tubes. Retention of the pink color (*p*H 7.5) by the phenol red in the M solution, indicates removal of excess sodium hypochlorite. The washed cysts were then divided into several test tubes with 3 ml of a 4 percent proteose peptone in M solution and placed on a rotating wheel (~ 25 rotations per minute) at 33°C in dim light. The proteose peptone solution served both to hatch the *Artemia* cysts and to test their sterility. Clarity of the peptone broth indicates that the hatched larvae are bacteria-free.

Normal hatching of the decapsulated cysts begins within 24 and 48 hours, and up to 75 percent of the cysts will hatch. Larvae were collected aseptically with a Pasteur pipette, washed three times in sterile M solution and offered to the hydra. Larvae of *Artemia* survive in the hypotonic M solution for several hours and are readily captured by the hydra. Dead larvae and unhatched cysts do not decay in the bacteria-free medium, but slowly undergo autolysis. Although we did not find any ill effects on the hydra from accumulated dead larvae, we transferred our hydra into fresh dishes about once a week.

Ten clonal cultures of bacteria-free symbiotic and five of bacteria-free aposymbiotic *H. viridis* (Swiss strain) were initiated from single hydra placed in 10 ml of M solution in standard test tubes. The feeding response of the hydra was normal, and their number doubled about every 4 days. Some hydra synchronously formed two or three buds. As the number of hydra in the test tubes increased (> 30), we transferred them into Erlenmeyer flasks (150 ml) or into test tubes (36 by 160 mm) containing 50 ml of M solution to prevent inhibitory effects of crowding (*8*), and to initiate mass cultures. Controls, consisting of 60 bacteria-free green hydra starved for 30 days, formed only six buds during this period. We concluded that our *H. viridis* obtained all of their nutritional requirements from the bacteria-free larvae of *Artemia* and that no additional factors or bacterial products are required for their vegetative reproduction.

We tried to initiate similar bacteria-free cultures from ten hydra specimens of each of two nonsymbiotic strains of *H. vulgaris*. These hydra, like *H. viridis*, were fed bacteria-free larvae five times a week, but in 3 to 4 weeks, no buds had formed. Some of these hydra were then fed nonsterile *Artemia*, and others were inoculated with bacteria isolated from budding stock cultures; in all of these hydra, normal budding was resumed after 1 to 3 days (Fig. 1).

We conclude that the nonsymbiotic species of hydra that we studied lack a budding factor that is endogenous to *H. viridis*. This missing budding factor, which may be a nutrient, a vitamin, or a hormone, can be provided exogenously by either nonsterile *Artemia* larvae or by some bacteria. Our method of producing an aquatic bacteria-free predator-prey system may be useful in providing similar bacteria-free systems for nutritional and developmental studies.

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Cyclic Biological Expression in Mouse Mammary Tumors

Abstract. *Biological characteristics were assessed in GR mouse mammary tumors during 22 serial transplantations. Although unidirectional progression from hormone dependency to independency was observed, other biological markers such as progesterone receptors, polyploid frequency, and thymidine kinase activity demonstrated cyclic phenomena every fourth to sixth transplant generation, suggesting the continued presence of regulatory mechanisms among various cell subpopulations.*

Breast cancers, even of the same histologic type, show a wide spectrum of biological behavior and consist of heterogeneous cell populations. Treatment can provide temporary improvement in some patients, but nearly all patients with advanced breast cancers die as a result of the disease. Continuous changes in the biological behavior of tumors could account for the failure of many clinical therapies (*1*).

Most tumors show a gradual and unidirectional biological change toward a more undifferentiated state, a process usually referred to as tumor progression (*2*). Study of tumor biological markers has demonstrated a decrease in steroid receptor levels during the progression of human breast cancers (*3*) or after serial transplantations of rodent mammary tumors (*4*). Undifferentiated tumors also are associated with a polyploid chromosomal composition (*5*) and high thymidine kinase activity (*6*).

To gain a better understanding of the evolution of mammary tumors we assessed the changes of steroid receptor concentration, thymidine kinase activity, and chromosomal pattern in GR mouse mammary tumors during their transition from hormone-dependent to hormone-independent states.

