A mutational analysis of *Dictyostelium discoideum* multicellular development

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We have collected *Dictyostelium* mutants that arrest in development after aggregation, but before first finger formation. A total of 118 mutant strains were isolated and are referred to as mound (*mnd*) mutants. Nine complementation groups (*mndA–mndI*), containing 46 of the mutant strains, were defined by parasexual methods. A statistical analysis suggested that there are about 118 genes which, when mutated, confer the mound phenotype. Of these genes, about 60 are predicted to be mutated in our collection: the 9 assigned to complementation groups and another 51 unassigned mutants. *mndA*, *G*, *H* and *I* were assigned to linkage groups VII, IV, II and VI, respectively. Development of the mutant strains was characterized by terminal morphology, neutral red staining and expression of marker mRNAs for prespore and prestalk cells. Three broad classes were recognized. (1) Post-aggregative mutants – those blocked early in multicellular development. They did not express any of the prestalk or prespore marker mRNAs and generally arrested as low mounds or ridges. (2) Pathway mutants – those blocked specifically in either prestalk or prespore differentiation. They expressed either prestalk or prespore marker mRNAs, but not both, and generally proceeded further morphologically than post-aggregative mutants. (3) Morphogenesis mutants – those apparently blocked in morphogenesis rather than cell differentiation. They expressed all the cell-type marker mRNAs tested. Most arrested as tight mounds lacking a tip and of defined upper size, but some mutants produced aberrant tips. The majority of mutants tested synergized with wild-type: 24/28 strains which cannot make spores when developed alone, were able to do so when allowed to develop with an equal number of wild-type cells. We suggest that some of the morphogenesis mutants have a cytoskeletal defect which prevents first finger formation and that these mutants can be physically carried through development by the wild-type (synergy by 'piggy-backing').

Keywords: *Dictyostelium discoideum*, development, mound mutants, gene numbers

INTRODUCTION

Molecular genetic analysis has allowed access to a whole range of master regulatory genes in developmental biology (Horvitz & Sternberg, 1991; Mayer *et al.*, 1991;

Nusslein-Volhard & Wieschaus, 1980). There are two major prerequisites for its success. Firstly, a genetic system allowing the isolation and maintenance of large numbers of strains carrying mutations which disrupt development. Secondly, some way of cloning the genes so identified. *Dictyostelium discoideum* has a relatively simple developmental programme which nevertheless displays many of the key phenomena seen in other organisms: cell differentiation, pattern formation, gradients, regeneration and complicated morphogenetic manoeuvres (Bonner, 1967; Loomis, 1982; Raper, 1940).

Since *Dictyostelium* is haploid and its development facultative, many sorts of mutants blocked in development

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Abbreviations: DIF-1, differentiation-inducing factor-1; GBF, G-box binding factor; Ig, linkage group; REMI, restriction enzyme-mediated integration.

can be readily isolated and maintained. Using parasexual genetics, it is also easy to place them into complementation groups, though the low frequency of recombination is inimical to further genetic analysis (Loomis, 1987; Newell, 1978; Welker & Williams, 1982). Despite these advantages, the possibilities of genetic analysis have not been greatly exploited because there has been no clear route from mutation to cloned gene.

Recent advances have now made a molecular genetic analysis entirely feasible. The restriction enzyme-mediated integration (REMI) technique for insertional mutagenesis, in which a restriction enzyme targets a transforming plasmid to its recognition sites in the genome, enables genes to be mutated and then readily cloned (Dynes *et al.*, 1994; Harwood *et al.*, 1995; Insall *et al.*, 1994; Kuspa & Loomis, 1992). Alternatively classical mutants may be complemented using genomic clone banks (Dynes & Firtel, 1989). Though this method is not routine, it may become so with further improvements in transformation techniques. In our experience, chemical mutagenesis has the advantage that many more mutants can be isolated than by REMI. With these possibilities in mind, we have made a large-scale mutational analysis of early multicellular development in *Dictyostelium* using chemical mutagenesis.

