Regulation of stalk and spore antigen expression in monolayer cultures of *Dictyostelium discoideum* by pH

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SUMMARY

The terminal differentiation of *Dictyostelium discoideum* cells plated as monolayers with cyclic AMP is dramatically affected by developmental buffer conditions. High pH and addition of weak bases induces spore differentiation while low pH and weak acids favour stalk cell formation. In order to analyse the timing and nature of this regulation we have raised and characterized an anti-stalk serum which we have used together with an anti-spore serum to monitor developmental progression in the monolayer system and to detect the phenotypic effects of pH at earlier stages of development. The stalk serum detects both polysaccharide and protein antigens expressed during the terminal stages of normal development. In monolayer culture, the stalk-specific protein antigen appears precociously, while the timing of prespore vacuole appearance is unaffected. Expression of stalk polysaccharide antigens in monolayer cultures occurs as early as 12 h and is localized in a single subset of cells or region of extracellular space within the small cell clumps that form. The effects of pH (and acid/base) on these phenotype-specific antigens can be detected early in development, shortly after their first appearance. In monolayers of wild-type V12 M2 cells, the low pH regimes appear to act more by suppressing the spore than enhancing the stalk pathway, while the high pH regimes both suppress stalk and enhance spore antigen expression. In monolayers of the sporogenous mutant HM29, low pH regimes both enhance stalk antigen and suppress spore antigen expression. These results show that extracellular pH regulates phenotypic expression during a large part of the differentiation process and is not simply restricted to terminal cytodifferentiation.

INTRODUCTION

The cellular slime mould *Dictyostelium discoideum* is a simple eukaryote which feeds on bacteria in the soil. Starvation initiates a developmental process in which individual amoebae aggregate chemotactically to form multicellular masses which then elongate into finger-like structures which may either transform into a migrating slug or proceed directly to terminal differentiation. Differentiation into alternative cell types (i.e. prespore and prestalk cells) can first be detected shortly after formation of these multicellular aggregates. When an appropriate environmental stimulus is received (e.g. reduced humidity, overhead light, etc.), culmination occurs involving cell maturation into spores and stalk cells within a characteristic fruiting structure.

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Cell differentiation in *Dictyostelium discoideum* can also be studied in monolayer cultures of amoebae starved and allowed to differentiate in the presence of 1–5 mM-cAMP (Town, Gross & Kay, 1976). Under these conditions, cells of strain V12 M2 plated at high cell density all differentiate into stalk cells, while 'sporogenous' mutants derived from this parental strain can form both stalk cells and spores (Town et al., 1976; Kay, Garrod & Tilly, 1978). We, and others, have used this *in vitro* system to identify factors that influence the relative expression of the stalk and spore phenotypes. Thus low extracellular pH (alone or in conjunction with weak acid), low ionic strength, certain proteases, certain sterols and an endogenous differentiation-inducing factor (DIF) all favour terminal stalk cell formation; while high pH (alone or with weak base) and high ionic strength inhibit stalk cell formation and in sporogenous mutants promote spore differentiation (Gross et al., 1981, 1983; Peacey & Gross, 1981; Town, 1984). These observations are of both conceptual value in suggesting possible mechanisms for proportion regulation during normal development and of practical value in generating cell populations expressing only one phenotype. The dramatic regulation of phenotype by extracellular pH represents superficially one of the simplest modes of phenotype switching and we have studied this process in greater detail.

Previous studies on the pH effect have been restricted to observations on terminal cell differentiation, with no information as to when during monolayer development the effects of pH on phenotypic regulation can first be observed. Such information is important for both practical and mechanistic reasons. Since no suitable markers for prestalk differentiation were available at the time this study was begun, we raised and characterized a stalk-specific antiserum and have used it, along with the analogous anti-spore serum (Takeuchi, 1963) to characterize further the development of cells in the monolayer differentiation system and to follow the timing and extent of phenotypic regulation by pH under these conditions.

