SHORT COMMUNICATION

Attempts to Isolate Mutants of *Dictyostelium discoideum* Which Express α -Mannosidase-2 Constitutively

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Evidence was sought for a gene-specific negative regulatory element of *Dictyostelium discoideum* similar to the repressor/operator systems that control some operons in prokaryotes and their viruses. Mutants of *D. discoideum* that constitutively express the stage-specific enzyme α -mannosidase-2 could provide such evidence. Reconstruction experiments demonstrated that the screening technique developed would detect mutants of that type. Over 2×10^5 survivors of heavily mutagenized cell populations were grown and their progeny tested. No mutants of the desired type were obtained. The possible implications of this finding are discussed.

INTRODUCTION

The accumulation of a considerable number of gene products is regulated in the *Dictyostelium* life cycle (Sussman, 1965; Loomis, 1975; Loomis *et al.*, 1977). Several enzymes only appear at specific stages in development and their accumulation requires concomitant RNA and protein synthesis. There is direct evidence that the accumulation of some of these gene products is the consequence of an increase in the specific mRNA (Alton & Lodish, 1977; Ma & Firtel, 1978; MacLeod *et al.*, 1980). Studies in prokaryotes have shown that transcription is controlled by the binding of regulatory proteins to operator sites on DNA close to the structural genes (for reviews, see Beckwith & Zipser, 1970, and Lewin, 1975). Mutations affecting regulatory proteins or their operators result in alterations in the control of the specific gene products. In negative control systems the regulatory protein blocks gene transcription and mutations inactivating the protein give rise to constitutive expression of the gene. We sought similar types of mutants affecting a developmentally regulated gene of *Dictyostelium discoideum* so as to define genetically the mechanisms of control.

α -Mannosidase-2 is an enzyme which is barely detectable in growing *D. discoideum* cells. The specific activity increases 30- to 40-fold late in development apparently as the result of *de novo* synthesis (Free *et al.*, 1976a). A constitutive mutation affecting α -mannosidase-2 would be expected to result in a high level of this enzyme in growing cells. Since a sensitive chromogenic assay (Loomis, 1970) can be used to detect α -mannosidase activity, the characteristics of the regulation of this enzyme presented a particularly favourable opportunity to screen for negative regulatory mutations. The approach described here would identify mutants which abolish repressor activity or operator mutants which abolish repressor binding.

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METHODS

Cell strains, culture and media. Strain Ax3 (Loomis, 1971) is the parent of strain M3 used in these studies (Free *et al.*, 1976b). Strain M3 lacks α -mannosidase-1 activity. The cells were grown axenically in HL-5 medium (Watts & Ashworth, 1970) on a gyratory shaker or in Multitest trays at 22 °C, or in association with *Klebsiella aerogenes* on SM plates and were allowed to develop according to Sussman (1966).

Mass screening. Approximately 10^7 cells of strain M3 were mutagenized with 500 μ g *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine ml⁻¹ and dispensed automatically into Multitest trays at a cell concentration that would either maximize the number of wells receiving a single viable cell or would give an average of 6 viable cells per well. The trays were incubated for 16 to 20 d until an average cell titre of 2×10^6 to 4×10^6 ml⁻¹ was reached. The trays were replicated and the replica trays were incubated at 22 °C. An equal volume (0.05 ml) of screening solution containing 16 mM-*p*-nitrophenyl- α -D-mannopyranoside, 0.3 % (w/v) Triton X-100 and 7 mM-acetic acid was automatically dispensed into the test trays. The final pH in the wells was 5.5. The trays were incubated at 35 °C for 8 h. After incubation, 0.05 ml 1.5 M-Na₂CO₃ was added to raise the pH and thus visualize the hydrolysed substrate. The trays were inspected visually for the presence of yellow colour in the wells. Those which had perceptible colour were noted and cells were transferred from the replica trays into HL-5 medium for further characterization. Details of the mutagenesis, dispensing apparatus and replication methods have been described (Brenner *et al.*, 1975, 1976). The trays were gently vortexed to suspend the cells immediately prior to replication and screening. Under this regime the replication efficiency was always greater than 99.5%. To control contamination by fungi which contain α -mannosidase, 0.75 μ g Fungizone ml⁻¹ was added to the growth medium. This concentration did not inhibit the growth of *D. discoideum* and did not affect α -mannosidase activity.