The period of development analysed starts at the end of aggregation, when the cells have gathered into loose mounds, and ends with the formation of a standing first finger, when an independent, patterned, multicellular entity has been established. In this period, the aggregates first compact and sub-divide into a series of mounds of fixed upper size (Hohl & Raper, 1964). The final size of a mound is apparently regulated by a series of organizing centres (tips), one per mound, which inhibit each others formation, probably by a graded diffusible signal; we refer to this process as size control (Durstion, 1976; Kopachik *et al.*, 1983). The mounds secrete an extracellular matrix, the slime sheath, consisting of cellulose and a number of proteins, including the *ecmA* and *ecmB* gene products (McRobbie *et al.*, 1988). Within the mounds prestalk and prespore cells differentiate. The prespore cells are relatively homogeneous in both expression of markers and eventual fate (they become spores), but the prestalk cell population is heterogeneous. Prestalk A cells express the *ecmA* mRNA and eventually produce the stalk proper of the mature fruiting body, whereas prestalk B cells express the *ecmB* mRNA and produce the basal disc (Jermyn *et al.*, 1989; Williams *et al.*, 1989). Prestalk A cells differentiate at scattered positions in the mound and later sort to populate the protruding tip that forms at the top (Williams *et al.*, 1989).

cAMP signalling controls cell movement (Siegert & Weijer, 1992) and is essential for the continued differentiation of cells after aggregation. Differentiation-inducing factor-1 (DIF-1), a chlorinated alkyl phenone (Kay & Jermyn, 1983; Morris *et al.*, 1987), induces prestalk and stalk cell differentiation (Town *et al.*, 1976; Williams *et al.*, 1987). Little is known of its mode of action, though there is a cytoplasmic binding protein (Insall & Kay, 1990) and

DIF-1 is inactivated by dechlorination, catalysed by DIF-1 dechlorinase (Nayler *et al.*, 1992; Traynor & Kay, 1991). DIF-1 biosynthesis, the binding protein and the metabolic enzymes are developmentally regulated and show a large increase at the end of aggregation.

The aim of our mutational analysis is to eventually identify genes involved in cAMP and DIF-1 signalling, determining the size of an aggregate, cell sorting and morphogenetic movements.

METHODS

Strains and cell growth. All strains ultimately derive from strain NC4; their genotypes and origins are shown in Table 1. Strains HM48, HM138 and HM140 are spontaneous cycloheximide-resistant mutants isolated from strain NP2 by selection on plates containing 500 µg cycloheximide ml⁻¹. Cells were grown at 22 °C in association with *Klebsiella aerogenes* on SM agar plates (Kay, 1987). To initiate development, they were freed from bacteria by repeated centrifugal washes in KK2 (16.6 mM KH₂PO₄, 3.8 mM K₂HPO₄, 1 mM MgSO₄, pH 6.2) and plated on 1.8% (w/v) L28 agar (Oxoid) containing KK2.

Mutagenesis was performed with NTG (Sigma) at 200 µg ml⁻¹ (usually from a 10 mg ml⁻¹ DMSO stock solution) for 20 min, giving a survival of 1–10% or with UV irradiation (15 s at 302 nm and 7 mW cm⁻²). Cells were plated clonally on SM agar in association with bacteria and colony morphology examined 5–8 d later for mutant development.

Genetics. Diploids were formed by making a mixture of about 10⁶ cells (a loop from a growth zone) of each of the two strains to be crossed in 1 ml sterile 20 mM CaCl₂ and incubating it in a multiwell plate overnight on an orbital shaker (180 r.p.m.) at 22 °C. Next day the contents of each well were distributed amongst four SM plates and spread in association with bacteria. Diploids were selected at the restrictive temperature of 26.8 °C. Plates were kept in plastic boxes, with a small air hole, to minimize evaporative cooling. Generally each cross gave at least one diploid.

Complementing diploids were immediately obvious from their wild-type fruit and spore head colour (yellow); further confirmation was not normally necessary. Non-complementing diploids were generally segregated to give haploids by growth on thiobendazole (Welker & Williams, 1980a) (3 µg ml⁻¹ in SM agar) and the segregants checked for the presence of outside markers from both parents (normally methanol and cycloheximide resistance).

