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4. Seventeen confluent plates (3×10^6 cells per plate) of human diploid fibroblasts (CRL-1121, American Type Culture Collection) were placed on serum-free medium for 2 days with ascorbate supplement (75 μg/ml). The combined medium (136 ml) was made 0.5*M* in acetic acid, centrifuged at 20,000*g* for 20 minutes, dialyzed against distilled water, and lyophilized. The product was suspended in 0.05*M* tris(hydroxymethyl)aminomethane hydrochloride (tris-HCl) buffer, *p*H 7.5, and stored at −20°C until used. Individual rabbits received approximately 0.5 mg of this material in complete Freund's adjuvant (Difco), divided among the four footpads. After 3 and 6 weeks, animals were given booster injections with the same amount of antigen without adjuvant, and pooled serums from two rabbits were prepared 1 week after the second booster.

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Hormonal Control of Sexual Morphogenesis in Achlya: Dependence on Protein and Ribonucleic Acid Syntheses

Abstract. *The induction of the male sexual organ primordia (antheridial hyphae) by the steroid hormone antheridiol in the water mold* Achlya ambisexualis *requires both transcription and translation. Inhibition of either of these processes eliminates the expected increase in the production and release of the enzyme cellulase, which accompanies the formation of the antheridial hyphae.*

The hormonal regulation of sexual morphogenesis in the water molds, especially in the genus *Achlya*, was revealed by Raper's experimental demonstrations of this system (*1*). The first morphological phase is initiated by the secretion of hormone (or hormones) by the vegetative female thallus and results in the induction of numerous lateral branches, called antheridial hyphae, on the male thallus. In a regular mating this sexually induced male secretes a hormone which induces the female thallus to produce oogonial initials. The antheridial hyphae then grow to the oogonial initials where they make physical contact and delimit an antheridial cell. As originally conceived, a complex of four hormones controlled the initiation of antheridial hyphae. The major hormone of this complex was called hormone A, and a highly concentrated extract from the culture medium was used for some chemical and physical characterization (*2*). The preparation of a crystalline compound has been described and named antheridiol (*2*), and a chemical structure has been proposed (*2*). Two possible isomers were synthesized, one with the same range of activity and physical properties as the natural extract (*2*). It is a normal tetracyclic steroid nucleus with a hydroxyl at C-3, a carbonyl at C-7, and a side chain at C-17 consisting of a ten-carbon chain in an α,β-unsaturated γ-lactone ring with three oxygen atoms. The chemical structure of sexual hormones in lower plants has been reviewed (*2*).

Thomas and Mullins (*3*) proposed that a prerequisite for the hormonal induction of these antheridial hyphae is a localized softening of the wall by the enzyme cellulase, which leads to the formation of lateral pegs by turgor pressure.

Although the structural formulas of sex hormones in lower plants have been studied (*2*), little is known about the synthesis of proteins and nucleic acids in terms of their relation to sexual differentiation. The requirements for the formation of antheridial hyphae should give direct information on processes required for this initial phase of sexual morphogenesis in a eukaryote. We now report on the question of whether protein or RNA synthesis, or both, are required for the hormonal induction of antheridial hyphae, and on their relation to the production of cellulase.

The strain E87 (male) of *Achlya ambisexualis* Raper was grown on a chemically defined medium (*4*). Mycelia in the early log phase of growth were obtained by inoculating zoospores into a nutrient medium and allowing these to grow for 48 hours at 24°C on a reciprocating shaker. The mycelia were then harvested, divided into equal lots, resuspended in the spent culture media (*3*), and equilibrated for 1 hour on a shaker at 24°C.

For experiments with inhibitors the mycelia were subjected to preliminary treatment with cycloheximide (final concentration, 1 μg/ml) for 1 hour or to preliminary treatment with actinomycin D (final concentration, 15 μg/ml) for 20 minutes. These inhibitors and the concentrations were chosen after tests were made on normal matings to find those concentrations which were fungistatic and would prevent antheridial hyphae production, but would not be fungicidal (*5*). Radioactive labeling was done by incorporation of a mixture of amino acids uniformly labeled with ^{14}C (specific activity, 1 mc/mg) into proteins (*6*), and by incorporation of uridine uniformly labeled with ^{14}C (specific activity, 160 mc/mmole) into RNA (*6*). The hormone antheridiol was a purified extract from culture media and was used at 5 or 15 unit/ml (*6*).

Protein was separated by a trichloroacetic acid method (*7*), dissolved in 2.0 ml of 0.1*N* NaOH, and analyzed by the Folin method and liquid scintillation counting (*7*).

For extraction of RNA, frozen mycelia were processed according to the method of Holdgate and Goodwin (*8*). The resulting powder was hydrolyzed in 0.5*N* KOH at 30°C for 12 to 15 hours. Protein, DNA, cell wall material, and $KClO_4$ were precipitated by acidifying the KOH digest to *p*H 2 with 1*M*

