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Morphogenesis in Trichoderma: Suppression of Photoinduction by 5-Fluorouracil

Abstract. *Sporulation in the fungus* Trichoderma viride *is inducible with a short light pulse. 5-Fluorouracil applied prior to photoinduction and removed thereafter suppressed sporulation without greatly affecting growth. This compound also halved the rate of incorporation of uracil-C^{14} into RNA but did not change the ratio of uridylic to cytidylic acid. The effect of 5-fluorouracil was counteracted by uracil but not by thymidine. This supports the hypothesis that 5-fluorouracil affects RNA rather than DNA.*

The biochemical control of differentiation can be studied best in systems in which differentiation can be artificially induced. In these systems, the changes caused by external stimuli can be followed and causal relationships between induction, biochemical change, and differentiation can be established. Although the correlation between induced enzyme synthesis and new specific messenger ribonucleic acid (mRNA) synthesis was made a few years ago (*1*), it was only recently found that, in *Bacillus* species, there exists a transcription of new and different mRNA's upon morphogenetic change (sporulation and germination) (*2*).

Most isolates of the fungus *Trichoderma viride* Pers. ex Fries produce flat, septate, hyaline mycelia when cultured in the dark. They sporulate when illuminated for more than 30 seconds, but only the narrow region of the mycelium produced just prior to illumination sporulates. The requirement of light for morphogenesis is continuous, as a short illumination given to a round colony of *T. viride* induces only a ring of mycelia bearing dark green spores (*3*). We therefore chose this organism to study the role of RNA in induced morphogenesis.

5-Fluorouracil (FU) has been shown to prevent induced enzyme synthesis without decreasing the synthesis of constitutive enzymes in *Escherichia coli* (*4*). Champe and Benzer (*5*) concluded that "FU acts mainly by incorporation into mRNA in place of uracil, there acting partially like cytosine." As this compound has such a pronounced effect on induced enzyme synthesis, we tested its effect on induced morphogenesis.

The culture conditions were as follows. Sterilized liquid potato-dextrose medium (2.7 ml) was soaked into a 7-cm sheet of sterilized Whatman No. 1 filter paper in each 10-cm petri dish, and a sheet of sterilized hardened filter paper (Whatman No. 50) was placed on the lower sheet. A small agar block was removed from an actively growing region of a culture of *T. viride*, isolate No. M 2042 (*6*), and put in the center of the hardened filter paper. Cultures were kept in the dark at 24°C. A 3-minute induction (about 5500 lu/m^2 of fluorescent lighting) was given after 26 to 36 hours of growth (culture diameters, 30 to 50 mm). Under these conditions a ring of conidiophores with dark green conidia (spores) was visible the following day; growth and sporulation were very uniform. Four cultures were used for each treatment. Since the mycelia adhered firmly to the hardened filter paper, rinsing the cultures and transferring them from medium to medium was facilitated.

We found that when cultures were transferred to FU 30 minutes before induction and left in it for 1 day, photoinduced sporulation was prevented. Sporulation was completely prevented but growth was only slightly affected by $7 \times 10^{-6}M$ FU. Even $7 \times 10^{-3}M$ FU did not stop growth completely.

Table 1. Uracil-2-C^{14} incorporation in *Trichoderma viride* RNA. Mycelia were grown to a diameter of about 5 cm in basal medium, then transferred to a dish with 2.5 μc uracil-2-C^{14} ($5 \times 10^{-5}M$), with or without $10^{-4}M$ FU, and kept there for either 1 or 6 hours. The mycelia, attached to the hardened filter paper, were then fixed in cold ethanol–acetic acid (3:1), rinsed with 70 percent ethanol, and air dried. Four half sheets per treatment were cut up in pieces (1 cm^2) and incubated 16 hours at 37°C in 6 ml of 0.3*N* KOH to which 1.5 mg of yeast RNA was added as carrier. After hydrolysis the KOH was neutralized and the product was precipitated with cold $HClO_4$. A portion was removed for total RNA determination and uridylic and cytidylic acids were separated on Dowex 50W × 4 according to the method of Katz and Comb (*14*). The $KClO_4$ precipitate (which included the DNA) was dissolved and found to contain less than 3 percent of the counts. Incubation of the mycelia on the filter paper with deoxyribonuclease did not reduce radioactivity, whereas incubation with ribonuclease removed over 95 percent of it. Results in the table are expressed in counts per minute per dish. FU, 5-fluorouracil; UMP, uridylic acid; CMP, cytidylic acid.

