One of the three promoters that were examined in this study was not strongly activated by the 35*S* enhancer, and other plant promoters may in turn prove to be partially or completely nonresponsive. It is also possible that the pattern of expression of a regulated promoter could be disrupted by the influence of the constitutively active 35*S* enhancer. If loss of regulation is to be avoided, high levels of transcription from such promoters might best be achieved by duplicating the sequence elements that are responsible for their regulated activity. This procedure may be effective for increasing the transcription rates of a wide variety of genes from all life forms, particularly when promoter or enhancer strength has not been maximized by natural processes. As exemplified by this study, it is a strategy that can be successfully applied even when the sequences involved in activating transcription are not fully characterized.

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36. We thank N.-H. Chua and J. Odell for providing their clone of 35*S* promoter DNA, and S. Rogers for the vector pMON178, *Agrobacterium* strains, and advice on transformation procedures. We thank S. Rogers and R. Fraley for critical reading of the manuscript. Funded by Natural Sciences and Engineering Research Council of Canada operating grant A2832 (to J.C.M.). M.D. was the recipient of a Challenge '86 University of British Columbia award.

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Propagation in Cell Culture of the Dinoflagellate *Amyloodinium*, an Ectoparasite of Marine Fishes

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***Amyloodinium ocellatum*, a common dinoflagellate ectoparasite of marine fishes, was successfully propagated on a fish gill cell line. In vitro infections were similar in cytopathology and development to those reported on natural hosts, and large numbers of parasites could be produced. Exposure of parasites in cell culture to an antiprotozoal drug produced a dose-dependent inhibition of infectivity that was much more sensitive than a motility assay previously used to assess the toxic effects of a drug on protozoan ectoparasites. This propagation system may be a useful model for studying the biology and control of protozoan skin parasites of fishes and for quantitatively studying host-parasite interaction at cellular interfaces.**

ECTOPARASITIC PROTOZOA, MAINLY ciliates and flagellates, commonly infect wild populations of fishes (*1*) and also are important and ubiquitous pathogens in aquaculture (*2*). They damage their hosts by feeding on or within the skin and gill epithelium, which results in osmoregulatory imbalance, respiratory impairment, or secondary infection (*2*). Some ectoparasitic protozoa are facultative commensals capable of a free-living existence, but most are obligate parasites that cannot survive away from the host for an extended period of time (*3–5*). In the latter group is *Amyloodinium ocellatum*, a blastodiniid dinoflagellate that parasitizes warmwater marine and estuarine fishes. Virtually nonspecific in host selection, it is commonly found in natural populations of fishes (*6*) and also is one of the most devastating parasites in warmwater mariculture (*7*).

Amyloodinium has a triphasic life cycle (*8*). In the parasitic stage (Fig. 1) it feeds as a stationary trophozoite (trophont) on the skin and gill surfaces. After several days it detaches, forming a reproductive cyst or tomont on the substrate (Fig. 1). This tomont divides, forming up to 256 free-swimming individuals (dinospores) that can infect a new host (Fig. 1). Under the proper conditions, *Amyloodinium* can complete its life cycle in less than 1 week; it thus can decimate a host population very quickly. Clinical signs of amyloodiniosis include anorexia, hyperventilation, pruritus, and behavioral changes (*6*). Therapy must be directed at the free-swimming infective (dinospore) stage, since dinospores are much less resistant than the tomonts and trophonts (*7, 9*). Infections are asynchronous; thus dinospores do not emerge simultaneously and therapeutic drug levels must be maintained for at least several days to be effective.

Studies of *Amyloodinium* and other ectoparasitic protozoa have been hindered by the lack of in vitro culture systems. This report describes the continuous propagation of *Amyloodinium* in cell culture, in which the entire life cycle of a protozoan ectoparasite of fish has been supported in vitro.

