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Cell-Fate Choice in *Dictyostelium:* Intrinsic Biases Modulate Sensitivity to DIF Signaling

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Cell fate in *Dictyostelium* development depends on intrinsic differences between cells, dating from their growth period, and on cell interactions occurring during development. We have sought for a mechanism linking these two influences on cell fate. First, we confirmed earlier work showing that the vegetative differences are biases, not commitments, since cells that are stalky-biased when developed with one partner are sporey with another. Then we tested the idea that these biases operate by modulating the sensitivity of cells to the signals controlling cell fate during development. Cells grown without glucose are stalky-biased when developed with cells grown with glucose. We find, using monolayer culture conditions, that they are more sensitive to each of the stalk-inducing signals, DIFs 1–3. Mixing experiments show that this bias is a cell-intrinsic property. Cells initiating development early in the cell cycle are stalky compared to those initiating development later in the cycle. Likewise, they are more sensitive to DIF-1. Assays of standard markers for prestalk and prespore cell differentiation reveal similar differences in DIF-1 sensitivity between biased cells; DIF-1 dechlorinase (an early prestalk cell marker enzyme) behaves in a consistent manner. We propose that cell-fate biases are manifest as differences in sensitivity to DIF.

Key Words: Dictyostelium; **cell fate choice; intrinsic bias; cell signaling; DIF.**

INTRODUCTION

The interplay between cell–cell signaling and intrinsic cell fate biases is often required for an equivalent group of cells to adopt different fates during development (Jan and Jan, 1995; Cepko, 1999). In *Dictyostelium* development, a population of starving cells aggregates to form a mound of some 10^5 cells and in this mound the precursors of the two main cell types (stalk and spore) can be first identified as prestalk and prespore cells.

There is strong evidence that this cell fate choice is influenced by intrinsic biases present in vegetative cells, before development starts (Takeuchi, 1969; Leach *et al.*, 1973; Maeda and Maeda, 1974). For example, cells grown in the absence of glucose (G^- cells) preferentially become stalk cells when mixed with cells grown in the presence of glucose (G^+ cells; Leach *et al.*, 1973). Similarly, cells around S/M phase of the cell cycle (Weijer *et al.*, 1984a; McDonald and Durston, 1984; Araki *et al.*, 1994), or with high intracellular calcium (Maeda *et al.*, 1973; Azhar *et al.*, 1996), are also "stalky" when developed with tester cells.

There is equally compelling evidence that cell fate is also controlled by cell–cell interactions during development. For instance, when a migrating slug is cut into anterior (prestalk) and posterior (prespore) zones, both pieces can regulate to restore the missing cell type and produce a normally proportioned fruiting body, with both stalk and spore cells (Raper, 1940; Sakai, 1973). In addition a number of diffusible signals which could control cell fate have been identified (Gross *et al.*, 1983; Schaap and Wang, 1986). Notable among these are DIF-1, a chlorinated alkyl phenone, and the closely related DIF-2 and DIF-3 (Morris *et al.*, 1987; Kay *et al.*, 1999). In cell culture, DIF-1 induces prestalk and stalk cell differentiation, while inhibiting prespore and spore cell differentiation (Kay and Jermyn, 1983). Furthermore, in normal development, the pattern of slugs becomes substantially more stalky when they are treated with DIF-1 (Wang and Schaap, 1989; Kay *et al.*, 1989).

To develop a model of pattern formation in *Dictyostelium*, it is important to identify the mechanism by which intrinsic biases affect cell fate (MacWilliams, 1982; Loomis, 1993; Gross, 1994; Aubry and Firtel, 1999; Kay *et al.*, 1999). One key observation, confirmed here, is that the biases can be arranged into a hierarchy: though G^- cells are more stalky than G^+ ones, cells grown to stationary phase are more stalky yet. Hence G^- cells preferentially become stalk cells in mixtures with G^+ cells, but become spores in

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mixtures with stationary phase cells (Leach *et al.*, 1973). Thus, the fate of a particular biased cell is not fixed but depends on the nature of the other cells in the aggregate.

There seem to be two possible ways in which a vegetative bias might affect cell fate, depending on how the prestalk/prespore pattern is itself produced. If pattern is produced by localized positional signals in the aggregate then, for instance, a stalky bias might work by causing cells to move preferentially into a region where they will later experience prestalk-inducing signals, and conversely for a sporey bias. However, prestalk and prespore cells probably do not first differentiate in defined positions (Williams *et al.*, 1989; Esch and Firtel, 1991), except possibly for prestalk-A cells (Early *et al.*, 1995), and differently biased cells are initially intermingled in the aggregate (Tasaka and Takeuchi, 1981; Araki *et al.*, 1994), so this mechanism seems unlikely. Alternatively, if positional information does not guide the first differentiation of prestalk and prespore cells, then it has been proposed that the fate biases might represent differences in sensitivity of cells to the signals that control their fate (MacWilliams, 1982; Weijer *et al.*, 1984a). We have tested this idea by examining the sensitivity of various biased cells toward the DIFs, as the best characterized signals controlling cell fate in *Dictyostelium*.