Incubation (hr)	Incorporation in: RNA (count/min)	UMP (%)	CMP (%)
	Control mycelia		
1	23,930	78	22
	Mycelia plus FU		
1	11,365	79	21
	Control mycelia		
6	143,550	56	44
	Mycelia plus FU		
6	68,140	63	37

Other inhibitors of nucleic acid, protein, and steroid synthesis were tested on this system. These included actinomycin D, azaguanine, azauridine, thiouracil, puromycin, and tris-(2-diethylaminoethyl)-phosphate trihydrochloride. Of these, only azaguanine differentially inhibits sporulation, with little effect on growth, in a manner similar to FU.

In later experiments, colonies were subjected to 5-hour treatment with FU beginning 30 minutes before induction. They were then rinsed, transferred to new dishes with medium, and incubated for a day. The results (Fig. 1*A*) indicated that even a temporary exposure of the culture to $10^{-4}M$ FU prevented photoinduced sporulation, although growth was only slightly affected.

If the photoinduced sporulation is not mediated through *de novo* synthesis of RNA, there should be little difference in the effect of FU when it is applied before, during, or after photoinduction. If FU retarded sporulation only when applied during induction,

this would be evidence for a photoinduced transcription of specific RNA's needed for sporulation. No conidia were induced when mycelia were illuminated during FU treatment (Fig. 1*B*). Sporulation occurred when the FU exposure was for the same duration several hours before or after photoinduction. 5-Fluorouracil can be incorporated into RNA and, because of turnover, reincorporated into more newly synthesized RNA (*7*). This might have been the case in our system, and may explain the slight inhibition of sporulation by the very early treatment with FU. The photoinduced transcription of "spore-forming RNA" may continue for several hours after the induction. This may explain the incomplete sporulation caused by the late treatment with FU.

The possibility exists that FU may be metabolized into 5-fluorodeoxyuridine (FUdR) (*8*) and act by interfering with thymidylate synthetase and thereby inhibiting DNA synthesis. Thymidine is a specific antidote of this inhibition (*9*). If FU acts as FUdR, thymidine, but not uracil, given at high concentrations should counteract FU. The inhibition of both growth and sporulation caused by FU was counteracted by uracil but not by thymidine (Fig. 1*C*). It even seemed that there was less growth in FU plus thymidine than in FU alone. The retardation of growth caused by FUdR could be counteracted by thymidine and to a lesser extent by uracil. These results led to the conclusion that FU was not converted to FUdR. In our system FU is probably incorporated directly into RNA, replacing uracil.

In order to test the effect of FU on the incorporation of uracil-2-C^{14}, cultures were grown to about 4 cm in diameter and then transferred to either basal medium or to basal medium plus $10^{-4}M$ FU, and uracil-2-C^{14} was supplied. The procedure and results are detailed in Table 1. 5-Fluorouracil clearly reduced incorporation of uracil-2-C^{14} into RNA without affecting the ratio of uridylic to cytidylic acid. Action of FU in *T. viride* seems to be similar to that found in animal cell cultures (*10*), *E. coli* (*4*), and *Neurospora* (*11*). In *E. coli* it was also found that, while the synthesis of inducible enzymes was prevented by FU, the rate of synthesis of constitutive enzymes was not affected (*12*). This may explain why *T. viride* continues its growth in the presence of concentrations of FU that completely inhibit induced morphogenetic change. Inhibition of induced morphogenesis by FU was previously found in *Xanthium*. Floral induction was prevented by FU which was incorporated into the RNA, while the incorporation of orotic acid into both RNA and DNA was reduced (*13*). Although the details of the effect of FU on the specific type of RNA synthesis in *T. viride* are being clarified, the data presented above demonstrate that FU prevents the photoinduced spore