Mutations were assigned to linkage groups by crossing with the multiply marked tester strain HU407 (from Dr K. Williams, Macquarie University, Australia), segregating haploids and testing their genotypes by standard methods (Loomis, 1987; Newell, 1978).

Mutant strains were stored by freezing vegetative cells in liquid nitrogen and by storing diploid spores, from a cross with a parental strain, on silica gel.

Characterization of mutant strains. Cells were stained with 50 µg neutral red ml⁻¹ at the start of development (Bonner, 1952). RNA was extracted from cells that had developed on KK2 agar for different lengths of time and prestalk and prespore mRNAs detected by Northern blotting as before (Berks & Kay, 1990). In synergy experiments, cells from two different strains were washed free of bacteria and mixed, generally 1:1, and plated for development on KK2 agar. The genotype of any spores formed was checked by plating them on SM agar with

Table 1. Genetically marked strains used in this work

Strain	Parent	Genotype*										Origin
		<i>whi</i>	<i>bwn</i>	<i>tsg</i>	<i>cyc</i>	<i>acr</i>	<i>axe</i>	<i>spr</i>	<i>man</i>	<i>bsg</i>	<i>cou</i>	
X2		+	<i>A</i>	<i>A1</i>	+	+	<i>A1,B1</i>	+	+	+	+	Williams <i>et al.</i> (1974)
X22		<i>A</i>	+	<i>D12,E13</i>	+	<i>A1</i>	+,+	<i>A1</i>	+	+	+	Williams & Newell (1976)
NP2		+	+	<i>A1</i>	+	+	<i>A1,B1</i>	+	+	+	+	Williams <i>et al.</i> (1974)
HM48	HL6	+	+	<i>J2</i>	<i>902</i>	+	<i>A1,B1</i>	+	+	+	+	This study
HM138	NP2	+	+	<i>A1</i>	<i>903</i>	+	<i>A1,B1</i>	+	+	+	+	This study
HM140	NP2	+	+	<i>A1</i>	<i>905</i>	+	<i>A1,B1</i>	+	+	+	+	This study
HU407		+	+	<i>K21</i>	<i>A1</i>	+	+,+	+	<i>A2</i>	<i>A5</i>	<i>A351</i>	Welker & Williams (1980b)

* Phenotypes of mutations at the loci are as follows: *whiA*, white (rather than yellow) spore heads; *bwnA*, brown pigment produced; *tsg*, grows at 22 °C but not at 27 °C; *acrA*, grows on 2% methanol; *axeA* plus *axeB*, grows axenically; *sprA*, round (rather than oval) spores; *manA*, lack of α -mannosidase; *bsgA*, unable to grow on *Bacillus subtilis*; *couA*, sensitivity to 1.0 mM coumarin.

bacteria, after all non-spores had been killed by detergent treatment (0.3% Cemulsol for at least 30 min).

RESULTS

Mutant isolation

Mutations were induced by NTG or, occasionally, UV mutagenesis. Most mutants were isolated in strains HM140 and X22 (Table 1), which are marked for parasexual genetics and develop well. HM140 is axenic, allowing the possibility of DNA-mediated transformation; other axenic strains (X2, HM48 and HM138; Table 1) were used for a few experiments, but were not so satisfactory. X22 is non-axenic, but was used as the second parent because axenic strains do not cross well with each other in parasexual crosses (Dr P. C. Newell, personal communication, confirmed by our experience).

Mutants were chosen by their plaque morphology when grown on bacterial lawns. Wild-type plaques display the entire developmental sequence from growing cells around the edge of the plaque, to fruiting bodies in the centre. Mutants blocked at various stages of development are readily recognized; we chose ones which aggregated, but then arrested as mounds or ridges, in some cases with a tip. Because the common feature of the chosen mutants was their arrest in development at the mound stage, we refer to them as mound (*mnd*) mutants. Mutants that proceeded further and made a slug were not taken, nor were ones with a leaky phenotype. A total of 118 mutants were collected from more than 100 separate mutagenesis experiments in which about 200 000 plaques were observed.