**MATERIALS AND METHODS**

*Cell growth and development*

*Dictyostelium discoideum* strain V12 M2 or one of its sporogenous mutants, HM29 (Kay, 1982), were used in these experiments. Vegetative amoebae were grown either on SM agar plates (Sussman, 1966) with *K. aerogenes* or as suspension cultures with *E. coli* as described previously (Town, 1984). For normal development, washed amoebae were plated either on KK$_2$ (20 mM-potassium phosphate, pH 6.0, 2 mM-MgSO$_4$) agar at 2–3 x 10$^8$ cells per 100 mm plate (= 3–6–5.5 x 10$^6$ cells cm$^{-2}$) or on Millipore filters (10$^6$ cells per 47 mm filter; = 5.8 x 10$^6$ cells cm$^{-2}$) supported by glass beads in 3 ml Average Salts buffered with 10 mM-MES (2-(N-Morpholino)ethanesulfonic acid) and 10 mM-Tris (Tris (hydroxymethyl) aminomethane) (Town, 1984). In later experiments we used a DMG/Hepes developmental buffer (15 mM-3,3'-dimethyl glutarate, 15 mM-Hepes (N-2-hydroxyethyl piperazine-N'-2 ethanesulfonic acid), pH 6.3, 22.4 mM-Na$^+$, 22.4 mM-K$^+$, 1 mM-MgCl$_2$, 1 mM-CaCl$_2$, 10 μg ml$^{-1}$ gentamycin) because of its superior buffering capacity (pH change <0.2 units throughout development). Migrating slugs were collected from H$_2$O agar plates as described by others (Ellingson, Telser & Sussman, 1971). For development as monolayers, cells were plated at either 10$^5$ or 10$^6$ cells cm$^{-2}$ in 60 mm tissue culture dishes (Falcon no. 3002) containing 4 ml buffered salts plus 5 mM-3',5'-cyclic
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adenosine monophosphoric acid (cAMP). Separated populations of mature stalks and spores were obtained by suspending fruiting bodies from KK2 agar or growth plates in KK2 buffer (no Mg\(^{2+}\)) then repeatedly vortexing and filtering through cheesecloth or Nitex 100 mesh (Tetko).

**Antibody production**

Anti-spore serum was produced using methods of Forman & Garrod (1977) and *D. mucoroides* spores as immunogen. Anti-stalk serum was produced in a New Zealand White rabbit using a similar protocol. Whole stalks and stalk fragments from 12 KK2 agar plates were used as the immunogen. Boost injections containing a similar number of cells (1-0–2-8 mg protein) were given at 2-3 week intervals for approximately 6 months with test bleeds performed prior to each injection.

**Antibody absorptions**

For initial cytological work, removal of antibodies that were not stalk-cell-specific was achieved by absorbing the sera with vegetative cells (5 x 10^9 cells ml^-1) and spores (0-5 ml packed spores ml^-1 serum). Anti-spore serum was absorbed in a similar fashion using vegetative and stalk cells. In experiments involving protein analysis, complete removal of all cross-reactive proteins was accomplished by incubating antisera with appropriate protein extracts bound to nitrocellulose filters. Approximately 10 mg of vegetative, spore or whole fruiting body proteins, extracted by boiling in SDS lysis buffer (0-0625 M-Tris, pH6-8, 1-25 % SDS (sodium dodecyl sulphate)), were incubated in this buffer with filters for 2 h at room temperature. After rinsing in phosphate-buffered saline (PBS, 140 mm-NaCl, 10 mm-phosphate buffer, pH 7-2) and incubation in 3 % gelatin in PBS (1 h, 37°C), appropriate filters were incubated with the serum to be absorbed (e.g. anti-stalk serum with vegetative, spore or fruiting body protein filters) for 90 min at room temperature or overnight at 4°C. The serum was collected and the absorption process repeated with fresh filters until removal of unwanted reactivity was complete.

**Cell fixation and immunofluorescent staining**

To prepare cells undergoing normal development for immunofluorescent staining, cell masses were first disagggregated by incubation in 0-1 % NaN\(_3\) and 1 mM-PMSF (phenylmethylsulphonyl fluoride) (10^8 cells 5 ml^-1) in H\(_2\)O for 10 min at room temperature followed by trituration with a 20 g needle. Cells were fixed with 60 %, then 100 % methanol and stained for indirect immunofluorescence (Forman & Garrod, 1977). In some cases, cells developing as submerged monolayers were methanol-fixed in situ on culture dishes.

**SDS polyacrylamide electrophoresis and immunostaining of proteins**

Vegetative cells, whole slugs, prestalk and prespore fractions dissected from the anterior and posterior of slugs, respectively, and mature stalks and spores were collected in SDS lysis buffer containing 1 mM-PMSF. Following protein determination (Lowry, Rosebrough, Farr & Randall, 1951), equal amounts of protein (10–20 µg) were loaded and separated on 5–17.5 % SDS polyacrylamide gels (SDS-PAGE) (Laemmli, 1970).

