates were dried, sprayed with EN^{3}HANCE (NEN), and exposed to x-ray film. Results were quantitated by scintillation counting, after scraping off labeled bands into 0.5 ml of ethanol followed by 4 ml of Quickszint scintillation cocktail (Zinsser Analytical, Frankfurt).

Protein was determined by the Bio-Rad dye-binding assay with bovine serum albumin used as the standard.

Northern analysis of mRNA was done as before (33), and the intensity of bands on the autoradiograms was quantitated by using a computing densitometer (Molecular Dynamics, Sunnyvale, CA).

RESULTS

DIF-1 Dechlorinase Is Located in the Anterior of the Slug. DIF-1 dechlorinase enzymatic activity can be determined in the high-speed supernatants of cell lysates by following the conversion of ^{3}H-labeled DIF-1 to ^{3}H-labeled DIF-3, with substrate and product separated by TLC. Using this assay, we determined the distribution of DIF-1 dechlorinase in the slug.

Slugs were disaggregated into separate cells, and prestalk and prespore fractions were separated by centrifugation through Percoll density gradients. As shown in Fig. 1 and Table 1, DIF-1 dechlorinase activity is ≈14-fold enriched in the prestalk fraction compared with the prespore fraction. The prestalk fraction was 85–91% pure, and the prespore fraction was 97–98% pure, as determined by staining prespore cells with a specific antibody (32). When allowance is made for this cross contamination, the enrichment rises to 30-fold (Table 1).

The prestalk cell fraction from the gradient contains AL cells from the posterior of the slug (34) as well as prestalk cells

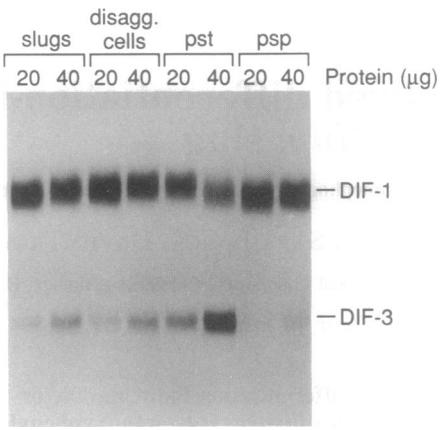

FIG. 1. TLC illustrating the localization of DIF-1 dechlorinase to prestalk cells. DIF-1 dechlorinase was assayed in cell lysates prepared from slugs of strain V12M2; their disaggregated (disagg.) cells and the prestalk (pst) and prespore (psp) fractions were obtained after centrifugation of these cells through a Percoll gradient.

from the anterior. We therefore confirmed that DIF-1 dechlorinase is in the anterior of the slug by dissection experiments. When slugs were cut at ≈40% of their length from the tip, the anterior fragment contained 87% of the total slug DIF-1 dechlorinase activity, at 13-fold higher specific activity than the posterior fragment. A more refined dissection, in which slugs were cut closer to the tip, showed that the anterior 13% (by protein) contained 76% of the dechlorinase, 25-fold enriched over the rest of the slug (Table 1).

We also compared the distribution in the slug of DIF-1 dechlorinase to standard mRNA markers: ecmA for prestalk A cells, ecmB for prestalk B cells, and PsA/D19 for prespore cells (35, 36). DIF-1 dechlorinase and the prestalk mRNAs were enriched in the prestalk fraction of gradient-separated cells to a similar extent, >10-fold in both cases (compare Table 1 and ref. 35). However, they did not colocalize in dissected slugs: ecmA mRNA was ≈4-fold enriched and ecmB mRNA was slightly de-enriched in the anterior 40% of the slug, compared with the 13-fold enrichment of DIF dechlorinase (Table 2). Comparable results have been obtained previously for the distribution of ecmA mRNA (35) and ecmB mRNA (K. Jermyn, personal communication) in slugs. Evidently the ecmA mRNA, and especially the ecmB mRNA, are found in prestalk cells throughout the slug (posterior AL cells as well as anterior prestalk cells), whereas DIF-1 dechlorinase must be essentially confined to the anterior prestalk cells. These experiments show that DIF-1 dechlorinase is located in prestalk cells and is largely confined to the anterior of the slug. We therefore regard the anterior of the slug as a localized sink for DIF-1.