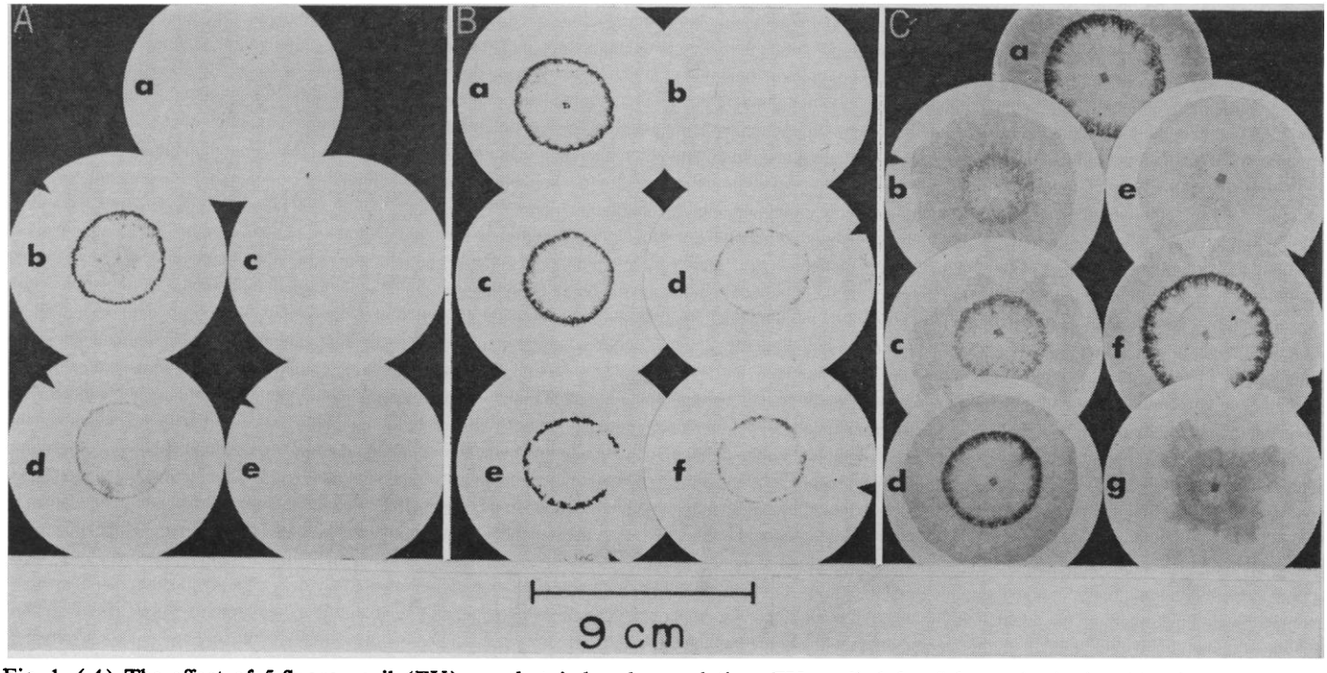

Fig. 1. (*A*) The effect of 5-fluorouracil (FU) on photoinduced sporulation; FU applied for 5 hours beginning 30 minutes before a 3-minute illumination. The average diameters of mycelial growth, in centimeters ± standard deviations, are in parentheses: *a*, dark control (7.4 ± 0.1); *b–e*, illuminated: *b*, no FU (7.1 ± 0.1); *c*, $10^{-5}M$ FU (6.8 ± 0.1); *d*, $3 \times 10^{-5}M$ FU (6.7 ± 0.1); and *e*, $10^{-4}M$ FU (6.6 ± 0.1). (*B*) The effect of time of FU treatment relative to the time of photoinduction. Mycelia were transferred for 5 hours to either $3.4 \times 10^{-5}M$ FU (*b, d, f*) or to fresh basal medium (controls: *a, c, e*) and then returned to fresh basal medium; *a* and *b* were transferred 30 minutes before illumination; *c* and *d*, transferred 12½ hours before illumination; and *e* and *f*, transferred 9 hours after illumination. FU cultures were illuminated 32 hours after planting. Final average diameters, ± standard deviations, of mycelial growth: *a*, 6.5 ± 0.1; *b*, 6.4 ± 0.2; *c*, 6.8 ± 0.2; *d*, 6.6 ± 0.0; and *e*, 6.9 ± 0.1. (*C*) Effects of uracil and thymidine as antidotes for FU and 5-fluorodeoxyuridine (FUdR). Treatments listed below were from time of planting to termination of the experiment. Average diameters of mycelial growth in centimeters, ± standard deviations, are in parentheses: *a*, control (8.6 ± 0.3); *b*, FUdR ($2 \times 10^{-5}M$) (6.6 ± 0.3); *c*, FUdR plus uracil ($2 \times 10^{-2}M$) (7.3 ± 0.1); *d*, FUdR plus thymidine ($2 \times 10^{-2}M$) (7.0 ± 0.1); *e*, FU ($5 \times 10^{-5}M$) (7.2 ± 0.3); *f*, FU plus uracil (8.3 ± 0.3); and *g*, FU plus thymidine (5.5 ± 0.3). In preliminary experiments neither uracil nor thymidine alone affected sporulation although thymidine ($2 \times 10^{-2}M$) slightly inhibited growth. Parts (*A*) and (*B*) after alcohol fixation and (*C*) after staining with cotton blue.

formation and strongly reduces incorporation of uracil, while only slightly affecting growth. We therefore conclude that the process of induced morphogenesis we have described is mediated by *de novo* synthesis of RNA's.

