Induction of Macrocyst Germination in the Cellular Slime Mould *Dictyostelium mucoroides*

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Despite the usefulness of the macrocyst cycle for genetic analysis of slime mould cells, a great difficulty has been the extremely poor germination of the macrocysts. We describe culture conditions for reliable, high-frequency macrocyst germination, using *Dictyostelium mucoroides* 7 (Dm7) and a mutant MF1 derived from it. Irradiation with relatively short-wavelength light (blue-near-UV) was particularly important for the induction of germination, and only a short period of irradiation was required to obtain a high percentage of germination. There was a critical temperature between 19 and 22 °C for efficient induction of germination; a relatively high pH, around 8.0, also favoured germination. Germination competence increased with the age of the macrocysts.

INTRODUCTION

Many cellular slime moulds have two developmental pathways: sorocarp formation and macrocyst formation (Raper, 1951; Blascovics & Raper, 1957). Macrocysts are large, thick-walled, resting structures formed after the fusion of starving amoebae, and are probably sexual zygotes (Erdos *et al.*, 1973*a, b*; MacInnes & Francis, 1974; Francis, 1975; Wallace & Raper, 1979). Some species, like *Dictyostelium mucoroides*, are homothallic, and the first sign of macrocyst formation is the formation of large cell aggregates in a process showing some resemblance to asexual aggregation. The aggregate is subdivided into smaller masses (precysts), each surrounded by a fibrillar sheath. A cytophagic cell (giant cell) arises at the centre of the precyst and engulfs all the other cells in the precyst to form finally the mature macrocyst surrounded by a thick wall. The environmental conditions that favour macrocyst formation differ from those favouring sorocarp formation (Weinkauf & Filosa, 1965). Ethylene may function as a macrocyst-inducing hormone in *D. mucoroides* 7 and a mutant derived from it (MF1) (Amagai, 1984; Amagai & Filosa, 1984).

A great difficulty with using this sexual cycle for genetic studies has been the extremely poor germination of the macrocysts in the laboratory, possibly because of their thick wall. Nickerson & Raper (1973) demonstrated that light irradiation and temperatures around 15 °C can induce macrocyst germination in *D. mucoroides* 7. However, there have been no further reports on germination, probably because it takes a relatively long time to induce it. The present study was undertaken to determine the environmental conditions for reliably obtaining a high rate of macrocyst germination.

METHODS

Organisms and cultivation. A spontaneously occurring mutant, MF1, isolated from the wild-type strain *Dictyostelium mucoroides* 7 (Dm7) forms macrocysts with and without light (Filosa, 1979). MF1 was mainly used, and Dm7 for comparison. *Escherichia coli* B/r was used as a food source (Bonner, 1947). Spores of MF1 were obtained from sorocarps formed either over activated charcoal on nutrient agar (Filosa, 1979), or on lactose/peptone (LP) agar without charcoal. Approximately 1 ml of a suspension of MF1 spores together with *E. coli* was incubated at 22 °C for 24 h. Cells were then harvested and washed twice in 20 mM-phosphate buffer (pH 6.2). The washed cells were re-inoculated into a suspension of *E. coli* in an Erlenmeyer flask (100 ml) double

wrapped with aluminium foil, and shaken on a rotary shaker at 22 °C. The shake culture system used was quite effective for synchronizing macrocyst formation (Filosa & Dengler, 1972). Morphologically mature macrocysts were formed after 7 d of shake culture; they were washed twice in various solutions to be tested and diluted to a density of about 1000 macrocysts ml⁻¹. Samples (1 ml) of suspension were transferred into 3.5 cm diameter plastic dishes (Falcon, no. 1008). The washing and transferring procedures were done under room light as quickly as possible in order to avoid the effect of light. The preparations were sealed with Parafilm, double wrapped with aluminium foil and incubated at 22 °C in the dark. The age of macrocysts was counted from the start of this incubation.

Light irradiation. Macrocyst samples were irradiated for various times in an incubator equilibrated at 19 or 22 °C. White fluorescent lamps (30 W) were mainly used in addition to blue monochromatic fluorescent lamps and black-light fluorescent lamps. The light intensities to which the samples were exposed were adjusted to be almost the same for each light source.

Determination of the germination rate. After certain periods of incubation, more than 1000 macrocysts for each sample were counted under an inverted phase-contrast microscope to estimate the germination rate. In completely germinated macrocysts, only the thick wall remained as a stable structure after many cells had been released by germination. Germinating macrocysts often had a cracked wall from which part of the cell mass had moved out. These structures were regarded as germinated macrocysts. The germination rate (%) was calculated as: (no. of germinated macrocysts/total no. of macrocysts) × 100. Amoebae produced by macrocyst germination again seemed to re-form macrocysts via cell aggregation during culture. Therefore, the above germination rate is considered to be an underestimate.