Amyloodinium ocellatum strain DC-1 was originally isolated from aquarium-reared clown fish (*Amphiprion ocellaris*) and has been serially passaged since 1981 on clown fish and hybrid striped bass (*Morone saxatilis* and *M. chrysops*) (*10*). Studies (*11*) had shown that microbial contaminants could be eliminated from harvested parasites by serially transferring individual tomonts through sterile artificial seawater that contained antibiotics. The dinospores that emerged from these tomonts could infect and be serially propagated on gnotobiotic fish. Some parasites in this germ-free system continued to grow and develop even after the death of their host fish (*11*), suggesting that *Amyloodinium* might be adaptable to cell culture.

To test this hypothesis, approximately 50 decontaminated tomonts were added to confluent cultures of G1B cells, an established fish gill cell line (*12*). Each flask

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contained 5 ml of either SWG, HG, or SWG/HG medium (*13*).

Trophonts attached to G1B cells in SWG/HG and HG were visible within 24 hours of dinospore emergence. The G1B cells incubated in SWG alone died within 24 hours, and no trophonts were observed in these cultures. Trophonts in HG medium eventually produced low yields of noninfective dinospores. In contrast, trophonts in SWG/HG produced many tomonts and dinospores, and after approximately four cycles of infection the cell monolayer was completely killed. The G1B cells incubated in SWG/HG without dinospores were normal. Dinospores serially transferred to fresh G1B cultures in SWG/HG have been passaged more than 70 times in vitro.

Synchronous infections (*14*) of G1B monolayers produced 20,000 to 50,000 trophonts and up to 500,000 dinospores. Trophonts formed tomonts 2 to 6 days after attachment, and tomonts produced motile dinospores after two to four additional days at 25°C. The length of the life cycle was determined by the size of the tomonts; it could be as brief as 4 days if dinospores were produced after only one or two tomont divisions. This development rate is similar to that reported for experimental infections of *Amyloodinium* in fishes (*6, 8*).

Dinospores adhered to the monolayer as soon as 5 minutes after being added to the G1B culture. Within 5 to 20 minutes of their initial adherence, they began to elongate and acquire the irregular shape of a trophont. Damage to G1B cells was evident 30 minutes after adherence. The first signs were a darkening and retraction of individual G1B cells adjacent to attached parasites. As the infection progressed, a widening zone of cell necrosis surrounded the trophonts (Fig. 2), which penetrated host cell cytoplasm with rhizoids (*15, 16*) (Fig. 2, c and d). Trophonts constantly twisted and turned slowly although they did not appear to change location. The stomopode, a whiplike process emanating from the base of the parasite, continually waved over the adjacent host cells.

The precise mechanism by which *Amyloodinium* feeds on and damages host cells is unknown. Lom and Lawler (*17*) found that a single *Amyloodinium* trophont damages and kills several host cells, which probably accounts for the severe injury that the trophonts inflict on the host. Rhizoids anchor the parasite to the host cells, but Lom and Lawler (*17*) believe that rhizoids are incapable of absorbing the nutrients required by the parasite. The stomopode may function as a source of digestive enzymes that are injected into host cells or as a feeding tentacle that can gather up fragments of damaged cells that have been severed off by the pulling motion of the rhizoids (*17*). The stomopode activity in G1B cultures suggests some role in the feeding process. The constant twisting movement of the parasites may facilitate the severance of host cell fragments, which may then be phagocytized. (Food vacuoles full of dense granular and membranous material were visible inside the trophonts.)

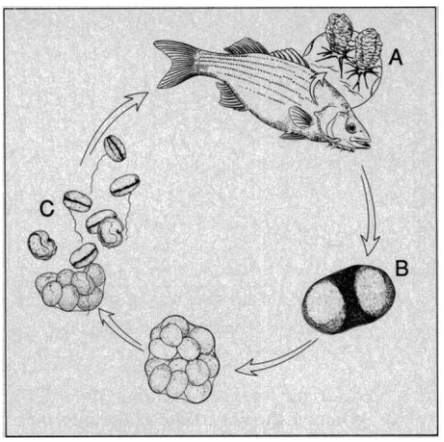

Fig. 1. Diagram of the life cycle of *Amyloodinium*. (**A**) Parasitic trophont stage that feeds on the skin and gill epithelium. (**B**) Encysted tomont stage that divides to produce free-swimming infective dinospores (**C**).