Proteins were electrophoretically transferred to nitrocellulose sheets using the method of Towbin, Staehelin & Gordon (1979). The blotted samples were immunostained using a horseradish peroxidase–anti-peroxidase (PAP) complex (Glass, Briggs & Hnilica, 1981), then scanned with a Shimadzu CS-930 Dual Wavelength TLC Scanner at 460 nm.

**RESULTS**

**Production of anti-stalk antibodies**

In order to obtain an immunological probe of stalk differentiation to complement the frequently used spore antiserum (Takeuchi, 1963), we raised antibodies
against mature stalk cells. Initial characterization of stalk antibody activity was by indirect immunofluorescence. The preimmune rabbit serum reacted weakly with all fixed *D. discoideum* V12 M2 cells. However, this reactivity could be completely removed by absorption with a mixture of vegetative cells and spores. Stalk-specific antibodies in absorbed immune serum were evident by the bright fluorescence of individual stalk cells and stalk sheath and the lack of reactivity with spores (Fig. 1A,B) or vegetative cells (Fig. 5A,B). The specific staining of the nascent stalk tube in a culminating sorocarp could be demonstrated as shown in Fig. 1C,D.

**Nature of the stalk antigens**

To determine the nature of the antigens reacting with the antiserum, a variety of extractions and digestions designed to selectively remove protein and polysaccharide components were performed on stalk material (Table 1). Following each extraction, washed residual material was examined using indirect immunofluorescence. The amount of cytofluorescence did not change following treatments designed to remove proteins from the cells (pronase digestion; various detergent extractions (Geltosky, Weseman, Bakke & Lerner, 1979), boiling in SDS–urea

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**Fig. 1.** Cell specificity of immunofluorescence using anti-stalk antibodies. (A,B) Mechanically dissociated V12 M2 fruiting body containing stalk cells (*st*) and spores (*sp*), body plus intact stalk (*stk*) (×250). (C,D) Culminating cell mass showing stalk cells and a nascent stalk tube (*stt*). (A,C) Immunofluorescence; (B,D) phase contrast. ×120.
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Table 1. Effects of enzymatic or chemical digestions or extractions on immunofluorescence of stalk material

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Material extracted</th>
<th>Immunofluorescence of residual material</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>none</td>
<td>+++</td>
</tr>
<tr>
<td>NP40 (0.5%), sodium deoxycholate (0.5%) and SDS (0.1%), 15 min, 25°C or SDS-PAGE buffer, 4 min, 100°C</td>
<td>proteins, lipids</td>
<td>+++</td>
</tr>
<tr>
<td>Pronase (10 mg ml(^{-1})) + trypsin (10 mg ml(^{-1})), 16 h, 37°C</td>
<td>proteins</td>
<td>+++</td>
</tr>
<tr>
<td>Cellulase (3%), 16 h, 21°C</td>
<td>cellulose</td>
<td>++</td>
</tr>
<tr>
<td>Sequential extraction:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) 9 M-urea + 2% SDS, 48 h, 21°C, then new urea/SDS, 100°C, 1 h, urea/SDS rinse, then ddH(_2)O, 100°C, 1 h</td>
<td>proteins*</td>
<td>+++</td>
</tr>
<tr>
<td>(b) 1 mg ml(^{-1}) cellulase, 18 h, 38°C, then 10 mg ml(^{-1}) cellulase, 6-5 h, 40°C</td>
<td>cellulose</td>
<td>++</td>
</tr>
<tr>
<td>Sequential extraction:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) ddH(_2)O, 3×3 h, 100°C</td>
<td>proteins, polysaccharides</td>
<td>+++</td>
</tr>
<tr>
<td>(b) Ethanol:benzene (1:2), 70°C, 6 h</td>
<td>phenolics, lipids</td>
<td>+++</td>
</tr>
<tr>
<td>(c) 0.5% EDTA in 0.5 M-NaPO(_4), pH 6.8, 100°C, 1.5 h</td>
<td>polysaccharides</td>
<td>±</td>
</tr>
<tr>
<td>(d) 4.4 M-NaOH, 0.65 M-boric acid, pH 13.4, 25°C, 16 h</td>
<td>polysaccharides</td>
<td>±</td>
</tr>
<tr>
<td>(e) 6 N-H(_2)SO(_4), 25°C, 3 h, then 3 N-H(_2)SO(_4), 95°C, 4 h</td>
<td>polysaccharides</td>
<td>−</td>
</tr>
</tbody>
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* This procedure effectively removes ~90% of the proteins as determined by Lowry measurements.