MATERIALS AND METHODS

Strains and Culture Conditions

Ax2 cells were maintained in axenic medium containing 86 mM glucose at 22°C (Watts and Ashworth, 1970). Actin15-GFP (Gerisch *et al.*, 1995) or ecmAO-lacZ Ax2 transformants (Early *et al.*, 1993) were initially grown in tissue culture plates with axenic medium containing 20 μg/ml G418 and then transferred to shaken culture without G418 for use in experiments. For G^+/G^- experiments, cells were grown for 2 days to midlog phase in medium with or without glucose and then diluted and allowed to grow to 1–2 $\times$ 10^6 cells/ml in the same medium. Cell cycle synchronization was by release from cold shock (Maeda, 1986; Araki *et al.*, 1994).

In Vivo Localization of Biased Cell Populations

For all sorting experiments, 10% GFP-labeled cells were mixed with 90% unlabeled Ax2 cells. Slug formation was encouraged by plating 10^7 cells as thin streaks on 1.5% L28 agar plates (Oxoid) containing 10% NS (100% is 20 mM KCl, 20 mM NaCl, 1 mM $CaCl_2$) and allowing migration toward a unilateral light source. Where the proportion of labeled spores was to be scored, culmination was encouraged by developing 4 $\times$ 10^7 cells on 5-cm-diameter black Millipore filters, supported on prefilters containing KK2. At the end of development, all cells were harvested and detergent-resistant spores scored by microscopy.

Monolayer Assays

In all assays, washed cells were plated in stalk medium (10 mM 2-(*N*-morpholino)ethane sulfonic acid, 10 mM KCl, 2 mM NaCl, 1 mM $CaCl_2$, pH 6.2, with 200 μg/ml streptomycin sulfate and 15 μg/ml tetracycline) with various additions and kept in the dark at 22°C (Kay, 1987). Stalk and spore cell differentiation was scored by phase-contrast microscopy. For stalk cell assays by cAMP removal (Berks and Kay, 1988), cells were incubated at 2 $\times$ 10^5 cells/ml (1.5 ml/3.5-cm-diameter tissue culture dish) with 5 mM cAMP for 24 h, washed twice in KK2, and then incubated for a further 24 h without cAMP, but with various concentrations of DIFs. Alternatively, cells were incubated with 15 mM 8-Br-cAMP with or without DIF-1 and stalk and spore cells scored after 48 h. EcmAO-lacZ expression was induced (Early *et al.*, 1995) and detected (Dingermann *et al.*, 1989) as described. For prespore marker gene expression, cells were plated at 1 $\times$ 10^6 cells/ml (10 ml/9.5-cm-diameter dish) with 5 mM cAMP for 6 h before adding DIF-1. After a further 2 h 1 $\times$ 10^7 cells were harvested, RNA was extracted, and Northern was analysis performed (Berks and Kay, 1990), with blots initially probed for psA mRNA and then probed and normalized to IG7 mRNA.

DIF Dechlorinase Assay

Cells were harvested, washed twice with KK_2, and resuspended at 2 $\times$ 10^7 cells/ml in KK_2. After shaking for 1 h in KK_2, cells were pulsed with 50 nM cAMP at 6-min intervals. DIF-1-dechlorinase was assayed in the high-speed supernatant (Nayler *et al.*, 1992) except that 5 mM DTT and 20 mM GSH were present in the assay mix.

RESULTS

Growth Conditions and Cell Cycle Position Bias Cell Fate in Vivo

We first confirmed that cell fate biases could be imposed under our conditions. Cells labeled by constitutive GFP expression were mixed in a 1:9 ratio with unlabeled cells and their distribution (or fate) was followed in the slugs that formed. With labeled and unlabeled cells grown under identical conditions, the labeled cells become randomly distributed throughout the slug (Figs. 1A and 1B). However, in agreement with previous results (Leach *et al.*, 1973) when G^+ and G^- cells are mixed, the G^- cells tend to localize to the anterior (prestalk) and the G^+ cells to the posterior (prespore) zone of the slug (Figs. 1C and 1D).

Furthermore, there is a hierarchy of biases. GFP-labeled G^- cells were mixed with unlabeled G^+, bacterially grown or stationary-phase cells and allowed to develop under conditions favoring culmination. The proportion of GFP-labeled spores in the resulting fruiting bodies was counted as a quantitative measure of bias. G^- cells can be shifted from a stalky bias (when mixed with G^+ cells) to an increasingly sporey bias when mixed with bacterially grown or stationary-phase cells (Table 1) (Leach *et al.*, 1973). These results confirm that cell fate is not predetermined but depends on interactions with other cells in the developing aggregate.

