Role of Enzymatic Wall-Softening in Plant Morphogenesis: Hormonal Induction in Achlya

Abstract. *Sexual hormone A, which induces antheridial branching in male strains of* Achlya, *also elicits a rise in cellulase. The peak of induced cellulase corresponds in time with the appearance of branches that are the male sexual organ primordia; only those strains that branch in response to the hormone show a concomitant rise in cellulase. The response to the hormone is inhibited by compounds that block protein synthesis, for example,* p-*fluorophenylalanine and puromycin. Vegetative branching, induced by substrates such as casein hydrolysate, is also accompanied by a rise in cellulase.*

The hormonal control of sexual reproduction in water molds, such as *Achlya ambisexualis* Raper, comprises a number of successive stages, each exhibiting complete dependence on that immediately preceding it. Four morphological phases, each initiated and controlled by one or more specific hormones, have been recognized (*1*). The first phase is initiated by the secretion of hormones A and A_2 by the vegetative female plant and results in the production of numerous antheridial branches on the male plant (*1*). A variety of chemicals will mimic the branch-induction (*2*) but only hormone A results in the delimitation of the branches by septa (*3*).

The vegetative plant body of *Achlya* consists of coenocytic hyphae, septa being formed only to delimit reproductive organs. The hyphal walls are composed of about 15 percent cellulose, with a microfibrillar structure, and about 85 percent of an amorphous noncellulosic polysaccharide complex (*4*).

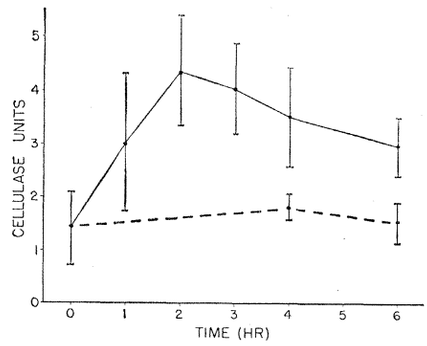

Fig. 1. Time sequence of cellulase with (——) and without (– – – –) the addition of hormone A at time zero. Branch primordia begin to appear about 1 hour after addition of the hormone. Each point represents the mean of five replicates; vertical lines indicate the standard error of each mean.

A prerequisite for the induction of branches might be a "softening" of the primary wall structure. The production of "weak spots" in the cell walls, as a result of enzymatic action, could allow the hyphal contents to be flow-oriented in the direction of maximum strain into finger-like laterals. This appears to be the case.

We have assayed the cellulase activity from mycelia induced to form branches and compared it with similar assays from noninduced mycelia.

The mycelium was grown in a defined medium under standard conditions; the extraction methods were similar to those reported previously; and the cellulase activity was determined by a standard viscometric method (*5*). The substrate was carboxymethylcellulose, a soluble cellulose derivative. Each viscometer tube contained 1 ml of enzyme extract which represented 1 g fresh weight of mycelium. A 10-percent reduction in the flow time over a period of 1 hour represents 1 unit of enzyme activity. Branching was induced by several agents, including hormone A, casein hydrolysate, and an amino-acid mixture. Since the results were similar in regard to the correlation between branching and the rise in cellulase, this report will be limited to induction by hormone A.

The cellulase activity from mycelia harvested at different times after adding hormone A is illustrated in Fig. 1. We found that induced mycelia show a rapid rise in cellulase activity to a peak that coincides in time with the appearance of branch primordia. This is followed by a decline to the noninduced level after about 24 hours.

The decline in cellulase activity, which begins about 2 hours after the addition of the inducer, is attributable to the release of the enzyme into the medium (*6*). This may suggest that the site of action of the enzyme is outside the plasma membrane.

The rise in cellulase activity appears to depend on protein synthesis. There is a marked increase in the incorporation of C^{14}-leucine into protein following addition of the inducer (*7*). Preliminary screening of inhibitors of protein synthesis which might interfere with the response to hormone A showed that puromycin and DL-*p*-fluorophenylalanine completely inhibited branching and the rise in cellulase (Table 1). Other compounds, such as chloramphenicol, dihydrostreptomycin sulfate, ribonuclease, 2-thiouracil, 8-azaguanine, DL-norleucine, and DL-ethionine, failed to inhibit the responses of *Achlya* to hormone A.

Table 1. Dependence of cellulase and branching on protein synthesis. Mycelium harvested 2 hours after addition of inducer. Puromycin, 1.5 mg/ml; *p*-fluorophenylalanine (FPhe) and phenylalanine (Phe), 0.4 mg/ml. Numbers of replicates are shown in parentheses.

Treatment	Cellulase units (mean ± S.D.)	Branch induction
Control	1.27 ± 0.14 (2)	No (6)
Hormone A	3.99 ± .65 (2)	Yes (6)
Hormone A + puromycin	1.05 ± .29 (2)	No (2)
Hormone A + Phe	4.04 ± .32 (2)	Yes (2)
Hormone A + FPhe	1.22 ± .51 (2)	No (6)
Hormone A + FPhe + Phe	3.33 (1)	Yes (5)

Strains of *Achlya* that do not branch in response to hormone A also fail to show the rise in cellulase.

Wall-softening by cellulase or other enzymes may prove to be a fundamental morphogenetic process in a wide variety of plant materials. The stimulation of cell expansion by indoleacetic acid in plant tissue appears to be mediated by cellulase (*8*). Certain aspects of fruit-ripening apparently result from cellulase (*9*). Pollen grains show a readily diffusible cellulase which may lead to wall-softening prior to tube emergence (*10*). The induction of colonial growth in *Neurospora* by snail digestive juice appears to involve the creation of "weak spots" in the cell wall (*11*). In the present report, the induction of branching in aquatic fungi by substrates or by sexual hormones has been shown to be accompanied by increased cellulase activity and probably depends upon wall-softening by cellulase.