Complementation testing

Dictyostelium is a naturally haploid organism in which parasexual genetics depends on the rare fusion of two cells to form a stable diploid. Diploids were selected by using parental strains with complementing temperature-sensitive growth mutations and selecting at the restrictive temperature (Katz & Sussman, 1972; Loomis, 1987;

Table 2. Complementation groups

Mutants raised in an axenic background were crossed with those raised in a non-axenic background. The parent of all non-axenic strains is X22; of the axenic strains, X2 is the parent of strains HM51–HM90, HM48 is the parent of HM91, NP2 is the parent of HM142 and HM144, HM138 is the parent of HM153 and HM140 is the parent of the rest.

	Axenic strains	Non-axenic strains
<i>mndA</i>	HM57 HM142 HM61 HM153 HM62 HM158 HM72 HM160 HM73 HM183 HM79 HM202 HM91 HM203	HM98 HM120 HM108 HM123 HM109 HM128 HM110 HM224
<i>mndB</i>	NP127	HM113 HM 137 HM114
<i>mndC</i>	HM52 HM205 HM56	HM133
<i>mndD</i>	HM59	HM130
<i>mndE</i>	HM81	HM157
<i>mndF</i>	HM144	HM161
<i>mndG</i>	HM173 HM208	HM122 HM228 HM135
<i>mndH</i>	HM155 HM182	HM212
<i>mndI</i>	HM148	HM213

Newell, 1978). All mutants were first crossed with a parental strain to test for dominance. All were recessive except for the mutations in strains HM74 and HM226, which were not used further.

Essentially, all mutants in the non-axenic parent, strain X22, were crossed with the mutants in the axenic background (strains HM140, HM48, HM138, HM140, X2) in a 59 × 57 matrix. Once it was established whether or not a mutant belonged to a particular complementation group, further crosses with other members of the group

Fig. 1. Statistical estimate of the number of genes essential for development of a loose aggregate into a first finger. This estimate is based on the frequency of non-complementing diploids in almost 2000 crosses between the mutants and takes into account the observed differences in mutation frequency between loci. The broad curve without symbols gives the likelihood of different numbers of additional genes, over the 9 already established (i.e. assigned to complementation groups). It peaks at 109 extra genes and, with the 9 established genes, this gives 118 as the likeliest number of *mnd* genes. Of the 109 extra genes, some will be already present in our mutant collection (in one parent, but not in both) and the likelihood plot for this number is shown in the second curve (●): it is most likely to be 51.

were not generally made. Overall, almost 2000 crosses were made. A few diploids could not be isolated after several attempts and crosses with a few strains were discontinued for various reasons; in the statistical analysis it is assumed that these crosses would all have given complementing diploids. To the extent that this assumption is wrong, we will have slightly over-estimated the number of target genes giving the mound phenotype. The diploids either developed as wild-type or mutant, intermediate forms were not generally seen. Thus a clear pattern of complementation and non-complementation was apparent and nine complementation groups were

established (Table 2). Of the 116 mutants analysed by complementation, 46 (40%) were placed in complementation groups. *mndA* is a notable mutational hotspot with 22 alleles recovered, compared to five in the next most populous complementation group.

Estimate of gene numbers

A statistical estimate, using a new method developed by Andrew McLachlan (from whom the computer program may be obtained on request at the above address), suggested that the total number of genes giving the mound phenotype was most likely to be 118 (Fig. 1). This method utilizes the observed spectrum of mutation frequencies at individual loci to estimate the likeliest number of such loci. Unlike earlier methods, which had to assume equal mutation frequencies at all loci (Coukell, 1975; Warren *et al.*, 1975), it is able to accommodate mutational hotspots such as *mndA*. From the complementation analysis, nine loci have been established; Fig. 1 shows that there are likely to be an additional 109 loci, not yet assigned to complementation groups, making 118 loci in all. Of the extra loci, we estimate that 51 are represented in one parent or the other (but not both). Thus, we may have isolated mutants in about half of the genes giving the mound phenotype.