Mammary tumors were induced with estrone and progesterone pellets in oophorectomized 3-month-old female GR mice, as described by Sluyser and Van Nie (*7*). Mammary tumors usually developed in 2 to 3 months. For transplantation, tumor cell suspensions were injected into the flank region of (BALB/c × GR)F_1 hybrid mice, which had been castrated 1 week previously. In transplant generations 1 and 2 (TG1 and TG2), 1.5×10^7 cells were used, and for the later generations (TG3 to TG22), 2×10^6 viable cells were used. The viability of the tumor cells was determined by trypan blue exclusion.

The transplant recipients were divided into two groups: group 1, those that underwent oophorectomy without hormonal maintenance, and group 2, those that were maintained with the estrone-progesterone supplement (*7*) after oophorectomy. Two of the tumors that developed in the mice in group 2 were used for the next transplantation into four or five mice each in groups 1 and 2. Four to five of the tumors that developed in each of groups 1 and 2 at various transplant generations were used for cytogenetic study, steroid receptor assay, and thymidine kinase assay.

The rate of tumor development was expressed as the number of days between tumor cell inoculation (day zero) and tumor diameter reaching approximately 1 cm. The difference in tumor development rate between groups 1 and 2 was used as a criterion for hormonal dependency or independency.

The tumor growth pattern (Fig. 1A)

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demonstrated that the first four generations were hormone-dependent, since tumors that developed in the hormone-supplemented mice in group 2 developed much earlier (by more than 2 months) than those in group 1. In contrast, tumors beyond the fourth generation (TG5 to TG22) were hormone-independent; that is, the tumors developed at similar rates in both groups. The larger number of tumor cells used in the first two transplantations may account for the initial rapid growth rate. From TG5 through TG9 there was a steady acceleration of tumor growth rate. In the fourth generation, tumors took 70 days to reach 1 cm in diameter, whereas only 20 to 30 days were needed for TG9 through TG22.

We also examined the tumor chromosomal pattern in order to elucidate the process of clonal selection during serial transplantations. Each chromosome spread was analyzed for ploidy and the presence of marker chromosomes by a G banding technique. For each tumor sample, 50 metaphase spreads were analyzed, and the percentage of polyploids and marker chromosomes was determined.

In group 2, the incidence of polyploidy in the first tumor generation was 8 percent; the incidence steadily increased to 48 percent in TG5 (Fig. 1B). The incidence of polyploidy was higher in the tumors of group 1 than in the tumors of group 2 in TG2 and TG5.

In spite of the unidirectional change in hormone dependency, the percentage of polyploidy in tumors of TG6 unexpectedly decreased to 11 percent. From TG8 through TG20 a large acrocentric marker chromosome appeared in both diploid and polyploid cells (Fig. 2A), thus establishing the continuity between transplant generations. Through this continuity there were two more cyclic changes in polyploid percentages, each cycle lasting four to six transplant generations. Changes in polyploidy percentages were also observed in vitro. When the chromosome compositions of TG11 cells were analyzed in cultures less than 1 week old, there was a steady increase in polyploid percentage from 25 percent in the original passage to 77 percent in the seventh passage, and to 88 percent in the eleventh passage in the culture medium.

The transition from the hormone-dependent to hormone-independent state is frequently accompanied by changes in tumor steroid receptor content (*4*). Cytosol progesterone receptors of the 22 serial transplant generations were measured by a modified sucrose gradient method with ^{3}H-labeled R5020 being used as a ligand (*8*). The progesterone receptor patterns of TG2 and TG22 are shown in Fig. 2B.

Progesterone receptors increased initially and then declined from TG2 through TG5 and disappeared from TG7 and later generations (Fig. 1C). However, cyclic reappearances of progesterone receptors were demonstrated in TG6, TG10, TG16, and TG22. The tumor estrogen receptor concentrations (not shown) showed a similar but less conspicuous rhythm.