Characterization of strains. The putative mutants were picked to 2 ml HL-5 medium from the replica trays 1 d after the screening and grown to 2×10^6 cells ml⁻¹. In addition, we spread cells from replicas of the wells inoculated with an average of 6 viable cells per well on SM agar in association with *K. aerogenes*. Twenty-five clones from each population which grew on these plates were subsequently grown in 9 ml HL-5 medium. We calculated that if only 1 in 10 of the cells was constitutive, testing 25 clones should give 93 % confidence that the mutant will be rescreened as a pure population (there is 0.9 probability that a cell will not be mutant, so that the confidence of finding a mutant is $1 - 0.9^n$, where n is the number of clones sampled: $1 - 0.9^{25} = 0.93$). Cells from wells receiving fewer viable cells would be rescreened as pure populations with even greater confidence of recovery of constitutive mutants. The cells were washed, resuspended in 0.15 % Triton X-100, and assayed using *p*-nitrophenyl- α -D-mannopyranoside. The extracts were assayed for 6 h at 35 °C and the specific activity was determined. Protein was estimated by the method of Lowry, using crystalline bovine serum albumin as a standard. Cell extracts from Ax3 (that have α -mannosidase-1) and developed M3 (that have α -mannosidase-2) served as controls.

Materials. Sterile Multitest trays (Linbro, no. MRC-96-TC) were obtained from Bellco Glass (Vineland, N.J., U.S.A.). *p*-Nitrophenyl- α -D-mannopyranoside was obtained from Calbiochem, and Fungizone from Grand Island Biologicals (Grand Island, N.Y., U.S.A.).

RESULTS

Reconstructions

There are two stage-specific isoenzymes of α -mannosidase. The minor isoenzyme, α -mannosidase-2, can be detected in mutants such as strain M3 which lack the major isoenzyme activity (Free *et al.*, 1976a). α -Mannosidase-2 activity is undetectable in vegetative cells of M3 but appears late in development. The sensitivity of the assay in Multitest trays was measured in order to determine the appropriate screening conditions for detecting mutants which constitutively express high levels of α -mannosidase-2 in vegetative cells. Reconstruction experiments were carried out using growing and developed cells of M3. The cells were deposited in wells of Multitest trays in which cell populations were grown, lysed by detergent and the enzyme activity assayed. After 8 h incubation at 35 °C, wells containing extracts from as few as 10^5 developed cells were bright yellow while those containing up to 10^6 growing cells were colourless. The amount of product produced was directly proportional to the number of cells added and the assay time. Mixtures of growing and developed cells gave the amount of product expected from the developed cells indicating that no inhibition of α -mannosidase-2 activity occurs in the presence of vegetative extract. The trays were

Table 1. *Mutant screening of strain M3*

No. of cells per well	P ₁ * (clones)	Total clones screened*	Putative constitutive mutants rescreened
0.66	6528	11 000	13
0.8	7558	15 300	25
0.79	7000	15 000	9
0.63	6200	11 000	12
0.66	6528	11 000	9
6.0	204	76 000	12†
5.8	201	72 000	4†

* From the number of empty wells per tray (P₀), the average number of cells per well was derived from the Poisson equation, as was the P₁ class (those wells which received 1 cell per well) and the total number of clones in wells receiving 10 viable cells or less.

† The contents of each well containing a putative mutant were diluted and cloned; 25 clones from each were tested for α -mannosidase-2 activity.

screened for constitutive α -mannosidase when the wells contained 2×10^5 cells per well, at which titre the viability was always greater than 90 % (MacLeod, 1979).

The results of these reconstruction experiments indicate that if 10 % of the population which had grown to 3×10^5 cells per well had the specific activity of α -mannosidase found in developed cells, they could be detected. Likewise a clonal population of constitutive cells which, during the growth phase, had a specific activity 10 % of that found in developed cells could be detected in the Multitest wells under our assay conditions.

Screening for constitutive mutants

In seven separate experiments, cells of strain M3 were mutagenized under conditions which reliably result in mutations in a gene coding for a protein of 66000 daltons at a frequency of 10^{-3} (Loomis, 1978*b*). In each experiment, the level of mutagenesis was monitored by counting the percentage of aggregationless mutants among the survivors (Loomis, 1978*b*). The mutagenized cells were distributed into Multitest wells (Table 1). After growth the colonies were replicated and assayed for α -mannosidase-2 activity. Any colonies which gave an indication of activity were rescreened. No mutants were found which expressed α -mannosidase-2 at significantly higher levels during growth than the parental strain.