RESULTS

Effects of pH and temperature on macrocyst germination

Preliminary experiments using 20 mM-phosphate buffer at a pH range of 6.0–7.5 showed that increasing pH favoured germination, but that the rate was below 50%. The germination rate was improved by the use of Good buffers instead of phosphate buffers. In all cases, light irradiation was essential for induction of germination (see below). When macrocysts of age 30 d were irradiated with white fluorescent lamps for 32 h, the germination rate was low at all pH values examined. During prolonged irradiation (10 d), however, the germination rate was raised to more than 60%, the highest percentage being attained in 25 mM-Bicine buffer (pH 8.0). Thus, a relatively high pH seemed to favour macrocyst germination, as summarized in Table 1.

To determine the temperature dependency of macrocyst germination, macrocysts (age 40 d), washed and suspended in 25 mM-Bicine (pH 8.0), were irradiated with white fluorescent lamps (30 W) for 1 h at 22 °C, and then incubated in the dark at temperatures from 15 to 28 °C. After 48 h incubation, the percentage germination rates were as follows (means ± SD, *n* = 5): 15 °C, 65.8 ± 3.2; 19 °C, 71.6 ± 2.1; 22 °C, 28.6 ± 3.0; 25 °C, 28.4 ± 2.5; 28 °C, 16.7 ± 2.8. These results indicate that there is a critical temperature between 19 and 22 °C above which germination is inhibited.

Induction of macrocyst germination by light irradiation

The germination of macrocysts was quite sensitive to light, particularly of short wavelengths. When macrocysts (age 20 d) were irradiated with a blue or a red monochromatic fluorescent lamp of the same strength, the blue light was quite effective in inducing germination, but the red light was almost without effect, the same as complete darkness. A black-light fluorescent lamp

Table 1. *Effect of pH on macrocyst germination*

Mature macrocysts of MF1 were washed and suspended in the designated buffers (25 mM) and Bonner's standard salt solution (BSS; pH about 5.0). After 30 d incubation at 22 °C, the preparations were irradiated continuously with white fluorescent lamps (30 W) for the indicated times at 22 °C. The germination rates (%) were then determined, as described in Methods; the results are means of three experiments.

Duration of irradiation	MES (pH 6.0)	PIPES (pH 6.5)	PIPES (pH 6.8)	HEPES (pH 7.5)	Bicine (pH 8.0)	BSS
32 h	21.5	26.0	26.0	28.3	31.0	16.1
10 d	62.2	68.9	73.7	74.2	78.8	61.6

Fig. 1

Fig. 2

Fig. 1. Induction of macrocyt germination by light. Macrocysts of MF1 were incubated in 25 mM-Bicine (pH 8.0) for 20 d at 22 °C, as described in Table 1. At age 20 d they were irradiated continuously for the indicated periods with white fluorescent lamps (▲), blue monochromatic fluorescent lamps (■), and black-light fluorescent lamps (□) at 19 °C, followed by determination of the germination rate. As a control, the germination rate of macrocysts kept in the dark was determined (○). The bars represent SD ($n = 5$).

Fig. 2. Relation of irradiation time to germination rate. Macrocysts of MF1 (age 20 d) prepared as described in Fig. 1 were irradiated with blue monochromatic fluorescent lamps for the indicated periods (t) at 19 °C (●) or 22 °C (○). After 48 minus t h of incubation at 19 or 22 °C in the dark, the germination rates were determined. The bars represent SD ($n = 5$).

that emitted near-UV light was slightly more effective than blue light (Fig. 1). Light-induced germination began after 24 h of irradiation and the rates increased rapidly during the next 24 h. Although the wavelength of light was quite important for induction of macrocyt germination, a long period of irradiation was not necessary. The relationship between the irradiation period and germination rate is shown in Fig. 2, the germination rates being determined after 48 h (t h of irradiation, plus (48 minus t) h in the dark) of incubation at 19 or 22 °C. At both temperatures, almost maximal germination rates were induced by only 15 min irradiation, though the rate at 22 °C was considerably lower than that at 19 °C. The germination rates increased slightly with irradiation time after 15 min.

Relation of macrocyt age to germination

Nickerson & Raper (1973) reported that the germination rate increased with the age of the macrocysts. To examine this more precisely, macrocysts 20 d and 40 d old were compared under the same culture conditions. The final percentage of germination was always higher in the older macrocysts, and germination also occurred about 12 h earlier in the older macrocysts than in the younger macrocysts (Fig. 3). In addition, the germination rate seemed to increase slowly even in the dark, this being more evident in the older macrocysts. Incidentally, germination was never induced in macrocysts of age 10 d, at least within 48 h of incubation.