Because trophonts were easily counted (Fig. 2), we used this cell culture system to quantitatively evaluate the infectivity of dinospores after exposure to a toxic insult. The percentage of differentiated dinospores (that is, trophonts) in cell cultures containing different concentrations of amphotericin B (*18*) is shown in Fig. 3. Infectivity was inhibited in a dose-dependent fashion. After 48 hours, motile dinospores were not seen in any wells, indicating that all parasites capable of attachment had done so by this time. Inhibition of infectivity was evident at dosages that were not detectably toxic; this was assessed by using a motility assay, which scores whether any motile dinospores are present in individual wells.

Because the motility assay measures a positive response as the presence of any (as few as one) motile dinospores, it tends to underestimate the effect of a treatment on parasite infectivity. This is evident in Fig. 3, which provides data on motile dinospores that were in wells without G1B cells and were observed at all dosages in which parasites attached to the monolayer. Motility is the criterion commonly used to examine the effects of drugs, of environmental variables,

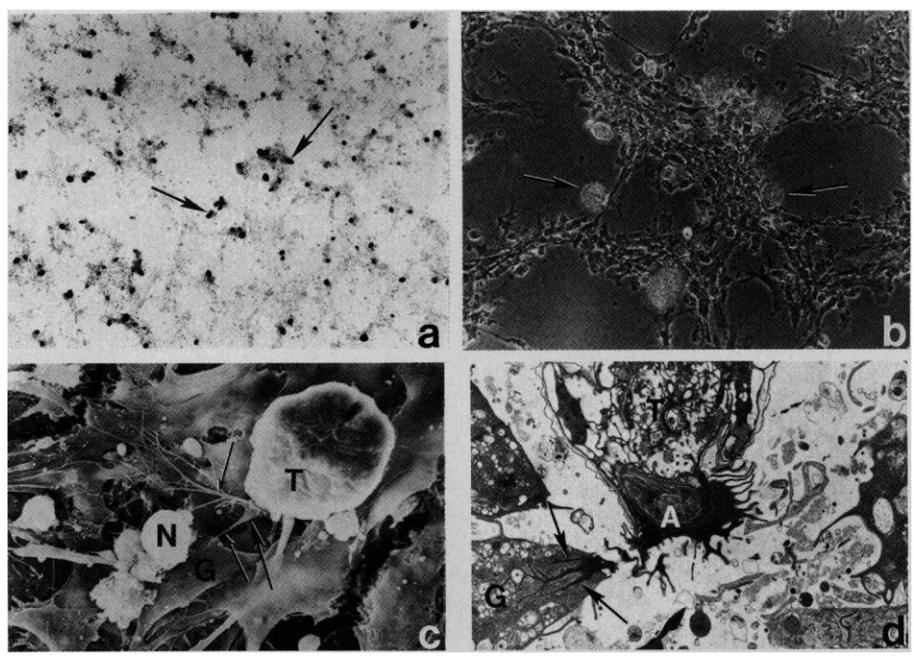

Fig. 2. Infection of G1B cells by *Amyloodinium* trophonts. All examples are from 24- to 48-hour infections. (**a**) Light photomicrograph showing a 1-cm^2 well containing a G1B culture infected with approximately 5000 parasites. The dark-pigmented trophonts (arrows) are easily distinguished. Magnification, ×20. (**b**) Phase-contrast photomicrograph showing the cytopathic effect from several trophonts (arrows). Note the large areas of cell loss and the retracted, damaged G1B cells. Magnification, ×100. (**c**) Scanning electron micrograph showing the attachment plate (large arrow) at the base of a trophont (T). Thin branching strands of cytoplasm, the rhizoids (small arrows), originate from the attachment plate to enter G1B cells (G). Necrotic G1B cell (N). Magnification, ×660. (**d**) Transmission electron micrograph showing the base of a trophont (T). Rhizoids (arrows) emanating from the attachment plate (A) penetrate a G1B cell (G). This mode of attachment is characteristic of *Amyloodinium* as described by Lom and Lawler (*17*) on naturally infected fishes. Magnification, ×3300.