( Freeze & Loomis, 1978) and decreased only slightly following cellulase digestion (Freeze & Loomis, 1978). However, when stalk cell material was extracted using methods originally designed to sequentially remove components of plant cell walls (lipids and phenolics, pectins, hemicellulose, cellulose and proteins) (Heath & Northcote, 1971; Cook & Stoddart, 1973), cytofluorescence decreased dramatically, indicating that serum reactivity was due primarily to the presence of polysaccharide antigens in stalk cells and sheath material. To confirm this, antiserum was absorbed with vegetative and whole fruiting body proteins immobilized on nitrocellulose filters. This removed all antibodies reactive with SDS-soluble material as demonstrated by the complete absence of bands when used in immunoblotting experiments. Cells incubated with such absorbed antiserum revealed the same fluorescent staining characteristics as those from earlier experiments using whole serum absorbed with spores and vegetative cells (data not shown).
Identification of stalk-specific protein antigens

Although removal of stalk cell proteins by detergents or proteases did not lead to a visible decrease in stalk cell immunofluorescence, the possibility remained that some stalk-specific proteins were also recognized by the polyclonal antiserum. When antiserum that had been absorbed with vegetative and spore cells was used to stain immunoblotted proteins from a variety of cell types, a large number of protein or glycoprotein bands were seen, many of which were common to vegetative, stalk and spore extracts. These cell type nonspecific antibodies could be removed by absorbing crude antiserum with vegetative cell and, or, spore proteins immobilized on nitrocellulose filters. Absorption of crude antiserum with filter-bound vegetative proteins removed all antibodies reactive with vegetative proteins (Fig. 2, lane 1), but numerous proteins were still recognized in both cell types at later stages. One band ($M_r \approx 40,000$; ST-40) was greatly enriched in stalk cells upon terminal differentiation (lane 5). A few other bands were often enriched in prestalk and stalk cells but their appearance on blots was less consistent. Numerous immunoreactive bands could be seen in spore extracts (lane 6), most of which were also found in stalk cell lysates (lane 5).
Absorption of antiserum with both vegetative and spore proteins bound to filters removed all but a few stalk protein antibodies (Fig. 2, lanes 7, 8). The only stalk band consistently identifiable with different batches of absorbed serum was that of ST-40. Serum absorbed in this way has been used to detect ST-40 in subsequent experiments. Removal of antibodies reactive with other prestalk and stalk proteins by this double absorption procedure may have been due either to stalk or prestalk contamination of the spore protein extract used to absorb the serum, or to antigenic determinants common to prestalk, stalk and spore proteins.

**Developmental expression of anti-stalk and anti-spore cytofluorescence**

We first determined the timing of expression of antigens recognized by our anti-stalk serum during normal development on filters, and then proceeded to examine its expression during cell monolayer differentiation. In each case, we also followed progress along the spore pathway using the traditional anti-spore serum.

1. **Normal development**

Cells were disaggregated at various times during normal development and examined by indirect immunofluorescence using absorbed antiserum (Figs 3, 4). Only low background fluorescence was observed in vegetative (Fig. 5A,B) or 9 h cells (Fig. 3A,B). Stalk antigen reactivity was first detectable at the onset of culmination, when cells exhibited a diffuse overall fluorescence which was quickly followed by the appearance of bright peripheral fluorescence. This probably reflected the deposition of cell wall material or secretion of extracellular matrix, though by phase contrast cells were still amoeboïd (Fig. 3C,D). The proportion of cells reacting with the anti-stalk serum is shown in Fig. 4. The characteristics and temporal appearance of spore serum-reactive material detectable by immunofluorescence was similar to that previously described (Hayashi & Takeuchi, 1976; Forman & Garrod, 1977) (Fig. 4) and is attributed to the presence of prespore vesicles (PSVs), which contain mucopolysaccharide (Takeuchi, 1972) and protein (Devine, Bergmann & Loomis, 1983) precursors of the spore coat.