Linkage analysis

Mutations in four of the complementation groups were assigned to linkage groups after crossing with the multiply marked tester strain, HU407 (Welker & Williams, 1980b; Table 1). The strains used (HM98, HM235, HM212 and HM213) all derive from the strain X22 parent and, when crossed with strain HU407, give diploids having all six established linkage groups marked. Haploids were segregated and cloned from each of the resulting diploids, and their genotypes determined (Table 3). Coumarin sensitivity (*couA*), the standard marker for linkage group VII (lgVII) (Welker & Williams, 1980b) proved very hard

Table 3. Assignment to linkage groups

Mutant strains (HM98, HM135, HM212 and HM213) were crossed with the multiply marked tester strain HU407, the resulting diploids haploidized and the genotypes of the resulting segregants determined.

	I		II		III		IV		VI		VII	
	<i>cycA1</i>	+	<i>acrA1</i>	+	<i>bsgA</i>	+	<i>bwnA1</i>	+	<i>manA2</i>	+	<i>tsgK21</i>	<i>couA</i> +
<i>mndA</i>	10	13	9	14	8	15	4	19	14	9	3	0
+	16	11	12	15	11	16	8	19	13	14	0	4
<i>mndG</i>	16	16	13	19	8	24	0	32	11	21	14	18
+	5	9	7	7	6	8	14	0	6	8	11	3
<i>mndH</i>	29	9	38	0	24	14	11	27	18	20	27	11
+	2	9	0	11	2	9	2	9	1	10	2	9
<i>mndI</i>	6	13	17	2	6	13	2	17	0	19	10	9
+	9	10	19	0	17	2	19	0	19	0	18	1

Fig. 2. Morphology of wild-type and mutants. All photographs are of stock plates of cells grown on SM agar in association with *K. aerogenes* and are taken from above (all $\times 45$). Terminal mutant phenotypes are shown. (a) Strain X22 (wild-type parent), mounds with tips just emerging; (b) strain X22, first fingers forming; (c) strain HM183 (*mndA*), tight mounds, no tip; (d) strain NP127 (*mndB*), mounds less tight than *mndA*, no tip; (e) strain HM205 (*mndC*), raised mounds, no clear tip; (f) strain HM130 (*mndD*), interconnected, irregular mounds, no tip.

to score. Instead, *tsgK* was used as the IgVII marker, after first eliminating strains having *tsgD* or *tsgE* by the linkage of these markers to *cycA* and *acrA*, respectively. Some of the segregation patterns of unlinked markers are strongly biased, occasionally with a whole class of expected segregant missing (as in *mndI*). This problem is frequently encountered in parasexual crosses and may be due to the

presence of deleterious, linked mutations (Newell, 1978; Newell *et al.*, 1977; Welker & Williams, 1980b). Nevertheless, Table 3 shows that in each cross, the mound mutation segregated independently of markers in five of the six linkage groups but was linked to the remaining marker. Thus, *mndA* was assigned to IgVII, *mndG* to IgIV, *mndH* to IgII and *mndI* to IgVI (with a little less

Fig. 3. Morphology of mutants. All photographs are of stock plates of cells grown on SM agar in association with *K. aerogenes* and are taken from above (all $\times 45$). Terminal mutant phenotypes are shown. (a) Strain HM81 (*mndE*), large, low mounds, no tip; (b) strain HM161 (*mndF*), mounds, sometimes with a tip-like protrusion; (c) strain HM208 (*mndG*) compact, roughly hemispherical mounds, no tip; (d) strain HM212 (*mndH*), scalloped ('brainy') mounds, no definite tip; (e) strain HM213 (*mndI*), irregular, interconnected mounds, no tip; (f) strain HM174 (unassigned), extensive mounds with multiple, arrested tips.

confidence due to the biased segregation of some markers).