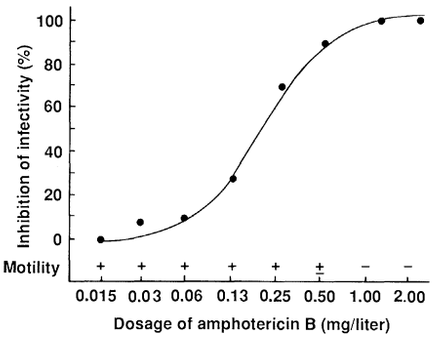

Fig. 3. The effect of amphotericin B on infectivity and motility of *Amyloodinium* dinospores. Twofold dilutions of amphotericin B (*23*) were prepared in a simple salt solution (HBSSM) (*13*) and added to triplicate confluent 0.5-cm^2 G1B monolayers that had been rinsed once with HBSSM. A 0.25-ml volume of drug dilution was added to each well. Synchronous dinospores (*14*) were diluted to 2000 dinospores per milliliter in HBSSM, and 0.25 ml of this suspension was added to each well. Cultures were sealed with plastic tape and incubated at 25°C. The number of trophonts in each well was counted after 48 hours by using an inverted phase-contrast microscope. Calculations for the percentage inhibition of infectivity are described in (*24*). Triplicate wells containing drug dilutions, but without G1B cells, were scored for the presence of motile dinospores according to the method of Gilbert *et al.* (*20*). Motility was scored at the same time as the infectivity assay was read. A (+) value indicates that motile dinospores were observed in all three wells; a (−) value indicates that no motile dinospores were observed in any of the three wells; a (±) value indicates that motile dinospores were observed only in one of the three wells.

and of the immune response on the infective stage of protozoan ectoparasites of fishes. This stage is generally considered to be most susceptible to therapy. Effectiveness is usually defined as immobilization of all parasites in a treatment group after a defined period of time (*9, 19, 20*). Such systems, however, are poor indicators of sublethal effects that may affect the infectivity of the parasites, but not their motility. Furthermore, some agents, such as copper, can reversibly inhibit the motility of dinoflagellates without killing them (*21*). It is difficult to quantify the proportion of parasites that are motile compared to those that are nonmotile in a treatment; therefore, the motility assay is used qualitatively.

Infectivity is a more valid index of a treatment's effect on parasite survival. For *Amyloodinium*, infectivity is expressed as attachment to the host and completion of development. The infectivity assay may be a sensitive detector of drug-resistant strains as well as an aid in studying host recognition and attraction.

This cell culture system also allows the observation of host-parasite interactions over many generations, thus integrating the combined effects of a treatment on all life stages. Long-term studies may reveal even more subtle effects not evident with the short-term infectivity assay. Maintenance of drug levels for several days would represent the typical course of in vivo treatment of *Amyloodinium* and other protozoan ectoparasites with similar life cycles. Quantitative evaluation of these effects on in vivo hosts is much more difficult.

Dinoflagellates are significant constituents of aquatic ecosystems. In addition to their role as parasites, they are important primary producers, consumers, and endosymbionts in many invertebrates. The approximately 2000 living species include about 140 parasites that mainly affect invertebrates (*22*). Many free-living and endosymbiotic dinoflagellates have been cultured in vitro, but to my knowledge, until now no parasitic species have been successfully adapted to cell culture. This in vitro system may help researchers study how the transition from free-living form to parasite is accomplished.