Tumors with a high percentage of polyploid cells appeared to have low progesterone receptor levels. Breast tumors lacking steroid receptors were previously reported to have a high thymi-

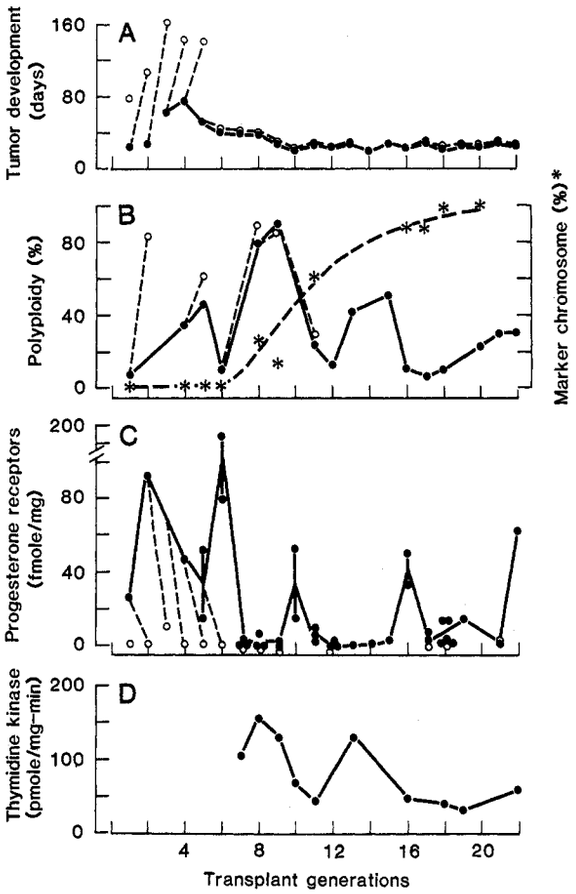

Fig. 1. Changes of biological characteristics in GR mouse mammary tumors during serial transplantation in oophorectomized (BALB/c × GR)F_1 recipient mice, either maintained with an estrone-progesterone supplement (group 2, ●) or without such maintenance (group 1, ○). (A) Tumor growth rate. The points represent the average number of days required in four or five recipients for the tumor to reach 1 cm in diameter. Tumor inoculation occurs on day zero. (B) Polyploidy and marker chromosome. Each point represents the average of analyses of 50 metaphase spreads of one tumor sample. (C) Progesterone receptors. Each point represents the 8*S* progesterone receptor content of one tumor. (D) The thymidine kinase activities of various transplant generations measured simultaneously from tissues stored in liquid nitrogen. Results from one of the two separate experiments with similar rhythms are depicted here.

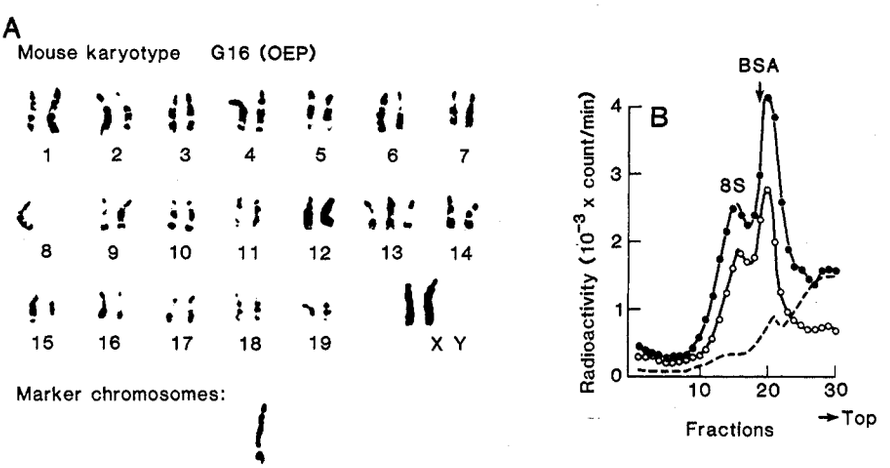

Fig. 2. Characteristics of the biological markers. (A) The marker chromosome by G banding technique. (B) The 8*S* and 4*S* types of progesterone receptors in tumor generations 2 (●) and 22 (○) measured by a sucrose gradient (5 to 20 percent) method with ^{3}H-labeled R5020 used as the ligand. The control curve (dashed line) was obtained when 100× concentrated R5020 was used as a competitor.

dine labeling index and proliferation rate (*9*), features commonly shared in tumors of high polyploidy. There was no ready explanation for the resurgence of progesterone receptors in every fourth to sixth transplant generation. A similar alternation between polyploid and diploid cells was previously observed by Makino in the Watanabe ascites hepatoma model (*10*).