Morphological classification

Many, though not all, of the mutants can be regarded as arrested stages in the wild-type sequence of morphological development. We, therefore, based our morphological classification on the wild-type sequence as seen in clones or streaks of cells growing and developing on SM agar, in association with bacteria (Figs 2 and 3, Table 4).

Morphological class 1 mutants. These arrest shortly after aggregation as low, loose mounds. There is no evidence for an upper fixed size for the aggregates or for a slime sheath.

Morphological class 2 mutants. These produce more tightly packed aggregates, which may have scalloped surfaces (*mndB*) and appear sub-divided, indicating that there is some sort of size control. A slime sheath is not normally present.

Morphological class 3 mutants. These produce discrete

Table 4. Phenotypes of mutants assigned to complementation groups

Complementation group	Example	Morphology*	mRNA expression†			Spores‡		Neutral red staining§
			<i>ecmA</i>	<i>ecmB</i>	<i>psA</i>	Alone	Synergy	
<i>mndA</i>	HM79	3	++	++	++	—	+	Red eye
	HM98	3	++	+	++	—	+	Red eye
	HM142	3	(+)	(+)	+			
<i>mndB</i>	NP127	2	(+)	++	+++	—	+	
	HM113	2	(+)	+	++	—	+	Sectors
	HM114	2	(+)	+	++	+	+	Sectors
	HM137	2	(+)	++	++	+	+	Sectors
<i>mndC</i>	HM52	4	+	+	+++	—	+	Uniform
	HM56	4	++	++	++	—	+	Uniform
<i>mndD</i>	HM59	2	0	+	0	—	(+)	Uniform
	HM130	2	0	0	0	—	(+)	
<i>mndE</i>	HM81	1	0	+	0	—	(+)	Uniform
	HM157	1	0	+	0	—	(+)	
<i>mndF</i>	HM144	2	(+)	++	0	—	+	
	HM161	2	+	+	0			
<i>mndG</i>	HM135	3	+++	+++	+++			
	HM173	3	+++	+	+++			
	HM208	3	+++	+++	+++			
	HM228	3	+++	+++	+++			
<i>mndH</i>	HM155	2	(+)	+	+++	—	+	
	HM212	2	(+)	+	++			
<i>mndI</i>	HM148	2	++	++	++	—	+	Uniform
	HM213	2	+	+	+++			

* The morphological classes are explained in the text

† Expression of the prestalk genes *ecmA* and *ecmB* and the prespore gene *psA* was determined by Northern blotting; expression levels of the mutants at 21 h were compared to the parental strains after 15 h of development. Approximate levels are as follows: + + +, control; + +, 20–50% of control; +, detectable, but less than 20% of control; (+), trace; 0 not detectable.

‡ Spore formation is indicated with the mutant cells developing alone or in synergy with equal numbers of wild-type cells. Synergy is scored as follows: +, mutant produced approximately the same number of spores in the synergy mixture as did the wild-type; (+), mutant produced less than 5% wild-type spore yield; —, mutant produced no spores.

§ Red-eye staining is a red central core, normally at the top of the mound; sector staining is staining of cells at the boundaries of sectors in large aggregates; uniform staining is as the name suggests.

mounds, roughly hemispherical or raised to resemble tree trunks, containing tightly packed cells. There is definite size control: large aggregates are visibly sub-divided into mounds of at most 0.5 mm diameter (though some X22-derived strains produce mounds of up to 1.5 mm diameter). A slime sheath is formed, as evidenced by a shiny surface, but there is no sign of a tip.

Morphological class 4 mutants. This is a more heterogeneous class, the common property being tip formation. Some strains have large aggregates, divided into sectors each having a tip-like protrusion; at the other extreme, single, compact mounds with tips are produced.