Similarly, mixtures of GFP-expressing and unlabeled cells were used to confirm the correlation between cell cycle position at starvation and cell fate (Weijer *et al.*, 1984a; McDonald and Durston, 1984; Araki *et al.*, 1994). Labeled cells were synchronized by release from cold shock,

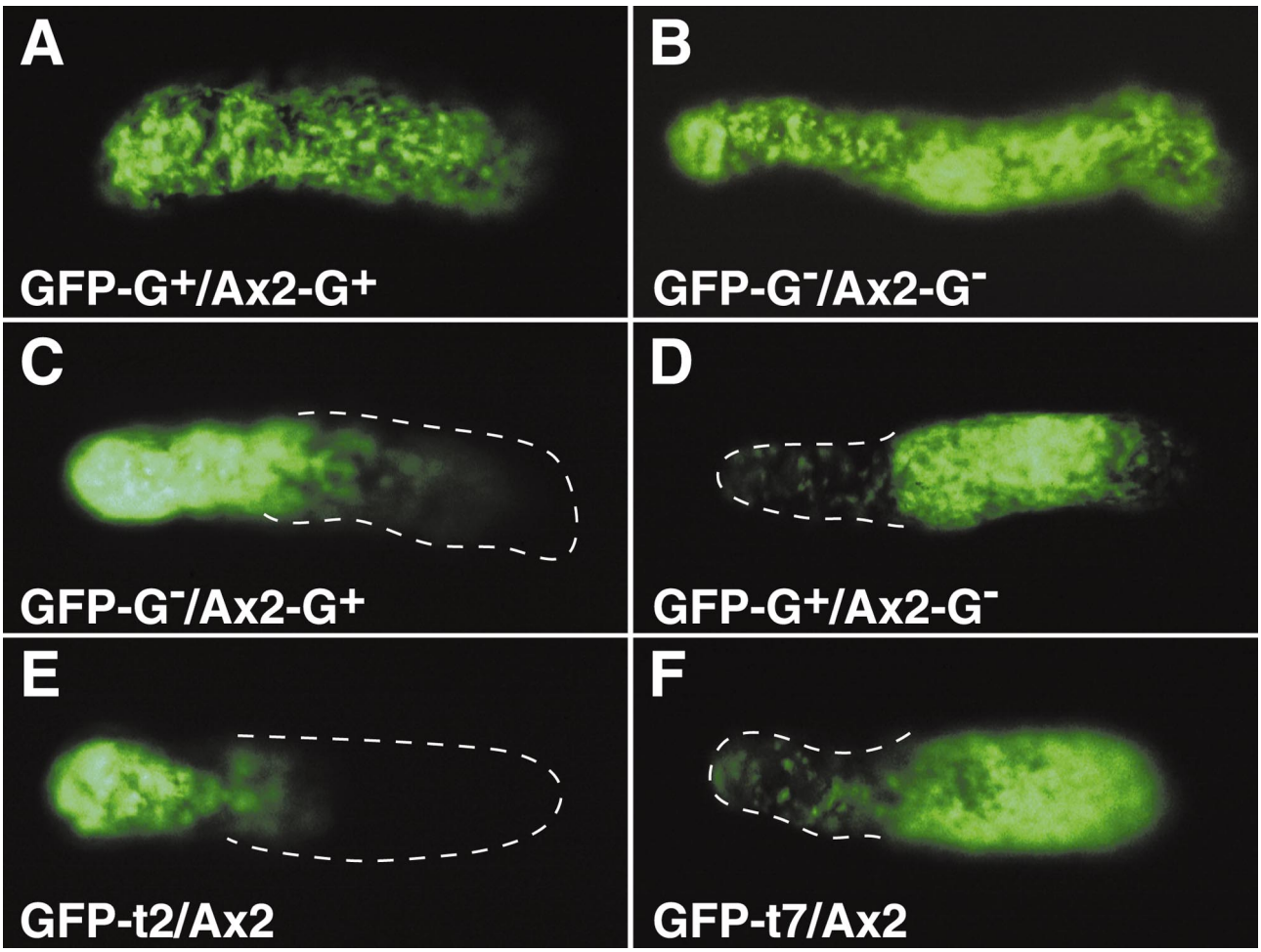

FIG. 1. The distribution of GFP-expressing cells mixed with unlabeled cells in chimeric slugs. GFP cells were grown with or without glucose and mixed in all combinations, in a 1:9 ratio, with unlabeled cells also grown with or without glucose (A, B, C, and D), or GFP cells were taken at different phases of the cell cycle and mixed in a 1:9 ratio with unlabeled unsynchronized cells (E and F; t2 is around mitosis/S phase, t7 is late G2). It can be seen that G^- and t2 cells are prestalk sorting (the prestalk zone of the slugs is to the left), whereas G^+ and t7 cells are prespore sorting.

mixed with unlabeled unsynchronized cells, and allowed to develop. In this case cells around mitosis at the start of development (taken 2 h after release from low-temperature growth arrest) tend to localize to the prestalk zone, whereas cells from mid-G2 phase (6 h after release from low-temperature growth arrest) tend to localize to the prespore zone (Figs. 1E and 1F).