We also observed a cyclic pattern in tumor thymidine kinase activity. Cytosol thymidine kinase activity was measured by the method of Breitman (*11*) in transplant generations where there was enough tumor tissue available (Fig. 1D). High enzymatic activity was observed in the generations with a high percentage of polyploidy. The undulating curve of enzyme activity, observed in two experimental series, was parallel to that of a polyploidy curve (Fig. 1B). The quality of the thymidine kinase activity judging by values for the Michaelis constant (2.2 to 3.7 μM) was similar in tumors of high or low enzyme activity.

Our results suggest that in the early stage of tumor development the environment (hormonal milieu) favors the growth of hormone-dependent clones. The cyclic changes in polyploidy, thymidine kinase activity, and steroid receptor content suggest the continuous presence of regulatory mechanisms among various cell subpopulations. Another possibility is that there is some variation among clones and that a new clone of cells emerges as an older clone declines and disappears after maximum proliferation. It is also possible that in the late stage of tumor development genetic changes occur that are selectively advantageous for the tumor in its own particular environment. Further studies are needed to elucidate whether genetic modification is involved in this cyclic manifestation.

The cyclic phenomena that we observed were not due to variations in the techniques used in our laboratories. Cytogenetic studies were performed with cells from the same tumor as that used for receptor assay as well as with cells from different tumors of the same transplant generation. It is unlikely that the cyclic patterns were due to unequal sampling of tumor subpopulations at the time of each transplant and that such sampling error recurred at fixed intervals of every four to six generations. Since all of the tumors serially transplanted were derived originally from one tumor, more work with different tumors will have to be done before we can be sure of the generality of the phenomena. Nevertheless, our observation is not an isolated one. In a previous report (*12*), an oscillatory pattern of progesterone receptor content, although not described, was clearly demonstrated in three of four different, independently arising GR tumors which had gone through eight to ten transplant generations. Similar receptor changes were also observed in our laboratory in other transplant series of GR tumors, although they were not studied in detail for other biological markers. A marked fluctuation of immunological characteristics of BALB/cf C_3H mouse mammary tumors during serial transplantation has also been described (*13*).

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5-Azacytidine-Induced Reactivation of a Herpes Simplex Thymidine Kinase Gene

Abstract. *Mouse cells transformed with herpes simplex virus and containing the viral thymidine kinase (TK) gene in an inactive state were treated with 5-azacytidine. The result was the reexpression of the viral TK gene. Two days of exposure to 5-azacytidine followed by 2 days of expression time was sufficient for maximal induction of the TK^+ phenotype. The induction of TK expression by 5-azacytidine was concentration-dependent, with maximal induction at 10 micromoles per liter. 5-Azacytidine also inhibited the decay of TK expression in TK^+ transformants removed from selective conditions. Analysis of the methylation patterns of the viral TK gene with restriction endonucleases Hpa II and Msp I showed the active gene to be unmethylated, the inactive gene methylated, and the 5-azacytidine-induced gene unmethylated.*

In studies on the transfer of the thymidine kinase (TK) gene of herpes simplex virus (HSV) into mammalian cells, we showed that the expression of the transferred gene in the transformed cells was unstable (*1*). This instability of gene expression subsequently was observed to be a characteristic of gene transfer systems in general (*2–5*). When the HSV-transformed cells were removed from hypoxanthine-aminopterin-thymidine (HAT) medium, in which only cells expressing TK activity can survive (*6*), there was an exponential decay in the proportion of cells expressing the transformed (TK^+) phenotype. The viral TK gene, however, was retained in the TK-negative (TK^-) cells and could be reexpressed spontaneously, although reversion to the TK^+ phenotype occurred with very low frequencies (around one cell in 10^6) (*1, 5, 7*). These changes in the expression of the viral TK gene in the transformed cells did not appear to involve soluble, trans-acting regulatory substances (*5*) or gross changes in the viral gene sequences present in the cells (*7*).

One possible mechanism for regulating the expression of foreign genes in transformed cells involves the modification of the genes by methylation. To test this possibility, we examined the effects of 5-azacytidine (azaC) on the expression of the viral TK gene in HSV-transformed cells. 5-Azacytidine causes hypomethylation of DNA, presumably by substituting for cytosine residues in DNA that can be methylated (*8*). Treatment with azaC induces "differentiation" of mouse

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