Neutral red staining

We used neutral red as a convenient way of detecting the differentiation and patterning of prestalk cells during development of the mutants. Previous work showed that

neutral red stains the prestalk cells in the anterior of the slug (Bonner, 1952) and a scattering of 'anterior-like' cells in the posterior, but does not stain the prespore cells (Sternfeld & David, 1981). However, we observed a complicated staining pattern that evolves in the mound prior to tip and slug formation (Carrin, 1990). During aggregation of the wild-type, all cells stain evenly with neutral red; distinct, more strongly staining cells do not appear until the low mound stage. They first appear at scattered positions in the mound but then gather into a central 'red-eye' at the top, before dispersing again, usually down the surface of the mound. A tip forms a little after the red-eye has dispersed. We assume that this staining reflects the appearance and movement of a class of prestalk cells before tip formation. Various of the mound mutants arrest at particular stages in the process. In *mndC*, *D* and *E* strains, neutral red-stained cells are uniformly distributed in the aggregate, in *mndB* strains

Fig. 4. Gene expression by selected mutants. Strains were allowed to develop on KK2 agar for 21 h (parental strains are shown at 15 h), RNA extracted and 5 µg per lane analysed in a Northern blot. Prestalk A cells expressed *ecmA* mRNA, prestalk B cells expressed *ecmB* mRNA (and possibly *ecmA* mRNA) and prespore cells expressed *psA* mRNA. Expression levels were nearly always lower in the mutants than in the wild-type, but clear differences were apparent between them. For instance *mndA* mutants clearly expressed *ecmA* and *psA* and some alleles also expressed *ecmB*, whereas *mndB* mutants did not express *ecmA* and *mndE* mutants expressed only *ecmB* and this with only low efficiency.

sectors form with stained cells at the borders and in *mndA* there is a very prominent red-eye (Table 4).

Prestalk and prespore differentiation

Specific mRNAs were used to monitor the differentiation of prespore and prestalk cells in the mutants. The *psA* mRNA (Barklis & Lodish, 1983; Early *et al.*, 1988) encodes a cell-surface protein of unknown function which is expressed in all prespore cells. *ecmA* and *ecmB* mRNAs are specific markers for prestalk cells and both encode proteins of the extracellular matrix (i.e. the slime sheath). Their expression patterns are complicated and define several subclasses of prestalk cell (Ceccarelli *et al.*, 1991; Early *et al.*, 1993; Jermyn *et al.*, 1987). Both *ecmA* and *ecmB* expression is induced by DIF-1 and expression of these markers therefore indicates that cells both produce and respond to DIF-1. Expression of all three markers commences in wild-type strains 1–2 h before tip formation and increases rapidly during first finger extension (Berks & Kay, 1990; Jermyn *et al.*, 1987). Cells were allowed to develop on non-nutrient agar and harvested after 15–17 h, the time when the parental strains would be making tips, and at 20–21 h, to allow for the possibility of slow development in the mutant strains. The RNA (Northern) blots revealed three very general classes of mutants, which correlated quite well with the morphological classification (Fig. 4, Table 4).

1. Post-aggregative block mutants (e.g. *mndE*), in which there was no, or very little, expression of prespore or prestalk mRNAs. These were generally in morphological class 1 and we assume that they have a general block to post-aggregative development.

2. Pathway mutants (e.g. *mndB*, *F* and *H*), in which there was expression of one or two cell-type-specific mRNAs but not of the remaining one(s). These mutants were generally in morphological class 2 and we assume that they have blocks in one or more specific pathways of cell differentiation.

3. Morphogenesis mutants (e.g. *mndA*, *C*, *G* & *I*), in which all three cell types differentiate. These mutants are generally in morphological classes 3 and 4. One possible explanation of their failure to develop is that they are defective in some morphogenetic process necessary for tip formation and elongation (see Discussion).

Synergy with wild-type

Many of the mutants were tested for synergy with wild-type. Equal numbers of mutant and wild-type cells were mixed and allowed to develop on non-nutrient agar. Spores were harvested from the resulting fruiting bodies and plated clonally to determine their genotype. Surprisingly, 24 out of 28 of our mutants could form

spores in synergy with wild-type, but not alone (see Table 4). Because stalk cells are dead, we were not able to determine the genotype of the stalk cells in synergizing mixtures and so are unsure whether synergizing strains could form stalk cells as well as spores.