2. **Development in cell monolayers**

Cells (V12 M2) plated as monolayers with 5 mm-cAMP first collect into small mounds containing 10–20 cells before proceeding to differentiate exclusively into stalk cells. When cultures developing under these conditions were fixed in situ, treated with anti-stalk serum and examined by indirect immunofluorescence, a unique staining pattern was observed. As early as 12 h in development, bright localized areas of fluorescence were observed in up to 50% of the cell clumps (Fig. 5C,D). This fluorescence was apparently associated with one or a small number of cells or extracellular material. As development proceeded, the number of the reactive clumps increased, and a greater proportion of the neighbouring cells within each clump became immunofluorescent (Fig. 5E–H). Eventually all cells that exhibited stalk cell morphology under phase contrast reacted with the antiserum (Fig. 5I,J).
Developmental expression of stalk and spore protein antigens

(1) Normal development

The time course of the SDS-soluble ST-40 antigen appearance in wild-type (V12 M2) cells developing on Millipore filters is shown in Fig. 6A. The ST-40 band first appears at low levels at 15 h when some aggregates within the population were forming Mexican hat structures and beginning culmination. The expression of antigens that react with anti-spore serum absorbed with vegetative and stalk cells is shown in Fig. 6B. Only one band of $M_r$ 95,000 (PSP-95) has been identified as

![Fig. 3. Expression of anti-stalk immunofluorescence during V12 M2 development. (A,C,E) Immunofluorescence. (B,D,F) Phase contrast. (A,B) 9 h amoebae (am) (×440). (C,D) Cells from culmination stage (×500). (E,F) Mature stalks (st) and spores (sp) (×250).](image)
being prespore specific based on its presence in lysates of cells from the posterior region of migrating slugs and its absence from anterior cell lysates and lysates from mature spores or stalks (Fig. 6C). A second prominent band recognized by the anti-spore serum ($M_r$ 100000) is developmentally regulated and enriched in prespore and spore lysates, but is also found in stalk lysates (and thus may not be exclusive to spore differentiation). The relationship between these spore antigens and the SP-96 spore coat protein antigen identified by Devine et al. (1983) or the SP-90, SP-97 or SP-103 spore coat antigens of Delaney, Wilkinson & Hames (1983) has not been determined.

(2) Cell monolayer development

In order to compare ST-40 expression during monolayer and normal development, V12 M2 cells were plated either as monolayers at $10^6$ cells cm$^{-2}$ in DMG/Hepes buffer containing 5 mm-cAMP or on filters in the same buffer with cAMP. Cells were harvested at various times and the expression of ST-40 under the two developmental conditions was compared by analysing lysates on the same western blot. As shown in Fig. 7, ST-40 is expressed at least 3 h earlier during monolayer development than during normal development at a time long before stalk cell differentiation can be detected morphologically. No PSP-95 is detectable at any time under monolayer conditions in which all developing V12 M2 amoebae go on to form stalk cells (Fig. 8), although the 100000 $M_r$ band recognized by the anti-spore serum is expressed.
Fig. 5. Expression of stalk antigens during development of V12 M2 cells as submerged monolayers with cAMP at 10^5 cells cm^{-2}. (A,C,E,G,I) Immunofluorescence. (B,D,F,H,J) Phase contrast. (A,B) 0 h; (C,D) 12 h; (E,F) 18 h; (G,H) 24 h; (I,J) 36 h. x340.
Effect of altered pH, weak acid and weak base on timing and level of stalk and spore antigen expression in cell monolayers

(1) V12 M2

Previous studies have shown that altered buffer pH alone or in conjunction with weak acids or bases dramatically affects terminal differentiation of cells in monolayer culture (Gross et al. 1981; Gross, Bradbury, Kay & Peacey, 1983; Town, 1984). We wished to determine when during monolayer development these regimes affect phenotypic expression since it is well known that Dictyostelium cells are not determined and can alter their developmental fate even at late stages. Amoebae were allowed to differentiate as cell monolayers with cAMP at $10^6$ cells cm$^{-2}$ in DMG/Hepes buffer at various pH values. Stalk cell differentiation was slower under these conditions, probably due to the higher ionic strength of the buffer (Town, 1984), but the final degree of differentiation was the same in both DMG/Hepes and MES/Tris buffers.