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Virus Induction of Osteosarcomas in Mice

Abstract. *A virus extracted from an osteosarcoma of a mouse produces similar tumors when injected into newborn mice. The original tumor appeared spontaneously in an untreated CF1 male. The time between inoculation with virus and detection of bone sarcoma has been as short as 23 days.*

Because of growing evidence from other laboratories that some mouse tumors are caused by virus (*1*), we began a search for a viral agent that might be associated with osteogenic sarcomas in CF1 mice.

So far we have examined seven radiation-induced and four spontaneous osteosarcomas for evidence of virus by use of a modification of the method developed by Gross for isolating a leukemogenic agent from AK mice (*2*). Definite oncogenic activity has been found in an extract prepared from one of the spontaneous bone tumors.

The osteosarcoma from which the active agent was extracted appeared in a 260-day-old stock male mouse of the CF1/Anl strain. The tumor, which involved the 10th, 11th, and 12th thoracic vertebrae and the 12th rib (Fig. 1*A*), was removed under sterile conditions, ground in a cold mortar, and diluted with about five parts saline. A portion of the resulting cell suspension was injected into a litter of 12-day-old CF1 mice, and the remainder was centrifuged at 2500 rev/min for 10 minutes at 0°C (International centrifuge, model PR-2). The supernatant was centrifuged again for 20 minutes at 3300 to 3500 rev/min (approximately 1500*g*), and the resulting supernatant was injected subcutaneously into a newborn litter of CF1 mice and intraperitoneally into a litter that was 33 days old.

One of the mice that had received the extract on the day of birth died with an osteosarcoma of the cervical spine 280 days later. At 337 days a large tumor mass extending from the lower thoracic to the midlumbar spine was observed in another mouse of the same litter (Fig. 1*B*). An extract of this tumor was prepared as before and injected subcutaneously into four newborn litters of CF1 mice. A hard mass was observed on the back of one of the recipients when it was 67 days old, and 4 days later four more of the mice, belonging to three of the litters, had palpable bone tumors.

These four mice were killed at 71 days of age, and each was found to have two osteosarcomas. Five of the eight tumors were in the spine, two were in ribs, and one was in a humerus. The combined tumors were extracted, and a portion of the extract was put through a 0.45-μ, HA type, Millipore filter. Four newborn litters were inoculated subcutaneously, two with the extract and two with the cell-free filtrate. The first tumor was noted at 35 days in a mouse that received extract and at 61 days in a mouse that received filtrate. The latter animal had osteosarcomas in the sternum, first lumbar vertebra, right ilium, and right tibia when it was killed at 70 days of age.

The results obtained in one line of passage from the first extraction on 11 March 1963 to 11 March 1965, are summarized in Fig. 2. In each case 0.1 ml of material was injected either under the skin of the back or into the peritoneal cavity of CF1 mice. After the third passage only filtrates were used, and tests of the procedures showed the filters to be impervious to *Escherichia coli*. The chart includes all mice that survived to weaning age; preweaning mortality was somewhat higher than among undisturbed litters.

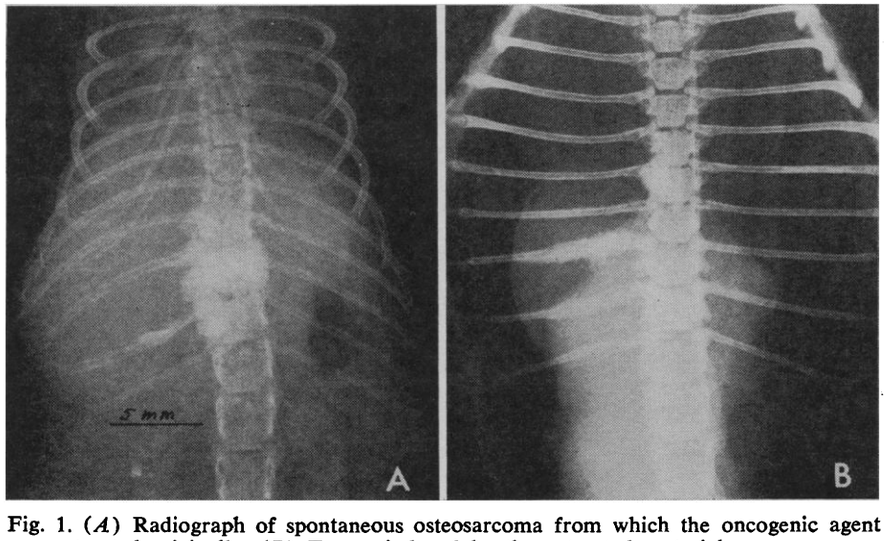

Fig. 1. (*A*) Radiograph of spontaneous osteosarcoma from which the oncogenic agent was extracted originally. (*B*) Tumor induced by the extracted material.