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6. The cellulase levels in media from noninduced and induced cultures were 0.57 and 1.61 cellulase units per milliliter of medium, respectively.
7. There was a 23 percent greater incorporation of C^{14}-leucine into protein in hormone A–induced mycelia as compared with untreated mycelia on the basis of three replicates and a 2-hour incubation period.
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Virus Particles and Murine Leukemia Virus Complement-Fixing Antigen in Neoplastic and Nonneoplastic Cell Lines

Abstract. *Twenty-seven lines of murine tissue cultures derived from 12 different cell pools and grown on various media were examined with the electron microscope for morphologically detectable virus particles. They were also tested for complement-fixing mouse leukemia virus antigens and for recoverable virus. A 100-percent correlation between results obtained by these two methods is reported. An additional 19 lines from 8 different cell pools were examined for either virus particles or complement-fixing antigens. All lines were assayed for neoplastic transformation. Seven cell pools gave rise to lines showing evidence of contamination with leukemia virus. Since most of these lines had also undergone "spontaneous" neoplastic transformation in vitro, this virus cannot be excluded as a possible cause of the neoplastic change, or of influencing it. The remaining cell pools gave rise to lines with no evidence of contamination with leukemia virus; but most of these lines also underwent similar transformation. These results suggest that "spontaneous" neoplastic transformation can occur in the absence of detectable mouse leukemia virus.*

It is a rather general consensus of investigators that cells maintained in long-term tissue cultures tend to undergo "spontaneous" neoplastic transformation. "Spontaneous" is understood to mean any transformation to which a known causative agent has not yet been definitely ascribed (*1*). As part of a long-term study of such transformation, we have made attempts to find a virus-free cell line that could be studied through several preneoplastic and neoplastic passages to gain some insight into the transforming process. Virus-like particles, morphologically similar to those identified as the murine leukemia virus, have been described in certain long-established mouse tissue culture cell lines, notably in Earle's strain L cells (*2*), but no biological activity has been associated with them. In the course of screening several cell lines, a strong correlation appeared between observation of C-particles with the electron microscope (EM) and detection of group specific complement-fixing (CF) antigens of mouse leukemia virus. Furthermore, an attempt has been made to correlate these findings with the neoplastic state of the cells in vivo.

Cell pools and cell lines examined by either or both testing methods and their respective sources of origin are listed in Table 1. Because of the recently reported (*3*) inhibition or delay of neoplastic transformation by fetal calf serum, a constituent of the culture medium, the medium we used is also included. The span of days in vitro and the subculture generations during which the cell testings were made are given for each of the cell lines. Some of the lines in medium containing horse serum were transferred to medium with fetal calf serum for two fluid renewals (about 4 days) before the tests for CF antigens, since certain lots of horse serum appeared to be anticomplementary. Table 1 also shows the neoplastic or nonneoplastic state of cell cultures at the time of the CF test and EM examination. All tests for neoplasia were carried out by intraocular or intramuscular implantation of cells into isologous mice, and the only ones considered positive were those that subsequently developed invasive, progressively growing sarcomas. For the CF test, as applied to the murine leukemia viruses, we used the method described previously (*4*), except that the serums were from Fischer rats bearing transplants of a thymic lymphoma induced by Gross virus or of a sarcoma induced by Moloney sarcoma virus. These serums were selected for their high reactivity with a wide variety of mouse leukemia antigens and their complete negative reactivity with control tissue and tissue culture antigens (*5*). Two test procedures were used, a direct CF test on the specific cell line under investigation and a virus-recovery test that utilized the ability of mouse leukemia viruses to induce a group-reactive complement-fixing antigen in Swiss mouse embryo tissue cultures (SMETC) (*4*). Cells scraped from one fully sheeted T-60 flask or from two T-15 flasks were resuspended in 2 to 3 ml of culture medium and the suspension was divided into two parts. Sufficient fluid was removed from one portion to leave an approximate 5-percent suspension, which was then frozen and thawed twice and tested at a 1 : 2 dilution for CF antigen. Antigen titers, ranging from 1 : 2 to 1 : 8, were determined in a few cases. The second sample was frozen and thawed once, and 0.2 ml of the undiluted suspension inoculated into SMETC for virus isolation; all samples giving a negative result were passed at least once to fresh SMETC. Antigen titers in the positive cultures were generally higher than in the original cell line, averaging 1 : 8 or more. All cell lines were tested under code numbers and results were tabulated independently of EM observations.

Some of the cell lines (those marked with asterisks in Table 1) had previously been tested and found negative (by testing for the production of antibodies in mice) for the following viruses: pneumonia virus of mice, reo 3, Sendai, polyoma, K, mouse adenovirus, lymphocytic choriomeningitis, SV_5, rat virus, and Theiler's virus (*6*).

Cells that were examined with the electron microscope were first fixed as a monolayer in the culture flask with 1 percent glutaraldehyde in 0.1*M* phosphate buffer (*p*H 7.3) for 1 hour at room temperature. Thereafter, cultures were rinsed three times with phosphate buffer and then allowed to remain in fresh buffer for 30 minutes. Finally, the buffer was changed again and the cells from two T-15 flasks were scraped loose with a piece of cellophane, combined in one conical centrifuge tube, and spun in an International centrifuge (rotor 269) for 10 minutes at 1800 rev/min. The resulting pellet was cut into 1-mm^3 pieces, fixed again with