The kinetics of appearance of anti-stalk antibody immunofluorescence in cells collected from dishes and dissociated were similar in all cultures except pH 7.5

Fig. 6. Immunostaining of lysates from V12 M2 cells sampled at indicated times during normal development on filters with DMG/Hepes buffer. (A) Absorbed anti-stalk serum stain showing ST-40 expression. (B) Absorbed anti-spore serum stain showing 95 K and 100 K $M_r$ bands. (C) Absorbed anti-spore serum showing 95 K band specificity. Lanes 1–6 are the same as 1–6 in Fig. 2; lane 7, whole fruiting structures. The lower $M_r$ bands reactive with anti-spore serum are common to all cell types.
with NH₄Cl, in which the proportion of fluorescent cells was reduced (Fig. 9A). The differences in relative proportions of fluorescent cells at 39h are real, but the absolute values are only approximate since it was impossible to completely

Fig. 7. Anti-stalk serum immunostaining of cell lysates from V12 M2 cells sampled at indicated times during normal and monolayer development. Cells were plated for normal development on filters or for monolayer development in dishes with DMG/Hepes buffer. Samples containing 20 μg protein were processed on the same blot and stained with absorbed anti-stalk serum.

Fig. 8. Anti-spore serum immunostaining of lysates from V12 M2 cells sampled at indicated times during monolayer development. Cell density was 10⁵ cm⁻² in MES/Tris buffer. Absorbed anti-spore serum showing 100K Mr bands and cell-type-nonspecific bands.
dissociate cells in later stages of development (21 h plus) for accurate scoring. All terminal cultures contained large numbers of fluorescent stalk cells except those at pH 7.5 with NH₄Cl in which the fluorescence seemed to be associated with the few stalk cells present and some extracellular material around the masses.

The proportion of PSV-containing cells in monolayer cultures was reduced by low pH and especially by low pH with propionate (Fig. 9B). Somewhat higher proportions of PSV-containing cells were observed in the cultures developing at pH 7.5, especially at later times, although inclusion of 5 mM-NH₄Cl delayed the appearance of PSVs by ~6 h, possibly due to its inhibitory effect on intracellular cAMP synthesis (Schindler & Sussman, 1979).

The expression of both ST-40 and PSP-95 was affected by the pH of the developmental buffer (Fig. 10). ST-40 was detected in this experiment at very
low levels at 12 h in cultures at all pHs (Fig. 10A). At 18 h, it was expressed at
higher levels in cells developing at pH 5.2 and at pH 6.1 than in those at pH 7.5.
Propionate (0.3 mM) did not apparently enhance ST-40 expression at pH 5.2, but
NH₄Cl (5 mM) in pH 7.5 buffer significantly decreased the levels of ST-40, more so
than pH 7.5 alone. PSP-95 was only found in cells developing at high pH, and its
expression was greatly enhanced by the presence of NH₄Cl (Fig. 10B). These
latter cultures formed tight cell masses with few if any stalk cells.

Fig. 10. Effect of pH on expression of (A) ST-40 and (B) PSP-95 during V12 M2
monolayer development. Densitometric scans of immunoblots were made, and data
are presented as the proportion of maximum absorbance observed for the specific band
recognized by each serum. Conditions and symbols were the same as in Fig. 9. In B,
PSP-95 was detectable only in the pH 7.5 cultures (with or without NH₄Cl).
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Fig. 11. The effects of extracellular pH, weak acid and weak base on terminal differentiation of HM29 cell monolayers. Cells were plated in DMG/Hepes buffer at the various pHs with cAMP. Cell density was 10^5 cm^{-2}. Results represent the proportion of each cell type observed by phase contrast after 4 days when development was complete.

(2) **HM29**

In wild-type strains developing in monolayers with cAMP, cells can undergo terminal differentiation into stalk cells but not spores. Similar experiments were therefore also performed with sporogenous mutant strain HM29, so that we could observe the effects of the extreme pH regimes in cultures where both stalk and spore pathways of differentiation could proceed to completion. The effect of altered extracellular pH on terminal differentiation of HM29 monolayers cultures is shown in Fig. 11. As shown previously (Gross et al. 1981, 1983; Town, 1984), low pH greatly favours stalk formation and high pH greatly favours spore differentiation. As with the wild-type V12 M2 cells, the effects of extracellular pH on differentiation can be observed as early as 8–12 h in development. The effect of pH on ST-40 expression in HM29 cells is more dramatic than in V12 M2 cells, and is evident at the time of its appearance (12 h) (Fig. 12A). High pH inhibits ST-40 expression compared with pH6.3 controls and, unlike V12 M2 cells, low pH enhances its expression relative to pH6.3 controls. Similar effects of pH are observed in the proportion of cells expressing stalk polysaccharide antigens (Fig. 12B). The pH effect on spore antigen cytofluorescence and spore differentiation is shown in Fig. 13, low pH, especially with the addition of propionate, inhibiting PSV expression and spore formation.