Growth Conditions Affect Responsiveness to DIF

Cells were grown in the presence or absence of glucose and assayed for responsiveness to DIFs 1, 2, and 3 in low-density monolayers, in which terminal differentiation of stalk cells and spore cells can be scored by microscopy. G^+ or G^- cells were incubated first with cAMP and then, after its removal, with various DIFs. Under these conditions spore cell formation is blocked and only stalk cells can form (Berks and Kay, 1988). Figure 2 shows that G^- cells are more responsive to each of the three DIFs than G^+ cells, with half-maximal induction achieved at a lower DIF concentration (for DIF-1, 3 nM compared to 40 nM).

To confirm that this result is not due to a general reduction in the ability of G^+ cells to differentiate, spore and stalk cell formation were examined simultaneously, using 8-Br-cAMP to induce spore maturation (Kay, 1989). Similarly, in these experiments, G^+ cells differentiate just as well as G^- cells, but are much less responsive to DIF-1. This is true whether DIF-1 is viewed as a stalk cell inducer (Fig. 3A) or as an inhibitor of spore cell differentiation (Fig. 3B).

To test whether the differences between G^+ and G^- cells are cell autonomous or not, the responsiveness of GFP-

TABLE 1

Quantification of Sorting of GFP-labeled Cells Grown without Glucose and Mixed with Unlabeled Cells Grown under Different Conditions

Growth conditions	GFP^+ spores (mean %)	Normalized to G^- (SD; *n*)
$Glucose^+$ (G^+)	2.5	0.38 ($\pm$0.16; $n = 5$)
$Glucose^-$ (G^-)	5.7	1.00 ($n = 5$)
Bacterially grown	10.7	2.48 ($\pm$0.60; $n = 3$)
Stationary phase	14.7	2.16 ($\pm$0.77; $n = 3$)

Note. 10% GFP-expressing G^- cells were mixed with 90% unlabeled cells grown under different conditions and the percentage of GFP-expressing spores was determined after 27–40 h of development. The results from five experiments are combined; three used a nonclonal population of actin15-gfp transformants and two used a strongly expressing clone (HM2088) from this population. In all cases the sorting hierarchy of $G^+ > G^- >$ bacterially grown/stationary phase was obeyed for directing the GFP cells away from spore formation. It was also found that the GFP-expressing cells, grown without glucose, were slightly stalky when developed with Ax2 cells grown under the same conditions (this bias is not detected in the more qualitative visual experiment shown in Fig. 1).

labeled cells was assayed in mixtures with unlabeled cells of a different physiology. The results show that the labeled cells behave according to their own growth conditions and independent of the growth conditions of the majority population with which they are mixed (Figs. 3C and 3D). Thus the sensitivity of cells to DIF in this assay is a cell-autonomous property, determined by their growth conditions.

Cell Cycle Position Affects Responsiveness to DIF-1

Cells were synchronized in the cell cycle by release from cold shock (Maeda, 1986) and samples taken hourly to both measure cell number and obtain dose–response curves to DIF-1 in the presence of Br-cAMP. Results are expressed in terms of either the induction of stalk cell differentiation by DIF-1 or the repression of spore cell formation. Figure 4 shows that, by either criterion, cells initiating development around mitosis are considerably more sensitive to DIF-1 than cells taken in the rest of the cell cycle (G2 cells) and that there is a fairly abrupt shift in sensitivity as the cell cycle proceeds. Before this shift, half-maximal induction of stalk cell differentiation is attained with about 4 nM DIF-1 and after it with about 10 nM DIF-1. The difference in responsiveness persists through a second round of division (albeit less markedly) and so is not likely to be due to an artifactual effect of cold shock (not shown). These results again show that prestalk-biased cells are more responsive to DIF-1 than prespore-biased cells.