The reconstruction experiments indicated that the screening conditions were sensitive enough such that if only 1 in 10 cells of the initial inoculum were constitutive for α -mannosidase-2, it could be detected. Therefore, in the last two experiments shown in Table 1, 27000 wells were inoculated with mutagenized cells of strain M3 such that on average each well received 6 viable cells. We calculated from the Poisson equation that about 1.5×10^5 clones were screened (85.5 % of the wells contained 10 cells or less). Sixteen populations were found which produced observable yellow product in the primary screening. Cells from replicas of these wells were grown clonally and the specific activity of α -mannosidase was determined in 25 separate clones from each of these populations. No strains which constitutively expressed α -mannosidase during growth were found.

DISCUSSION

Mutagenesis of populations of *D. discoideum* has reproducibly resulted in the recovery of structural gene mutants for four developmentally regulated enzymes at a frequency of about 10^{-3} (Brenner *et al.*, 1975; Dimond *et al.*, 1973; Free & Loomis, 1974; Dimond & Loomis, 1976; Loomis, 1978*b*). Thus, mutation in an equally mutable gene affecting the

stage at which an enzyme appears should be easily isolated if inactivation of its product resulted in unregulated expression of the enzyme. However, after screening more than 2×10^5 mutagenized clones, no strains which constitutively expressed α -mannosidase-2 were detected. The reconstruction experiments demonstrated that had such mutants occurred they would have been recognized by our screening procedure. The screening procedure would also have recognized operator mutations if an operator of 30 bases were involved in the negative regulation of α -mannosidase-2. The level of mutagenesis achieved in this study and others noted above (10^{-3} per gene of 10^3 bases) indicates a minimal mutation frequency of 10^{-6} per base. Using this value the probability is 99.7 % that an operator mutation would be recovered when 2×10^5 progeny of mutagenized cells were tested. If only 10 bases play an essential operator role, the probability of observing a constitutive operator mutation in 2×10^5 mutated cells is 86 %. Operator regions have been shown to comprise about 20 essential bases in prokaryotes (Sadler & Smith, 1971; Lewin, 1975). Fewer bases would not be expected to carry out this function in the more complex eukaryotic genome. Operator mutations would be expected to affect only contiguous genes and so would be unlikely to have pleiotropic effects, yet no constitutive mutants were recovered. We are forced to conclude that either α -mannosidase-2 is not negatively regulated or that constitutive expression of α -mannosidase is detrimental to vegetative growth. However, α -mannosidase-2 activity is unlikely to interfere with growth since the other isoenzyme, α -mannosidase-1, can be found at twice the specific activity in growing wild-type cells (Loomis, 1970).

One possible explanation we have considered is that α -mannosidase-2 is regulated by two independent mechanisms, mutations in either of which would be insufficient for constitutive expression. We would not expect to have recovered specific double mutants in the 2×10^5 clones we screened.

Although no definite conclusions can be drawn in the absence of mutations affecting the regulation of α -mannosidase-2, in light of the extensive and careful search, the lack of such mutants may be informative. At any given state of differentiation or growth condition, most potential genetic information in eukaryotes is not expressed. To keep a large number of specific genes repressed by negative control mechanisms would require a large proportion of the cellular protein to be repressors. There may be some upper limit on the genome size for which negative control is practical. Positive regulation would require regulatory proteins only for the active genes. For this and other reasons, Metzenberg (1979) makes an interesting prediction that most genes in eukaryotes will be subject to positive regulation. While a search for positive regulatory mutants was not technically feasible for α -mannosidase-2, it has been done for three other developmentally regulated enzymes. Five independent mutations affecting the specific activity of *N*-acetylglucosaminidase, four affecting α -mannosidase-1, and seven affecting β -glucosidase were all in the respective structural genes and none were in complementing second sites (Free *et al.*, 1976*b*; Loomis, 1978*a*, 1980). Some of these mutations resulted in no measurable enzyme activity. Thus, a gene for a positive regulatory protein is either much less mutable or does not regulate these enzymes. We are left with the impression that developmental regulation of genes in *Dictyostelium* utilizes mechanisms which are more complex than those in prokaryotes. The paucity of simple positive or negative regulatory mutants in other eukaryotic organisms suggests that this conclusion may not be restricted to *Dictyostelium*.

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