Regulation of DIF-1 Dechlorinase in Prespore and Prestalk Isolates. When a slug is cut into prespore and prestalk fragments, these fragments reorganize, restore the missing cell type, and eventually form a normal fruiting body (5). If the DIF-1 sink is an integral part of the patterning process in the slug, it would be expected to regulate in isolated slug fragments—perhaps to restore the original situation of the intact slug. Preliminary experiments revealed a complication: the level of DIF-1 dechlorinase in slugs is peculiarly sensitive to environmental disturbance. Opening the lid of the Petri dish for just 10 min to dissect slugs causes up to a 5-fold stimulation in DIF-1 dechlorinase specific activity 1 hr later (data not shown). Further investigation showed that this increase in enzyme activity is restricted to the prestalk zone of stimulated slugs and does not affect the prespore zone (compare the control curves in Fig. 2 *A* and *B*).

When a prespore zone is isolated, it first ceases migration and, over a period of ≈2 hr, rounds up and then forms a new

Table 1. Distribution of DIF-1 dechlorinase in migrating slugs

Separation method	*n*	DIF-1 dechlorinase, pmol/mg per hr			Prestalk/prespore ratio		Enzyme in anterior fraction, %
		Whole slugs	Prestalk	Prespore	Uncorrected	Corrected	
Gradient separation	6	36.4 ± 10.4	136 ± 35.5	8.4 ± 5.3	16.2	29.6	
Dissection front 36%*	12	29.4 ± 13	96.5 ± 45.8	7.4 ± 4.1	13.0		87
Dissection front 13%†	2	19.1	120	4.7	25.5		76

Slugs of strain V12M2, migrating in unilateral light, were either disaggregated and separated into prestalk and prespore cells by density-gradient centrifugation or cut into anterior (prestalk) and posterior (prespore) fractions with a microknife. Purity of prestalk and prespore fractions separated by gradient centrifugation was monitored by staining with an antibody against prespore cells to allow correction for cross-contamination (corrected column). Cell separations were done on 24- or 48-hr-old slugs; results did not differ significantly, so these results are combined. DIF-1 dechlorinase specific activity was determined as described.
*Anterior fragment = 36% total protein.
†Anterior fragment = 13% total protein.

tip. The isolate usually fruits on the spot, without forming a migrating slug. Fig. 2*B* shows that DIF-1 dechlorinase starts to reappear in prespore isolates within 30 min of amputation and rises to a peak at 2 hr, which is at least 30 times the level in the starting prespore zone. After this time, the level drops down to about the level of intact slugs. In contrast, the prespore zones of control slugs show no such rise.

DIF-1 dechlorinase levels rise in prestalk zones soon after they are cut from slugs, before eventually falling to about their initial level (Fig. 2*A*). However, as already mentioned, a large increase also occurs in the prestalk zone of intact slugs when the plates are opened for 10 min (as happens during slug cutting). Therefore, much of the increase in enzyme activity seen in isolated prestalk zones may be from this "open lid" effect and not from cutting the prestalk zone from the slug. The cause of the increase in DIF-1 dechlorinase activity in prestalk isolates, therefore, remains uncertain.

Repatterning of Prespore and Prestalk Isolates. We then asked whether the DIF-1 dechlorinase becomes properly localized in prespore and prestalk isolates. The plan and outcome of these experiments are shown diagramatically in Fig. 3. Prespore zones were cut from slugs, allowed 30–45 min to regulate, and then cut a second time into front and back halves. As shown in Table 3, the enzyme reappears in the front of the isolate and does not reappear in the back. In a wounding control, the rear of the prespore zone was cut off, and DIF-1 dechlorinase was assayed in the remaining prespore zone 30–45 min later. This wounding did not stimulate any enzyme activity in the prespore zone (data not shown).

Individual prestalk isolates were too small to dissect further, but we could demonstrate repatterning by combining them in mounds of ≈20 and allowing them to regulate for 4 hr, at which time long thin slugs had emerged. These slugs were then cut into fronts and backs, and again the DIF-1 dechlorinase was essentially restricted to the front (Table 3).

Prespore isolates are believed to reform their prestalk zones from AL cells, which sort to the anterior of the isolate (10, 16). However, we could not detect any such sorting of AL cells, stained with neutral red (30), in our prespore isolates until ≈2 hr of regulation, at the time of tip formation (data not shown).