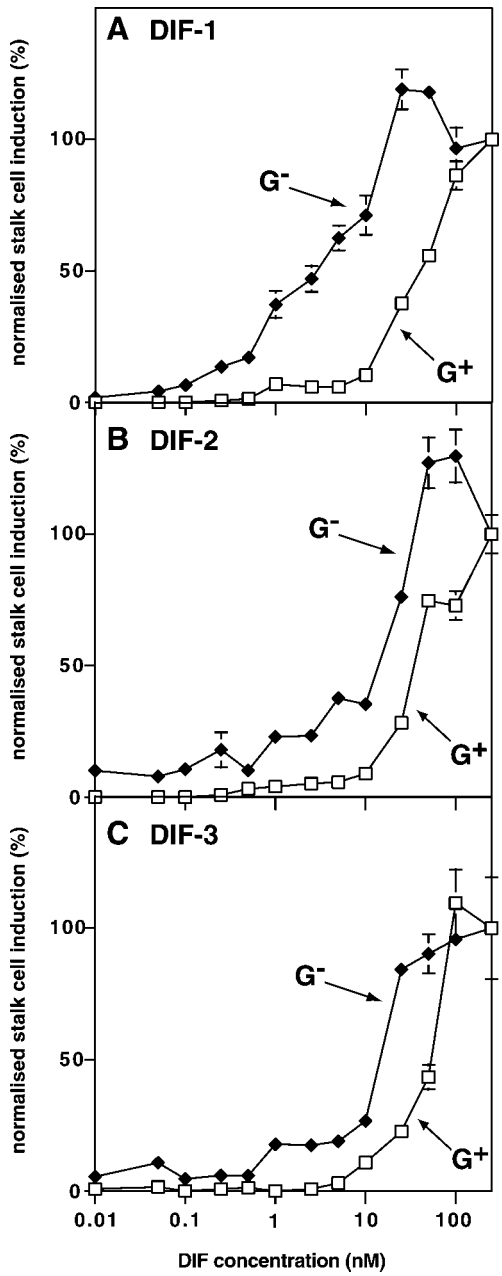

FIG. 2. Sensitivity of G^+ and G^- cells to DIF in cAMP-removal experiments. Ax2 cells were grown with (open symbols) or without (filled symbols) glucose, washed free of medium, and plated in monolayer culture at a density of 3.1×10^4 cells/cm^2 under stalk medium containing 5 mM cAMP. After 24 h the medium was replaced with fresh medium without cAMP and containing (A) DIF-1, (B) DIF-2, or (C) DIF-3 at the indicated concentrations. Stalk cell differentiation was scored 24 h later and results from three experiments are given, normalized to the percentage stalk cell differentiation at 250 nM DIF obtained in each case (G^+, DIF-1, 38%; DIF-2, 33%; DIF-3, 32%; and G^-, DIF-1, 41%; DIF-2, 33%; DIF-3, 36%).