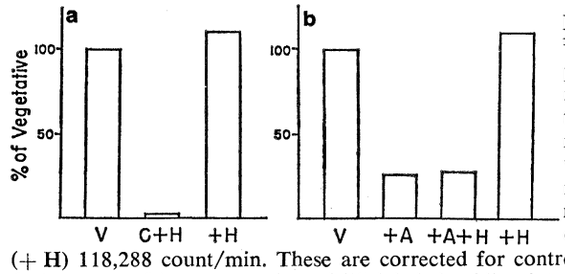

Fig. 1. (a) Incorporation of ^{14}C-labeled amino acids into protein (measured as counts per minute per microgram of protein from three separate experiments). The averages were: vegetative (V) 105,954 count/min; cycloheximide and hormone added (+ C + H) 1,025 count/min; and hormone added (+ H) 118,288 count/min. These are corrected for controls; the counting efficiency was 65.1 percent. (b) Incorporation of ^{14}C-labeled uridine into RNA (measured as counts per minute per microgram of RNA per minute of exposure to ^{14}C). The averages were: vegetative (V) 4.73; actinomycin added (+ A) 1.25; actinomycin and hormone added (+ A + H) 1.29; hormone added (+ H) 5.27. The V and + H values were from six separate determinations, and the + A + H values were from three separate determinations. The results were corrected for control counts, with a counting efficiency of 60 percent.

$HClO_4$ and adding $MgCl_2$ to a concentration of 2 m*M* (*8*). Two volumes of cold 95 percent ethanol were added, and the precipitate was removed by centrifugation at 15,000*g* for 10 minutes. The amount of RNA in the supernatant was determined by ultraviolet spectrophotometry (*8*), and the purity of the extract was determined by the absorbancy from 230 to 310 nm (*8*).

Cellulase was extracted from both the mycelium and the medium and assayed viscometrically (*9*). One unit of cellulase activity is defined as the amount of cellulase in 1 ml of extract, which causes a 10 percent decrease in flow time of the enzyme-substrate mixture after incubation for 1 hour at 30°C.

In all experiments a small sample of mycelium was removed and examined under a microscope for evidence of induction of antheridial hyphae and for comparison with control mycelium.

Cycloheximide added 1 hour before antheridiol effectively blocked the incorporation of amino acids into protein (Fig. 1a). The inhibition was 99 percent when compared with the controls. This inhibitor completely eliminated the visual appearance of antheridial hyphae normally induced by antheridiol. Thus, we concluded, on the basis of inhibition by cycloheximide, that translation is required for the hormonal induction of antheridial hyphae.

Actinomycin added 20 minutes before antheridiol blocked the incorporation of uridine into RNA to the extent of about 75 percent when compared with the controls (Fig. 1b). This inhibitor also completely eliminated the visual appearance of antheridial hyphae expected under induction by antheridiol. Thus, we concluded, on the basis of inhibition by actinomycin, that transcription is required for this hormonal induction.

A sharp rise in cellulase activity occurs prior to the appearance of antheridial hyphae after induction with antheridiol (*3*), and this increase has been proposed as a prerequisite to their formation. Therefore it was necessary to determine whether the inhibition of translation or transcription affects the hormone-induced rise in cellulase activity. Our experiments (Fig. 2) indicate that both translation and transcription are required for the induction of cellulase. Hormone-treated mycelia previously treated with cycloheximide or actinomycin are still capable of showing a delayed production of antheridial hyphae and cellulase when the inhibitors

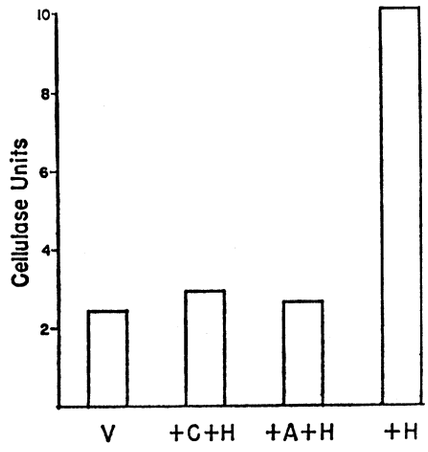

Fig. 2. Total cellulase activity from mycelia plus media after exposure for 1 hour to inducer. V, vegetative control; + C + H, prior treatment with cycloheximide, then hormone; + A + H, prior treatment with actinomycin, then hormone; + H, treatment with hormone only.

are removed, as was shown by washing the treated mycelia and then resuspending in media without additional hormone, an indication that the system for hyphal induction was still functional and that the uptake of hormone was not affected. The uptake of uridine appeared unaffected by the inhibitor because the amount of label remaining in the medium was similar in control and inhibited cultures.

While it is generally agreed (*10*) that the mechanism of action of steroid hormones in animals is a facilitation of gene transcription resulting in the induction of specific enzymes, the actual site of regulation within transcription is not yet settled. Some evidence (*10*) points specifically to transfer RNA while other (*10*) implicates the removal of a posttranscriptional repressor, which in turn promotes the accumulation of messenger RNA (mRNA). Our data for the steroid hormone antheridiol and the induction of antheridial hyphae in male strains of *Achlya* argues only for the concomitant synthesis of RNA and not necessarily for an increase in the rate of gene transcription. In addition, this synthesis of RNA is required for the increased production of the enzyme cellulase which accompanies the morphological expression of antheridial hyphae.

Studies with other aquatic Phycomycetes, namely *Blastocladiella* (*11*) and *Allomyces* (*11*) have implicated the presence of a stable mRNA that later directs the synthesis of enzymes necessary for differentiation during germination. This was not found in *Achlya* zoosporangial formation (*11*); nor have we found evidence for a stable mRNA during the induction of antheridial hyphae. The site of transcriptional control remains to be determined; classification of the *Achlya*-antheridiol-antheridial hyphae system should provide a model for studies on hormones and gene expression in eukaryotes.

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