Table 2. Distribution of DIF-1 dechlorinase and mRNA markers in dissected slugs

	n	Anterior/posterior ratio	Marker
DIF-1 dechlorinase	3	12.9:1	Prestalk cells
ecmA mRNA	4	4.4:1	Prestalk A cells
ecmB mRNA	4	1:1.5	Prestalk B cells
PsA/D19 mRNA	4	1:8.6	Prespore cells

Slugs were cut into anterior and posterior segments at ≈one-third of their length from the front. About 400 were collected for each experiment, and the pooled anteriors and posteriors were divided into two for enzyme and mRNA assays. An additional experiment in which the enzyme was not assayed is also included.

DISCUSSION

We have demonstrated the existence in the *Dictyostelium* slug anterior of a localized sink for DIF-1, the inducer of prestalk cell differentiation. The existence of this sink both explains and confirms the previous observation of a gradient in DIF-1 concentration in the slug with its lowest point in the anterior (8). The DIF-1 sink seems to be intimately tied into the patterning process in the slug because it is restored in prespore and prestalk isolates when they regulate to restore normal patterning.

Logically, the DIF-1 sink must also be the source of a gradient of DIF-1 metabolites in the opposite direction to the DIF-1 gradient. The first metabolite, DIF-3, is largely cell-associated, and only a small proportion of it is released into the medium (27, 37); any effects it might have are, therefore, likely to be local to the metabolizing cells. The other metab-

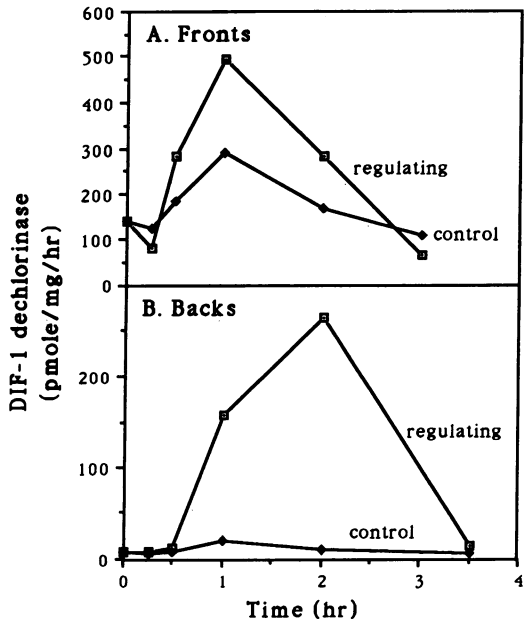

FIG. 2. Time course of regulation of DIF-1 dechlorinase during repatterning of isolated prestalk fragments (*A*) and isolated prespore fragments (*B*) from slugs. Migrating slugs of strain V12M2 were cut into prestalk and prespore fragments, and at the indicated times these fragments were collected (≈200 for each time point), and their content of DIF-1 dechlorinase was assayed. Each plate of slugs was open for only 10 min during cutting. Plates of control slugs were similarly opened for 10 min at the start of the experiment, and the prespore and prestalk zones were dissected at the indicated times afterward for enzyme assay.

