Temperature-Induced Synchronous Differentiation of Ascogonia in *Neurospora*

Cultivation of *Neurosposa crassa* or *N. tetrasperma* on a nitrate-sucrose synthetic medium at 35–37°C prevents any female ascogonial differentiation on mycelia which are richer in carotenoids but deprived of melanins[1–4]. However, the thermal block is reversible; if 37°C cultures are brought back to 25°C, ascogonia and then the protoperithecia are differentiated. It appears, therefore, that the increased temperature only prevents the production rather than destruction of the compound(s) necessary for ascogonial morphogenesis. The reverse experiments of serial transfer from 25 to 37°C have revealed the important fact that, after 36 h growth at normal temperature of 25°C (up to 66 h no ascogonia develop at 25°C), after a step-up to 37°C, ascogonia develop around 66–77 h. This provides evidence for the production of an informational substance between 36–48 h of incubation at 25°C, which is no longer affected after transfer to 37°C. It is, therefore, the process of biosynthesis rather than the product itself which is highly thermosensitive[5]. Three morpho-physiological stages have been described in the spontaneous developmental sequence of protoperithecia in the pseudohomothallic fungus *N. tetrasperma*[5]. Total RNA and protein levels during these stages have been compared with 37°C cultures where there was no differentiation of ascogonia. At the critical period, i.e. 48 h, the RNA and protein ratios of 25 and 37°C cultures were 17:1 and 3:1 respectively; whereas free amino acids ratio was 1:6. These results indicate defects in protein biosynthesis in non-differentiating cultures at 37°C[6]. There was also a report of qualitative differences in antigenic components in 25 and 35°C grown *Neurospora crassa*[7].

For further biochemical study, it was necessary to separate the vegetative growth from ascogonial differentiation. Temperature provided the means for synchronizing the ascogonial development. Cultures were initially made on plates of solid WESTERGAARD and MITCHELL synthetic medium[8] covered with sterile cellophane dialyzing membrane (Union Carbide Corp., 36 dialysis, wall thickness 0.025 mm, pore size 24 Å.). In further improvement the cultures were made in flat bottomed 500 ml Erlenmeyer flasks filled with 50 ml of liquid medium and inoculated with 2-day-old mycelial disk. The 7- to 10-day-old cultures at 37°C were then brought down to 25°C. In the case of liquid cultures, mycelial mats were harvested on to a filter paper in a Buchner funnel and transferred to Petri dishes containing sterile 0.1 *M* phosphate buffer (pH 6.8). Within 48 h after step-down, protoperithecia were produced synchronously. Microscopic examination showed the differentiation of ascogonial hooks at 18 h, ascogonial coils at 24 h and protoperithecia at 48 h.

This last technique enabled us to study whether there is any appearance of specific protein(s) associated with the differentiation of ascogonia. As there were reports of tyrosinase involvement in the protoperithecial development and its inhibition at 35°C paralleling with non-differentiation of protoperithecia[1,2], it was of interest to investigate the role of tyrosinase in synchronously developing ascogonial cultures.

We might also suspect, from these experiments, the implication of temperature-sensitive ascogonial genes which are not functional at 37°C, but are able to function at a permissive temperature of 25°C. We tested the function of these genes, using actinomycin D and cycloheximide, inhibitors of transcription and translation respectively. These inhibitors were introduced into the cultures at 0 time of step-down from 37 to 25°C. Ascogonial differentiation was completely inhibited at a concentration of 20 µg/ml of actinomycin D and at 10 µg/ml of cycloheximide (Figure 1).

Soluble proteins of treated and control cultures have been extracted after grinding in cold phosphate buffer (0.1 *M*, pH 6.8) with washed quartz sand, centrifuged at 20,000 *g* for 30 min and the supernatant was recentrifuged at 105,000 *g* for 1 h. This supernatant has been electrophoresed on polyacrylamide gels (250 µg protein/gel) according to ORNSTEIN and DAVIS[9] with a current of 4 mA per gel. The protein bands were made visible either with amidoblack or coomassie blue. One additional band at Rf 0.78 has appeared in differentiating step-down culture at 25°C. This band is inhibited by actinomycin D and cycloheximide treated step-down cultures and absent in nondifferentiating 37°C cultures (Figure 2).

Tyrosinase was stained with 50 mg DL-Dopa + 50 mg L-Proline in 50 ml of 0.1 *M* phosphate buffer pH 6.0. The anionic protein band associated with the differentiation of ascogonia does not stain for tyrosinase. In non-differentiating 37°C cultures, no tyrosinase was detected, but in step-down ascogonial differentiating 25°C cultures 2 bands of tyrosinase were detected.

In actinomycin D and cycloheximide treated step-down cultures 2 and 4 bands of tyrosinase were detected respectively, whereas ascogonial differentiation was completely inhibited (Figure 3). Thus, there is no correlation between the 'de novo' appearance of tyrosinase and ascogonial differentiation.