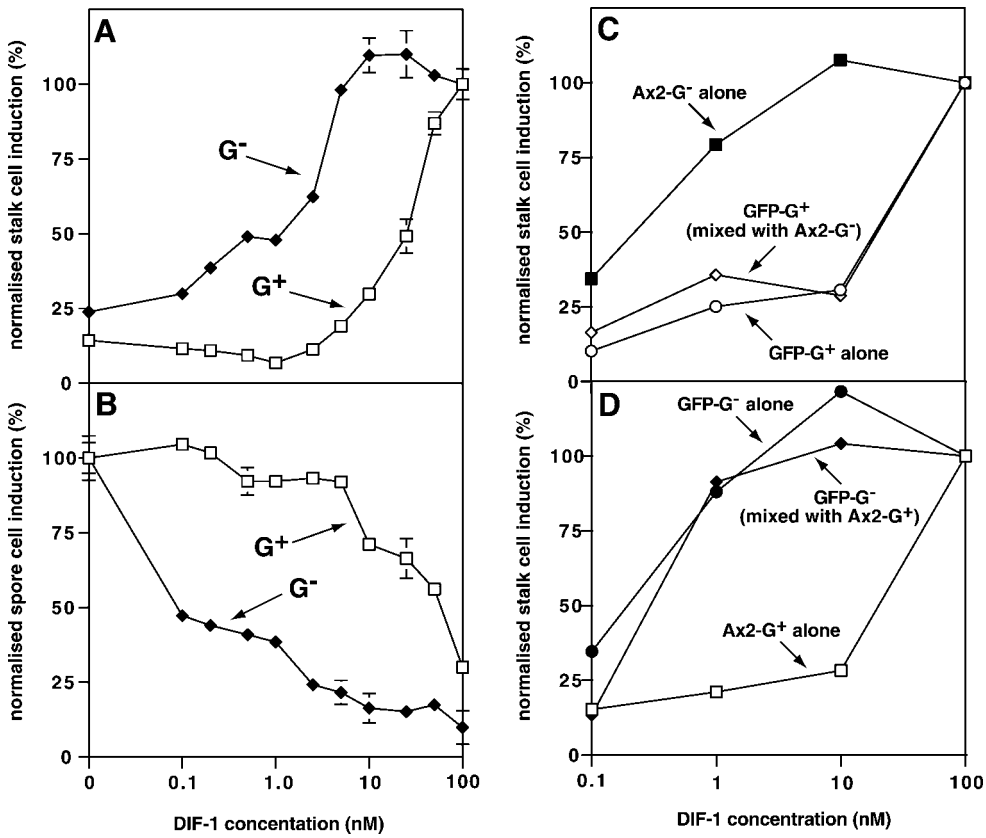

FIG. 3. Sensitivity of G^+ and G^- cells to DIF-1 in the presence of 8-Br-cAMP. (A and B) Pure cultures of G^+ and G^- cells are compared, scoring either induction of stalk cell differentiation or repression of spore cell differentiation by DIF-1. Results shown are normalized to stalk cell induction at 100 nM and spore cell induction at 0 nM DIF-1. By either measure, G^- cells are the more sensitive to DIF-1 (half-maximal stalk induction, G^- = 1.25 nM; G^+ = 25 nM DIF-1; half-maximal spore repression, G^- = 0.1 nM; G^+ = 60 nM DIF-1). Maximum stalk cells, G^- = 39% and G^+ = 37%. (C and D) The differences in sensitivity are cell-autonomous properties. A minority (10%) of GFP-expressing cells, grown with or without glucose, was mixed with a majority population of unlabeled cells, grown under the converse condition. Sufficient GFP remains in mature stalk and spore cells to allow the two populations to be scored separately. The labeled cells show the DIF sensitivity expected of their growth conditions and not that of the majority population with which they are developed. Spore differentiation was also scored with similar results, but is not shown. Results are typical of three experiments.

Induction of Prestalk/Prespore Markers in Biased Cells

As a more direct measure of cell fate bias, the DIF responsiveness of prestalk and prespore markers was compared in differently biased cells. To score prestalk cell differentiation on a cell-by-cell basis, we employed the ecmAO-lacZ marker. Cells were brought to a responsive state by prior incubation in monolayers with cAMP, the medium was changed, and DIF-1 was added. After a further 24 h of incubation, cells were fixed, stained, and scored. As expected, G^- cells were much more responsive to DIF-1 than G^+ cells (Fig. 5A).

A similar experiment, examining repression of the psA-lacZ marker for prespore cells, was not possible due to widespread expression of this stable marker in the cells during the preincubation period (Kubohara and Okamoto, 1993). However, direct measurement of psA transcript levels by Northern transfer revealed the expected principle: transcript expression was much more sensitively repressed in G^- cells by DIF-1 than in G^+ cells (Fig. 5B).

The prestalk-specific enzyme DIF-1 dechlorinase, whose function in inactivating DIF-1 makes it a good candidate to play a role in the initial cell fate decision, was also examined. Expression of this enzyme is rapidly induced by DIF-1 (Insall *et al.*, 1992), but the induction has several unusual properties and may be controlled at the level of translation (R.R.K. & R. Insall, unpublished observations), in contrast to the transcriptional control of ecmA and psA expression (Williams *et al.*, 1987; Early and Williams, 1988). Cells were brought to a responsive state by pulsing with cAMP in shaken suspension, DIF-1 was added, and enzyme activity was assayed in cell lysates. Two major differences between

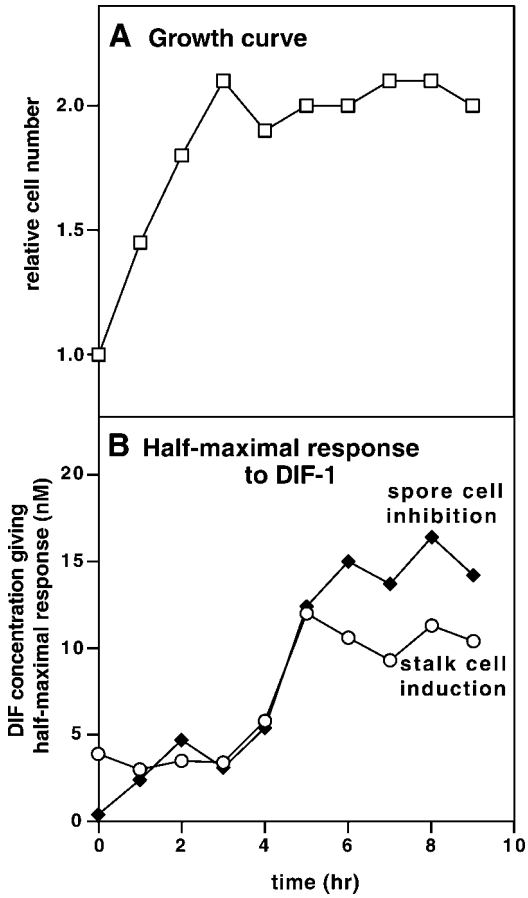

FIG. 4. Sensitivity of cells to DIF-1 through the cell cycle. Ax2 cells were synchronized by release from cold shock and DIF-1 titration curves performed on cells taken every hour through the first, synchronous, cell cycle. The relative cell number (A) shows that most cells divide within the first 3 h after release from cold shock. The sensitivity of cells to DIF-1 was determined in monolayer incubations in the presence of Br-cAMP, with both stalk and spore cells scored by microscopy. The concentration of DIF-1 giving a half-maximal response (measured either by the induction of stalk cell differentiation or by the repression of spore cell formation) is shown in (B). Results are typical of three experiments.