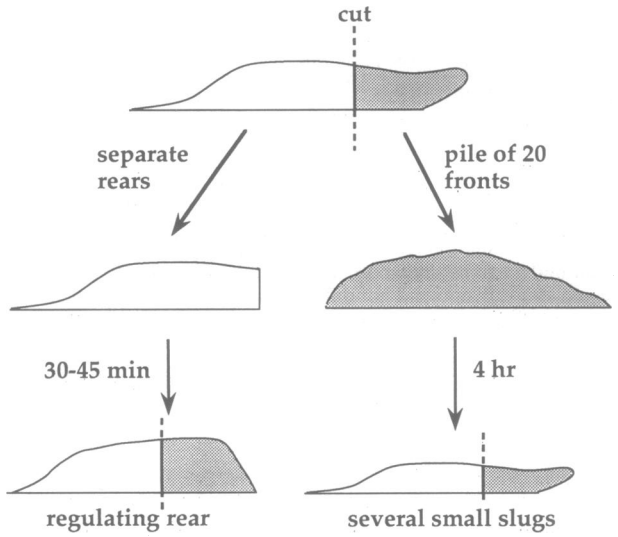

FIG. 3. Schematic diagram of plan and outcome of repatterning experiments. The objective of these experiments was to determine where the DIF-1 sink became localized in regulating prestalk (fronts) and prespore (rears) zones. Speckled areas show fragments containing DIF-1 dechlorinase: the enzyme is lost from the rear of prestalk isolates and gained in the front of prespore isolates. Slugs were cut into fronts and rears; the fronts were combined into mounds of ≈20 and allowed to regulate for 4 hr, by which time long thin slugs had emerged. These slugs were dissected into fronts and backs, and DIF-1 dechlorinase was determined. The rears were kept separate and dissected for enzyme assay after 30–45 min of regulation. At this time they were in the process of rounding up, but they still remained somewhat elongated.

olites are largely released into the medium and could potentially have more global effects in the aggregate.

Because DIF-1 induces prestalk cell differentiation and is metabolized by prestalk cells, there is the suggestion of a negative-feedback loop in the slug to control DIF-1 levels. This idea is strengthened by the finding that DIF-1 induces rapid expression of DIF-1 dechlorinase in responsive cells (38).

Although DIF-1 is an essential part of the patterning process, it is unlikely that the DIF-1 gradient can directly underlie the prestalk/prespore pattern in the slug. The gradient is in the wrong direction (8), and, in any case, the pattern does not appear to be made by a positional information mechanism (2) but by cell sorting, with cell-type proportions established by DIF-1 depletion (38–40). However, there are gradients in the slug: in tip activation and tip inhibition and in promoter activity within the prespore region (13, 41, 42). At least one of these gradients can regulate when a slug is cut in two, indicating that it responds to a dynamic signaling process (14, 43). In addition, there is evidence that

Table 3. Repatterning of prespore and prestalk isolates

Regulating tissue	*n*	DIF-1 dechlorinase, pmol/mg per hr		
		Newly cut	Regulated	
			Front	Back
Prespore	4	7	42	6
Prestalk	2	63	111	7

Isolated prespore regions of migrating slugs were allowed to regulate for 30–45 min and then were cut into halves, and the specific activity of DIF-1 dechlorinase was determined in the halves. Isolated prestalk regions were combined into piles of ≈20, and the slugs that emerged 4 hr later were similarly dissected. In both cases, the DIF sink was restored in the front of the isolate. *n* is the number of separate experiments.

patterning in both prestalk (15) and prespore isolates is regenerated by positional mechanisms (see below). Thus, we speculate that the DIF-1 and/or metabolite gradients may underlie the grading of properties in the slug and provide positional cues for the regeneration of the prestalk/prespore pattern in fragments of the slug.

When a prespore zone is cut from a slug, the DIF-1 sink rapidly reappears in the front of the isolate. This reappearance is accomplished before detectable sorting of AL cells (10, 16), indicating that the DIF-1 sink is not initially regenerated by sorting of the general population of AL cells. Our results, therefore, suggest that a positional signal is initially involved in regenerating the DIF-1 sink [and presumably the prestalk zone (17, 18)], although cell sorting may well become important later in the regeneration process.

Morphogen gradients can be generated in developing embryos by diffusion of a morphogen from a localized source; our results suggest that they can also be generated by diffusion of a morphogen into a localized sink.