**DISCUSSION**

Previous studies have shown that when *D. discoideum* cells are allowed to differentiate as monolayers with 1–5 mM-cAMP, the proportion of stalk cells and
Fig. 12. The effect of extracellular pH on stalk antigen expression during HM29 monolayer development. (A) ST-40 and (B) stalk polysaccharide antigens. Developmental conditions were as in Fig. 11. (A) Cell samples were lysed and immunblotted using absorbed anti-stalk serum. Blots were densitometrically scanned and data are presented as the proportion of maximum absorbance observed. (B) Cells were collected and clumps dissociated, then samples were immunofluorescently stained with anti-stalk serum absorbed with vegetative proteins and spore cells. Data represent the proportion of cells reacting with antiserum. (●) pH 5.4; (■) pH 6.3; (▲) pH 7.7.

Fig. 13. The effect of extracellular pH, weak acid and weak base on spore antigen expression during HM29 monolayer development. Developmental conditions and treatments were the same as in Fig. 12B except that samples were immunofluorescently stained with absorbed anti-spore serum. Data represent the combined proportion of PSV-containing cells (i.e. prespore) and spores. (○) pH 5.4; (□) pH 6.3; (△) pH 7.7; (●) pH 5.0+0.3 mM-propionate; (▲) pH 7.6+5 mM-NH₄Cl.
spores formed is strongly affected by extracellular pH. Low pH favours stalk cell formation and high pH spores, these effects being enhanced by the addition of weak acid and weak base, respectively (Gross et al. 1981, 1983; Town, 1984). Those studies, however, do not indicate at what stage of development pH can exert phenotypic regulation. This question is of interest both from a mechanistic standpoint, and also a practical standpoint if the pH (or similar) regimes are to be useful in generating cell populations expressing exclusively one phenotype. In order to investigate this mode of phenotypic regulation at earlier stages of development, we raised and characterized a new antiserum against stalk cells and have used it, along with the widely used anti-spore serum, to follow the effects of pH at both the cytological and molecular level.

The antiserum we have raised reacts strongly with polysaccharide components of the stalk cell wall and sheath, which constituted ~40% by weight of the initial immunogen. This polysaccharide component is responsible for the immunocyto-fluorescence. When suitably absorbed, the antiserum also recognizes several prestalk- or stalk-specific proteins on western blots, of which ST-40 is the most prominent. Both polysaccharide and protein antigens appear relatively late during normal development, being detectable only at or after the onset of culmination. Thus both antigens are specific for terminal stalk cell rather than prestalk differentiation. Our antiserum does not detect any of that class of prestalk proteins that appear early and are amplified in prestalk cells but not present in fully differentiated cells (Borth & Ratner, 1983; Morrissey, Devine & Loomis, 1984). Other monoclonal antisera reported while this work was in progress may be more suitable for analysing these earlier proteins (Tasaka, Noce & Takeuchi, 1983; Wallace, Morrissey & Newell, 1984). It is of interest that our ST-40 antigen appears earlier in monolayer cultures incubated with cAMP than it does during normal development. This observation, coupled with our repeated failure to detect any of the prestalk isozyme acid phosphatase II under these conditions (Town, unpublished data) suggests that under our monolayer conditions cells may bypass some of the earlier prestalk stages and proceed more rapidly to the terminal stages of stalk cell formation.