DISCUSSION

We have reported the first large-scale hunt for mutants blocked in the multicellular stages of *Dictyostelium* development. Previous work has either concentrated on the earlier aggregation stage (Coukell, 1975; Darmon *et al.*, 1977; Williams & Newell, 1976), or sought more specific classes of mutants, such as the sluggers (Newell & Ross, 1982), rapidly developing (Abe & Yanagisawa, 1983; Kessin, 1977) or stalky mutants (Morrissey & Loomis, 1981).

Statistical analysis suggests that about 118 genes are essential for the transition from a rather amorphous aggregate of cells to a series of separate unitary organisms, each with differentiated cells organized in a discrete spatial pattern and capable of complicated morphogenetic movements. More genes must be involved; those that are also essential for growth or block development at an earlier stage would not have been recovered, nor would mutations in important, but non-essential genes. Our estimate is comparable to that of Loomis, who compared mutation frequencies at known single loci with frequencies of various developmental mutants recovered in the same experiment. This suggested that about 150 genes are essential for aggregation and 300 for development as a whole (Loomis, 1978).

The mutants can be classified into three broad groups, based on terminal morphology and patterns of gene expression. This scheme is not definitive, but should assist in recognizing particular types of mutant.

Post-aggregative mutants arrest development as loose mounds and do not express prestalk or prespore mRNA markers, nor are prestalk cells detectable by neutral red staining. These mutants are very similar to the G-box binding factor (GBF) null mutant (Schnitzler *et al.*, 1994). The GBF protein is a DNA binding protein with sites in the promoters of many post-aggregative genes and these genes are poorly expressed in the GBF null mutant. The GBF protein is activated by extracellular cAMP signals using a transduction pathway distinct from the one used to activate gene expression during aggregation (Schnitzler *et al.*, 1995). Genes such as *mndD* and *mndE* may encode components of this transduction pathway, or other functions necessary for driving gene expression after aggregation.

The pathway mutants arrest as more compact mounds, though lacking a tip, and express either prestalk or prespore mRNAs but not both. They may therefore define genes which are directly involved in specifying cell fate. *mndH* cells express the prespore marker *psA* well, but the prestalk markers *ecmA* and *ecmB* poorly. Since *ecmA* and *ecmB* expression is induced by DIF-1 and the *mndH* cells do produce DIF-1 during development (unpublished

observations), they may be deficient in their response to DIF-1. Conversely, *mndF* cells appear to have a specific defect in prespore differentiation.

The morphogenesis mutants arrest as tight mounds, possibly with a rudimentary tip and express both prestalk and prespore markers. In the best studied example, *mndA*, neutral red staining shows that prestalk cells arise and sort to the top of the mound. These mutants raise a conundrum, since there is no obvious defect in gene expression to explain their failure to form a first finger. Cytoskeletal mutants, such as those in myosin 2 (*mhcA*) and multiple actin-binding proteins, have a similar phenotype to *mndA*, also expressing prestalk and prespore genes but arresting as tip-less mounds (De Lozanne & Spudich, 1987; Witke *et al.*, 1992). Evidently, elongation of the mound into a first finger imposes special demands on the cytoskeleton, which are not so apparent at other stages of the life-cycle. A further similarity is that both *mndA* and *mhcA* mutants can synergize well with the wild-type (unpublished observations). Thus, we propose that mutants such as *mndA* are defective in morphogenesis and that they may have defects in the cytoskeleton. However, *mndA* is not identical to *mhcA* as they map to different linkage groups [*mndA* to lgVII and *mhcA* to lgIV (Welker *et al.*, 1989)].

The majority of mutants synergized with wild-type to form spores. Synergy is normally assumed to result from the wild-type providing the mutant with some essential signal which it cannot make for itself. Such synergy is relatively rare in other organisms. But another type of synergy is also conceivable in which mutants with a defective cytoskeleton are physically carried through morphogenesis by the wild-type ('piggy-backing'). Piggy-backing is the likeliest explanation for the synergy of the myosin 2 null mutant. The relative abundance of piggy-backing mutants in our collection may contribute to the high overall frequency of synergy.

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