G^+ and G^- cells were observed. First, G^- cells reproducibly became responsive 1–2 h earlier in development than G^+ cells (Fig. 6A). This made comparisons of dose–response curves difficult, because by the time G^+ cells became responsive, G^- cells were already expressing appreciable DIF-1 dechlorinase. Second, G^- cells responded much more quickly than G^+ cells: maximal response was obtained in about 20 min, or half the time required by G^+ cells (Fig. 6B). Although this behavior is different from that of the other markers, it too could lead to preferential expression of the DIF-1 dechlorinase marker by G^- cells compared to G^+ cells.

DISCUSSION

It is well established that *Dictyostelium* cells can be biased toward becoming either stalk or spore cells by their growth history before development commences and long before prestalk and prespore cells actually differentiate. This is classically shown by mixing cells with differing biases at the start of development and finding that one population preferentially becomes stalk or spore cells. It is also clear (and confirmed here) that the biased states can be arranged in a hierarchy (Leach *et al.*, 1973), implying that bias is not a commitment to stalk or spore cell differentiation, but depends on the nature of the other cells in the

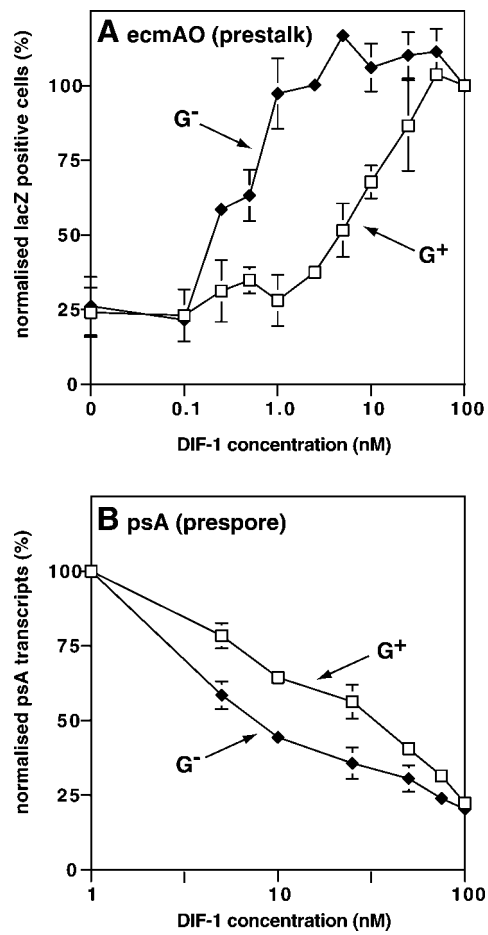

FIG. 5. Induction of prestalk and prespore markers in biased cells. The DIF-1 sensitivity of G^+ and G^- cells is compared in monolayer culture, in the presence of 5 mM cAMP. (A) Prestalk cell differentiation, using ecmAO-lacZ transformed cells to allow scoring on a cell-by-cell basis (half-maximal ecmAO induction for G^- and G^+ cells is 0.25 nM compared to 25 nM DIF-1). Maximum: $G^- = 13.6\%$ and $G^+ = 13.7\%$. (B) Prespore cell differentiation, using psA mRNA expression as marker (half-maximal psA repression for G^- is 8 nM DIF-1 compared to 33 nM DIF-1 for G^+).