From the above results the question arises, like in ty-1 mutant[10], whether a repressor protein is also produced in 37°C cultures which inhibits transcription of ascogonial genes. Our results with polyol (sorbitol, mannitol, xylitol or ribitol, conc. 0.005 to 0.5 *M*) supplemented WESTERGAARD and MITCHELL[8] synthetic media permitting the 37°C cultures to differentiate ascogonia-protoperithecia,

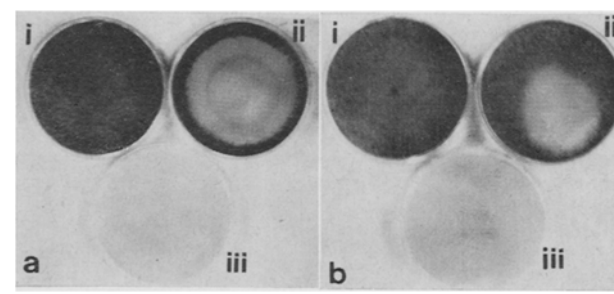

Fig. 1. Inhibition of protoperithecial morphogenesis in synchronized cultures (step-down from 37° to 25 °C) by: a) actinomycin D, i. control, ii. actinomycin D 10 µg/ml, iii. actinomycin D 20 µg/ml and b) cycloheximide, i. control, ii. cycloheximide 5 µg/ml, iii. cycloheximide 10 µg/ml.

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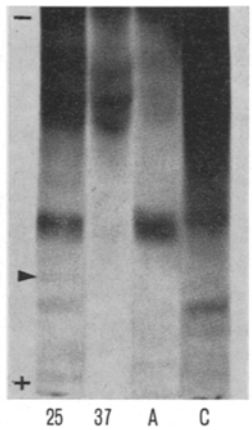

Fig. 2. Soluble protein profile on acrylamide gel electrophoresis during synchronous production of protoperithecia. 25 °C, note the band at Rf 0.78 with arrow mark which appeared in (step-down 37° to 25 °C) protoperithecial differentiating culture. 37 °C, absence of the band. A) actinomycin D treatment inhibits this band. C) cycloheximide treatment also inhibits the band.

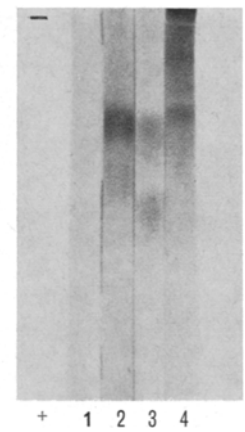

Fig. 3. Tyrosinase during synchronized development of protoperithecia. 1. 37 °C culture, no tyrosinase. 2. Step-down (37° to 25 °C) differentiating culture, 2 bands of tyrosinase. 3. Step-down (37° to 25 °C) actinomycin D treated, 2 bands of tyrosinase. 4. Step-down (37° to 25 °C) cycloheximide treated, 4 bands of tyrosinase.

is of interest. The possibility of this effect due to changes in cell wall permeability was checked by increasing the osmotic pressures of media with 1 to 10%, of NaCl, and polyethylene glycol '400'. There was no induction of ascogonia at 37°C. Thus the effect of polyols seems to be specific. Whether the induction of protoperithecia by polyols in non-permissive conditions (37°C cultures) is a phenomenon of derepression of genes or simply a metabolic effect providing an additional source of reduced nicotinamide coenzymes is under investigation.

Résumé. Une méthode pour la synchronisation de la différenciation des ascogones de *Neurospora* est présentée. Ce système de synchronisation basé sur la température facilitera l'analyse biochimique et génétique intégrée de la différenciation protopérithéciale. Il est déjà démontré qu'au moins une protéine est associée à ce processus et qu'il n'y a pas de corrélation entre la tyrosinase et cette différenciation.

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Sterilization of Paedogenetic *Heteropeza* Larvae with X-Rays

The gall midge *Heteropeza pygmaea* Winnertz (*Itonididae*, Diptera) can reproduce either bisexually or parthenogenetically in the adult stage, or paedogenetically[1-3]. Paedogenesis is parthenogenetic reproduction in the larval stage. It can be induced by defined nutritive conditions of the culture. In paedogenetically reproducing larvae, the ovaries produce small immature egg follicles which are released into the haemolymph. Within the larval blood the oocyte grows and after maturation embryogenesis goes to completion. Since no chorion is present the embryos can take up nutrients from the maternal haemolymph and grow continuously (see Figure). The increase in size of the embryos is at the expense of maternal tissues. At an age of 5 days a mother larva consists mainly of a stiffened body wall filled with offspring. The rearing conditions provided are such that the offspring, which hatch from the mother larvae, consist exclusively of daughter larvae which can reproduce paedogenetically.

The unusual mode of reproduction qualifies paedogenetic gall midges as interesting objects for in vitro culture of eggs and embryos in different culture media[4]. In vitro culture of *Heteropeza pygmaea* embryos is possible in a medium consisting of haemolymph and fat body cells obtained from *Heteropeza* larvae without progeny[5] (so-called sterile larvae). Such sterile larvae appear occasionally in standard cultures. For large scale culture experiments a convenient procedure for obtaining ample numbers of sterile larvae with large fat bodies and a maximum amount of haemolymph had to be developed. To this end we analyzed the effect of radiation on mother larvae, as well as on the eggs and embryos developing in their haemolymph. For all treatments we used 50 keV X-rays from a Müller-RT 100 machine.

In the first experiment mother larvae of different ages were irradiated. The age zero h is defined as the moment when a young larva hatches from its mother larva. With doses up to 3 kR no irradiated larvae died prematurely. But, with the exception of 96 h old larvae, the average of

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