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5. Obligate pathogens include the ciliates *Ichthyophthirius multifiliis, Cryptocaryon irritans, Chilodonella* spp., and *Trichodina* spp. and the flagellates *Ichthyobodo necator* and *Piscinoodinium pillulare*. Facultative commensals include the ciliates *Heteropolaria* spp., *Carchesium* spp., *Glossatella* spp., and *Scyphidia* spp. Species that produce a reproductive cyst, such as *I. multifiliis* and *P. pillulare*, can usually survive away from the host for several days. Parasites that complete their entire life cycle on the host, such as *Trichodina, Chilodonella,* and *Ichthyobodo*, usually die within minutes of removal.
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13. HG was a modified Ham's F-12 medium (Gibco, Grand Island, NY) in which all components were present at 52% of the standard concentration; in addition, it contained 23 m*M* Hepes buffer, 25 m*M* sodium bicarbonate, 9% fetal bovine serum, and gentamicin sulfate (200 μg/ml). SWG was an artificial seawater, 32 parts per thousand salinity (Instant Ocean Aquarium Systems, Mentor, OH), containing gentamicin sulfate (200 μg/ml); SWG/HG was a 50:50 mixture. The above media were filter-sterilized before use. HBSSM (Hanks balanced salt solution, modified), with Earle's salts containing an additional 6.5 g of NaCl per liter, was autoclaved before use.
14. Synchronous cultures of parasites were initiated by pelleting tomonts from cultures at 200*g* at room temperature. The supernatant was discarded and the tomonts were resuspended in 2 ml of SWG/HG and transferred to 1-cm^2 wells in a 24-well tissue culture plate (Costar Plastics, Cambridge, MA) and incubated at 25°C. Within 24 hours of sporulation, dinospores were aspirated from the supernatant fluid, leaving unsporulated tomonts behind. The dinospore suspension was transferred to a sterile siliconized glass tube and gently mixed, and a portion removed for counting. To count dinospores, 10 μl of the suspension were aspirated into a pipette tip rinsed in 10% neutral buffered Formalin and immediately loaded into a hemacytometer. A volume containing 100,000 dinospores was added to a confluent 25-cm^2 G1B monolayer in 5 ml of SWG/HG and incubated until 90% or more of the trophonts detached; the tomonts were then harvested in a similar manner.
15. For transmission electron microscopy, cultures with 24- to 48-hour infections were rinsed once with HBSS and fixed in McDowell-Trump's fluid for 12 to 24 hours. Cultures were scraped off the culture wells (Corning cell scraper, Corning Plastics, Corning, NY), pelleted at 10,000*g*, embedded in 4% agar, postfixed in 1% OsO_4 in 0.1*M* phosphate buffer, *p*H 7.3, dehydrated through an alcohol series, transferred to acetone, and embedded in Spurr's resin. Sections were cut at 70 nm on a Reichert-Jung Ultracut microtome, stained with methanolic uranyl acetate and Reynold's lead citrate, and then viewed on a Philips 410 transmission electron microscope. For scanning electron microscopy, cover slips with 24- to 48-hour infections were fixed in McDowell-Trump's fluid, rinsed with 0.1*M* phosphate buffer and then water, and passed through a series of alcohols from 50 to 100%. Specimens were dried under the conditions of the critical point, sputter coated with gold and palladium, and examined with a JEOL JSM-35 CF scanning electron microscope.
16. This mode of attachment distinguishes *Amyloodinium* from other parasitic dinoflagellates that infect fish. *Crepidoodinium* forms junctions with host cells but does not penetrate them, whereas *Piscinoodinium* produces rhizocysts.
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23. Amphotericin B (Fungizone, E. R. Squibb and Sons, Inc., Princeton, NJ) was reconstituted to 50 mg/ml in deionized water and stored at 4°C.
24. Percentage inhibition of infectivity was calculated from

$$\left(\frac{T_c - T_t}{T_c}\right) \times 100$$

in which T_c is the number of trophonts in a control well, and T_t is the number of trophonts in a drug-treated well. A nonlinear regression of log-transformed percentage inhibition of infectivity versus dosage indicated a significant relation ($P < 0.01$). This analysis was used to construct an estimation of the dose-response curve that is shown by using the following equation:

$$\text{Percentage inhibition of infectivity} = 100 \left\{1 - \exp\left[\frac{-(\log d - \log 0.015)^{3.9}}{2.7}\right]\right\}$$

where d is the dosage of amphotericin B.

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