The appearance of the polysaccharide antigen at distinct foci at early times (i.e. 12 h) might indicate the presence of organizer-like centres within each differentiating cell group. However, the level of resolution at which this early localized cytofluorescence was observed is insufficient to determine whether the antigens were strictly intracellular or also present extracellularly. It is possible that some antigen is also present in the extracellular space at early times (12–18 h) during normal development. If so, this might have gone undetected in immunofluorescence studies of normal development due to the disaggregation and washing procedures used. Given that such small amounts of antigen are present early in development, it is unlikely that this localized expression would be clearly detectable in histological preparations of the large cell masses that form during normal development.
Immunostaining with our antispore serum results in cytofluorescent pattern (punctate fluorescence within amoebae) which is temporally regulated (appearing between 9–12 h in development) in a manner similar to that described by others (Takeuchi, 1963; Hayashi & Takeuchi, 1976; Forman & Garrod, 1977), and thus effectively monitors expression of the spore pathway. The observation that our anti-spore antiserum detects fewer spore proteins on western blots than other anti-spore sera (Devine et al. 1983) might indicate that most of the reactivity of our serum is against mucopolysaccharide rather than protein antigens. Alternatively, fewer spore protein antigens may have been detected due to the methods used. The antisera described by others (Devine et al. 1983; Delaney et al. 1983) which react with numerous spore coat proteins were prepared using purified spore coat material as immunogens, thereby allowing a stronger response to these antigens. Our immunogen was a preparation of whole spores containing numerous antigens other than the spore coat proteins. In addition, some spore-specific antibodies may have been removed by spores contaminating the stalk cell preparations that were used to rigorously absorb stalk reactivity out of the crude spore serum.

Using our stalk and spore antisera, we have been able to determine when during monolayer differentiation extracellular pH affects the expression of these cell-type-specific markers, and whether these earlier effects are consistent with previous results on the modulation of terminal cell differentiation by extracellular pH. Although the details vary for each marker, some general conclusions may be drawn. In wild-type V12 M2 cultures, phenotypic modulation by pH can be observed quite early in development, shortly after the expression of these specific markers is first detected, and is more marked at later stages. During V12 M2 monolayer development in which all cells either form stalk cells or morphologically appear to remain as amoebae, the effects of low pH are more to inhibit the expression of spore markers than to enhance stalk markers based on comparisons made with pH6-1 controls. High pH, however, both inhibits stalk and enhances spore expression evident by advanced PSV expression and appearance of PSP-95. These effects are accentuated by the inclusion of weak acid or base at the appropriate pH. These results are entirely consistent with previously reported effects on terminal cell differentiation in monolayer cultures (Gross et al. 1981, 1983; Town, 1984) and extend them to demonstrate the suppression and, or, enhancement of these pathways at much earlier stages of the differentiation process. The expression of PSP-95 in monolayer cultures of wild-type cells at pH7-5 (with or without NH4Cl) shows that these conditions allow cells to progress further along the spore pathway than at pH6-1. However, they are still unable to undergo terminal differentiation into spores under monolayer conditions, a property acquired only by mutation of cells to the sporogenous phenotype.

During the development of the sporogenous mutant HM29, which can form both stalk cells and spores in monolayers, the effect of altered extracellular pH on stalk antigen expression is more dramatic. The altered expression of these antigens is consistent with the observed effects of pH on the proportion of terminally differentiated spores and stalk cells. In these cultures, stalk antigen expression is
both increased by low pH and decreased by high pH relative to pH 6.3 controls. There is no enhancement of PSV expression by elevated pH compared with pH 6.3 controls (which is consistent with similar proportions of terminal spores formed in these cultures), but low pH, especially in the presence of propionate, diminishes PSV expression.

Though the expression of antigens varies between strains, the effects of altered extracellular pH may be observed as early as 12 h in both V12 M2 (PSVs) and HM29 (stalk antigens) monolayer development. This is the earliest time of antigen appearance and corresponds to the time of tipped aggregate formation in normal development. From these and other results it is clear that cells respond to altered extracellular pH throughout the latter half of development. Together the spore- and stalk-specific antisera should thus be a useful means of monitoring differentiation in further studies of the regulation of phenotype by altered extracellular pH and other parameters.

The mechanism by which low and high extracellular pH (with or without weak acid or base) regulate stalk and spore differentiation is unknown. A popular model suggested that these regimes modulate intracellular pH, thereby initiating or stabilizing processes leading to spore or stalk pathway gene expression, respectively (Gross et al. 1981, 1983). However, recent results obtained in our laboratory using $^{31}$P nuclear magnetic resonance indicates that cytoplasmic pH varies little over a wide range of external pH conditions alone (Jentoft & Town, 1985) or with the addition of propionate or NH$_4$Cl (Town, Dominov, Karpinski & Jentoft, in preparation). Thus we feel that some other mechanism is involved in transmitting the external pH signal to the interior of these cells, thereby inducing a response. Whatever the mechanism, the phenomenon of phenotypic regulation by pH remains clear and dramatic and provides an excellent system in which to study the regulation of differentiation and cell type interconversion in Dictyostelium discoideum.

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