The above results were obtained with the mutant MF1. Essentially the same results were obtained with the wild-type strain Dm7 (data not shown).

DISCUSSION

Despite the usefulness of a true sexual cycle like macrocyt formation for genetic analysis of slime moulds, many genetic studies have been done by using the parasexual cycle, possibly because of the extremely poor germination of macrocysts. The data presented here show that a

Fig. 3. Age dependency of macrocyst germination. Macrocysts of MF1, 20 or 40 d old, were either irradiated continuously with blue fluorescent lamps or kept in the dark for the indicated periods at 19 °C, followed by determination of the germination rate. ○, Irradiated 20-d-old macrocysts; ●, irradiated 40-d-old macrocysts; □, 20-d-old macrocysts kept in the dark; ■, 40-d-old macrocysts kept in the dark. The bars represent SD ($n = 5$).

high rate (about 80%) of macrocyst germination can be reliably attained by using improved culture conditions. In addition to a relatively high pH, a temperature below 22 °C, and the use of aged macrocysts, irradiation with short-wavelength light (blue-near-UV) was particularly important for efficient induction of macrocyst germination. The shake culture system for obtaining macrocysts as employed in the present work was also effective for the synchronized formation and germination of macrocysts. In preliminary experiments, we used the method of Nickerson & Raper (1973) to induce macrocyst germination, but the germination rate varied from experiment to experiment and never exceeded 50%. Such a low rate of germination may at least partly be due to the relatively low pH of the agar plates used. It is also possible that the submerged macrocysts germinate more readily than macrocysts placed on an agar surface according to the method of Nickerson & Raper (1973).

The importance of light and temperature for macrocyst germination, as reported by Nickerson & Raper (1973), was confirmed by the present work. There seems to be a critical temperature around 20 °C for germination. This raises the possibility that there may be temperature-sensitive substance(s) involved in macrocyst germination, which is (are) unstable and/or not synthesized at higher temperatures. In this connection, it is interesting that several other forms of slime mould behaviour, such as the motility and differentiation of slugs, are modulated by temperature.

There is increasing evidence that cytoplasmic pH (pH_i) is important in the regulation of cell differentiation and proliferation in general (Busa & Nuccitelli, 1984; Whitaker & Steinhardt, 1982). Cytoplasmic alkalization has been shown to be a primary signal for the stimulation of protein synthesis after fertilization (Winkler *et al.*, 1980). It has been claimed that pH_i has a determinative effect on prespore–prestalk differentiation during asexual slime mould development (Gross *et al.*, 1983; Inoue, 1985) and that there is a measurable difference in pH_i between the two types of slug cells (Inoue, 1985). In our study, the germination of macrocysts was considerably affected by extracellular pH (pH_e), high pH_e favouring germination. Although it is not known whether pH_e may alter pH_i , thus resulting in a regulatory effect on germination, it would be of interest to examine the effects of pH_e , light and temperature on pH_i , in relation to the mechanism of germination. NH_4Cl acts as a weak base and elevates the pH_i of aggregating *D. discoideum* cells (Gross *et al.*, 1983); it would be interesting to determine the effect of NH_4Cl on germination.

A long period of light irradiation was not necessary to induce macrocyst germination. Recently, it was found that light-induced germination is completely dose-dependent, and that germination can be induced by a few seconds of irradiation (T. Abe and others, unpublished). Thus macrocysts are highly sensitive to light, and purely photochemical reactions seem to be involved in their germination. We are now analysing the action spectrum to identify photoreceptors for germination. The washing of macrocysts and their transfer into plastic dishes involved a brief exposure to room light in the present work. If this process was done either in complete darkness or under a red safety light, the maximal rate of germination was at most 40% (data not shown). This clearly indicates that even at age 0 d, some of the macrocysts are already photosensitive, brief light irradiation being a prerequisite for their germination.

The present report of conditions allowing a high rate of macrocyst germination will undoubtedly facilitate further genetic analysis and the accurate determination of the phase of meiosis during the developmental cycle of macrocysts. However, even under our conditions it still takes a relatively long time for macrocysts to acquire germination competence, and a shortening of this time is desirable. Although the detailed process of macrocyst formation in other species, such as *D. discoideum*, differs considerably from that in *D. mucoroides* and MF1, it will be of interest to determine whether the improved method of inducing germination is applicable to other macrocysts.

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