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1. Lawrence, P. A. (1966) *J. Exp. Biol.* **44,** 607–620.
2. Wolpert, L. (1969) *J. Theor. Biol.* **25,** 1–47.
3. Gierer, A. & Meinhardt, H. (1972) *Kybernetic* **12,** 30–39.
4. Driever, W. & Nusslein-Volhard, C. (1988) *Cell* **54,** 83–89.
5. Raper, K. B. (1940) *J. Elisha Mitchell Sci. Soc.* **56,** 241–282.
6. Hicklin, J., Hornbruch, A., Wolpert, L. & Clarke, M. (1973) *J. Embryol. Exp. Morphol.* **30,** 701–725.
7. Smith, J. C. & Slack, J. M. W. (1983) *J. Embryol. Exp. Morphol.* **78,** 299–317.
8. Brookman, J. J., Jermyn, K. A. & Kay, R. R. (1987) *Development* **100,** 119–124.
9. Town, C. D., Gross, J. D. & Kay, R. R. (1976) *Nature (London)* **262,** 717–719.
10. Sternfeld, J. & David, C. N. (1981) *Differentiation* **20,** 10–21.
11. Sternfeld, J. & David, C. N. (1982) *Dev. Biol.* **93,** 111–118.
12. Jermyn, K. A. & Williams, J. G. (1991) *Development* **111,** 779–787.
13. Durston, A. J. (1976) *Nature (London)* **263,** 126–129.
14. MacWilliams, H. K. (1982) in *Developmental Order: Its Origin and Regulation*, eds. Subtelny, S. & Green, P. (Liss, New York), pp. 463–483.
15. Gregg, J. H. & Karp, G. C. (1978) *Exp. Cell Res.* **112,** 31–46.
16. Takeuchi, I., Tasaka, M., Oyama, M., Yamamoto, A. & Amagai, A. (1982) *Prog. Clin. Biol. Res.* **85,** 283–294.
17. Pogge-von Strandmann, R. & Kay, R. R. (1990) *Dev. Genet.* **11,** 447–452.
18. Bonner, J. T., Chiquoine, A. D. & Kolderie, M. Q. (1955) *J. Exp. Zool.* **130,** 133–156.
19. Morris, H. R., Taylor, G. W., Masento, M. S., Jermyn, K. A. & Kay, R. R. (1987) *Nature (London)* **328,** 811–814.
20. Kay, R. R. & Jermyn, K. A. (1983) *Nature (London)* **303,** 242–244.
21. Kopachik, W. J., Dhokia, B. & Kay, R. R. (1985) *Differentiation* **28,** 209–216.
22. Williams, J. G., Ceccarelli, A., McRobbie, S., Mahbubani, H., Kay, R. R., Early, A., Berks, M. & Jermyn, K. A. (1987) *Cell* **49,** 185–192.
23. Early, A. E. & Williams, J. G. (1988) *Development* **103,** 519–524.
24. Kay, R. R., Berks, M. & Traynor, D. (1989) *Development* **107S,** 81–90.
25. Wang, M. & Schaap, P. (1989) *Development* **105,** 569–574.
26. Kopachik, W., Oohata, A., Dhokia, B., Brookman, J. J. & Kay, R. R. (1983) *Cell* **33,** 397–403.
27. Traynor, D. & Kay, R. R. (1991) *J. Biol. Chem.* **266,** 5291–5297.
28. Nayler, O., Insall, R. & Kay, R. R. (1992) *Eur. J. Biochem.* **208,** 531–536.
29. Kay, R. R. (1987) *Methods Cell. Biol.* **28,** 433–448.
30. Bonner, J. T. (1952) *Am. Nat.* **86,** 79–89.
31. Ratner, D. & Borth, W. (1983) *Exp. Cell Res.* **143,** 1–13.
32. Takeuchi, I. (1963) *Dev. Biol.* **8,** 1–26.
33. Berks, M. & Kay, R. R. (1990) *Development* **110,** 977–984.

34. Devine, K. M. & Loomis, W. F. (1985) *Dev. Biol.* **107,** 364–372.
35. Jermyn, K. A., Berks, M., Kay, R. R. & Williams, J. G. (1987) *Development* **100,** 745–755.
36. Barklis, E. & Lodish, H. F. (1983) *Cell* **32,** 1139–1148.
37. Kay, R. R., Taylor, G. W., Jermyn, K. A. & Traynor, D. (1992) *Biochem. J.* **281,** 155–161.
38. Insall, R., Nayler, O. & Kay, R. R. (1992) *EMBO J.* **11,** 2849–2854.
39. Williams, J. G., Duffy, K. T., Lane, D. P., McRobbie, S. J., Harwood, A. J., Traynor, D., Kay, R. R. & Jermyn, K. A. (1989) *Cell* **59,** 1157–1163.
40. MacWilliams, H. K. (1991) *Semin. Dev. Biol.* **2,** 119–128.
41. Kopachik, W. (1982) *J. Embryol. Exp. Morphol.* **68,** 23–35.
42. Haberstroh, L. & Firtel, R. A. (1990) *Genes Dev.* **4,** 596–612.
43. Lokeshwar, B. L. & Nanjundiah, V. (1983) *J. Embryol. Exp. Morphol.* **73,** 151–163.