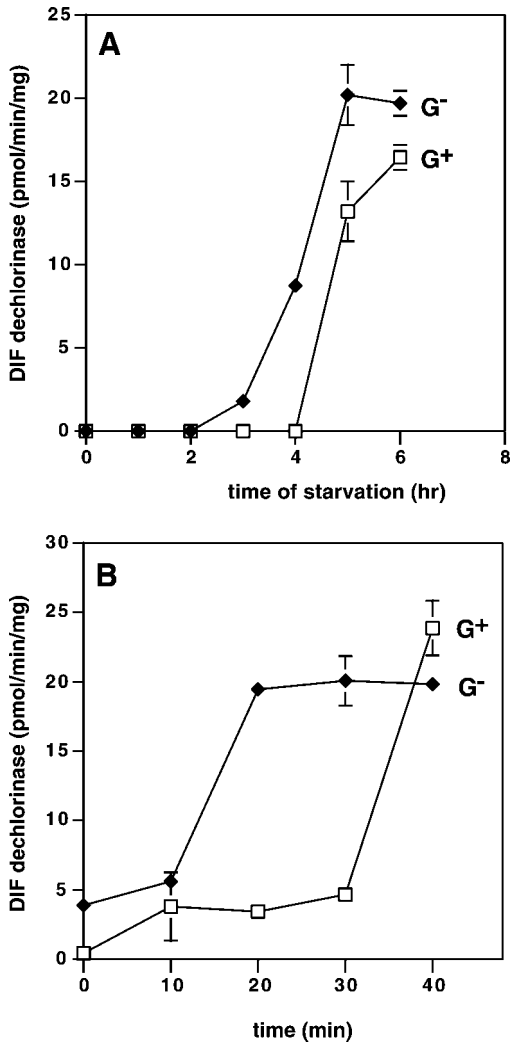

FIG. 6. Induction of DIF-1 dechlorinase, an early prestalk marker, in biased cells. G^+ and G^- cells were harvested and pulsed with cAMP in shaken suspension at a density of 2×10^7 cells/ml in KK2. (A) Cells were allowed to develop for the indicated times, and an aliquot was withdrawn and induced with 100 nM DIF-1 for 40 min before assaying. G^- cells become responsive to DIF-1 earlier in development than G^+ cells. (B) Cells were starved for 1 h and pulsed with cAMP for 5 h, then 100 nM DIF-1 was added and the time course of appearance of DIF-1 dechlorinase followed. G^- cells respond in about half the time required by G^+ cells, though not to a higher final level. Results are the means of two experiments.

aggregate (but see Gomer and Firtel, 1987; Clay *et al.*, 1995; Gomer and Ammann, 1996). Our results strongly suggest that vegetative biases work by modulating the sensitivity of cells to DIF at a later stage of development.

In the two most commonly investigated situations—bias by growth conditions and bias by phase of the cell cycle—we find that cells with a stalky bias are more sensitive to DIF than those with a sporey bias. This is manifest in several different assays, including by the scoring of morphological stalk and spore cells and by measuring expression of prestalk and prespore markers. The mean DIF-1 concentration in the mound is in the region of 10 nM (Kay, 1998) and the differences in sensitivity between biased populations roughly brackets this concentration. Thus, in monolayer culture, half-maximal stalk cell induction requires about 4 nM DIF-1 for cells early in the cell cycle (stalky biased), compared to about 10 nM DIF-1 for cells later in the cell cycle (sporey biased). The difference in sensitivity between G^- and G^+ cells can be as great as 100-fold, depending on the assay used, but in each case the half-maximal response with G^- cells is achieved at less than 4 nM DIF-1 and with G^+ cells at more than 10 nM DIF-1. It therefore seems likely that the differences in DIF sensitivity detected here are adequate to explain why stalky-biased cells tend to become prestalk cells in the mound and sporey-biased ones do not.

One technical aspect of the work not fully understood is the reason why some cells fail to differentiate into stalk cells in the monolayer assays, even at saturating DIF-1 concentrations. It has been suggested that some cells (presumably those that would become spores in normal development) are intrinsically unresponsive to DIF-1 in these assays (Clay *et al.*, 1995). However, this is inconsistent with the observation that spore formation can be completely suppressed by saturating levels of DIF-1 and with the efficient suppression of prespore markers by DIF-1 in other circumstances (Berks and Kay, 1990). Further, in strain V12M2, which forms beautiful, wild-type fruiting bodies, DIF-1 can induce essentially 100% stalk cell formation (Town *et al.*, 1976). We therefore think it more likely that some cells fail to differentiate under our conditions due to a combination of the fairly stringent scoring criteria employed and imperfections in the monolayer conditions, possibly due to requirements for additional factors (Mehdy and Firtel, 1985; Berks and Kay, 1988; Anjard *et al.*, 1998).

A wide range of differences between stalky- and sporey-biased cells has previously been described, but in the main it is difficult to discern how these differences could cause a cell fate bias. The most-studied difference is rate of early development, including expression of the surface cAMP receptors, but here the more rapidly developing population has been found to be stalky in some circumstances (Maeda and Maeda, 1974; Wang *et al.*, 1988), but sporey in others (Inouye and Takeuchi, 1982; Araki *et al.*, 1994; Abe and Maeda, 1994; Huang *et al.*, 1997), suggesting that rate of development is not directly linked to cell fate. Perhaps more interesting, in view of the evidence that DIF treatment stimulates Ca^{2+} uptake in responsive cells (Azhar *et al.*, 1997), is the finding that stalky biased cells have higher total and cytosolic Ca^{2+} levels (Maeda and Maeda, 1974; Azhar *et al.*, 1996). Other differences noted include glycogen content (Hames and Ashworth, 1974) and levels of various enzymes expressed early in development (Weijer *et al.*, 1984b). The attraction of the correlation that we have discovered between increased DIF sensitivity and a stalky

bias is that it offers a mechanism for the cell-fate bias of these cells. It will be very interesting to discover the physical nature of these biases (such as DIF-receptor density) and how they are transmitted from growing cells, through early development, to the point when prestalk and prespore cells differentiate.

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