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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fibroblasts into myotubes (*8*) and activation of genes on inactive human X chromosomes (*9*).

Cell lines LH-1 and LH-2 express HSV-TK activity and were derived by treating TK-deficient mouse cells [of the line LM(TK$^-$), clone 1D] with ultraviolet-inactivated HSV type 1 and selecting for TK$^+$ cells with HAT medium (*1*). Lines HT-12-1 and HT-Y, derived from LH-1 and LH-2, respectively, lack TK activity although they carry the HSV-TK gene (*1, 5, 7*). They were derived by prolonged propagation of HSV-TK$^+$ cells in nonselective medium, followed by cloning in nonselective medium (*1*). These cells are unable to grow in HAT medium, except for rare revertants that regain the HSV-TK activity.

In preliminary experiments to determine whether azaC treatment induces the reexpression of the inactive HSV-TK gene, HT-12-1 cells were cultured for periods of 1 to 4 weeks in the presence of 3 μM azaC. During this period, the cells were fed with fresh medium and azaC at least twice a week. The treatment with azaC caused a significant increase in the frequency of cells able to grow in HAT medium, suggesting the reactivation of the viral TK gene. However, the frequency of TK$^+$ cells induced did not show any further increase when the cells were exposed to azaC for longer than 1 week. Therefore, experiments were carried out to determine the optimal conditions for induction of TK activity in terms of the time of exposure to azaC and the expression time between azaC exposure and selection with HAT medium. Because of the results of the preliminary experiments mentioned above, these experiments focused on times shorter than 1 week.

Both the exposure time in the presence of azaC (3 μM) and the expression time (growth in the absence of azaC) before the cells were plated in HAT selective medium were varied (Table 1). The number of HAT-resistant colonies increased approximately sevenfold as exposure to azaC increased from 1 to 4 days. Varying the expression time after exposure to azaC also had a major effect. Giving the cells 2 days for expression after a 2-day exposure to azaC resulted in a threefold increase in the frequency of HAT-resistant colonies. In fact, the frequency of reversion with 2 days of azaC exposure plus 2 days of expression time was as high as the maximal reversion frequency that occurred with 4 days of exposure to azaC. Thus, it appears that 2 days of exposure to azaC is sufficient for maximal induction.

5-Azacytidine is a relatively unstable compound with a half-life of approximately 12 hours at *p*H 7.4 (*8*). Therefore, exposure times of 1 day or less might have been expected to be sufficient for maximal TK induction. The data in Table 1, however, clearly show that a 1-day exposure to azaC is not as effective as a 2-day exposure. A priori, the hydrolysis of azaC could account for the absence of any increase in the reversion frequency with exposures of more than 2 days. However, the data from the preliminary experiments mentioned above refute this point; that is, when the cells were exposed to fresh azaC at least twice a week for 4 weeks, there was no progressive increase in reversion frequency.

Results similar to those presented in Table 1 were obtained with HT-Y cells (data not shown). When the parental LM(TK$^-$) cells, which lack both cellular TK activity and the viral TK gene, were tested for inducibility of TK$^+$ cells by 3 μM azaC, no HAT-resistant cells were observed among more than 10^7 cells tested.

Using the optimal induction conditions defined above (2 days of azaC exposure plus 2 days of expression time), we conducted experiments with HT-12-1 cells to determine the effect of varying the azaC concentration from 0.1 to 30 μM. The frequency of HAT-resistant colonies increased progressively from 4.9 colonies per 10^6 cells at 0.1 μM to 30.4 colonies per 10^6 cells at 10 μM azaC. Concentrations of azaC above 10 μM gave inconsistent results, apparently complicated by the toxic effects of azaC at these concentrations. However, it appears that the induction of TK$^+$ cells is not further enhanced by increasing the azaC concentration above 10 μM. Similar results were obtained with HT-Y cells.

Table 1. Induction of TK activity by azaC in TK$^-$ (HT-12-1) cells. The cells (5×10^5) were inoculated in 100-mm dishes containing Dulbecco's modified Eagle's medium supplemented with 10 percent fetal calf serum (E medium) plus 3 μM azaC. After the appropriate exposure time, cells were either harvested immediately or rinsed, fed with E medium without azaC, allowed to continue growing for the appropriate expression time, and then harvested. Harvested cells were plated in triplicate at a density of 10^6 cells in 150-mm dishes containing HAT medium (*6*). After 8 to 9 days, the cells were fixed and stained, and the colonies were counted. Only colonies containing 50 or more cells were counted. Values represent the number of colonies in HAT per 10^6 cells, after correcting for plating efficiency in E medium and for background colonies developing in HAT medium from HT-12-1 cells not exposed to azaC (3.1 colonies per 10^6 cells). Relative to untreated controls, the plating efficiency of azaC-treated cells in E medium ranged from 85 percent (1 day exposure) to 77 percent (4 days exposure).

Exposure time (days)	Number of colonies per 10^6 cells in HAT medium after various expression times			
	0 day	1 day	2 days	3 days
1	2.1			6.8
2	5.0		15.4	
3	13.1	16.2		
4	15.0			

The ability of azaC-treated cells to grow in HAT medium suggests that they are phenotypically TK$^+$, but does not indicate whether the TK activity is determined by viral or cellular genes. Therefore, the nature of the TK activity was analyzed by polyacrylamide disk gel electrophoresis. The TK activity from azaC-induced TK$^+$ revertants (from both HT-12-1 and HT-Y) migrated with the TK activity from LH-1, which expresses the viral TK activity, with a relative mobility (R_F) of 0.5 (*1*). The activity from LM(TK$^+$) cells, which express the cellular TK ($R_F = 0.1$), migrated much more slowly than the viral TK, and such activity was not observed in azaC-induced revertants. Uninduced HT-12-1 cells showed essentially no TK activity. Thus the TK activity in azaC-induced revertants appears to have originated from the HSV gene.

Although these results indicate that azaC treatment leads to the reappearance of viral TK activity, they do not indicate whether azaC induced the TK$^+$ revertants or exerted selection pressure favoring spontaneous TK$^+$ revertants. Transformed TK$^+$ (LH-1) and TK$^-$ (HT-12-1) cells were mixed at a ratio of 1:100 and grown for 1 week in the presence of 3 μM azaC; the mixed population was then tested in HAT to determine the frequency of TK$^+$ cells. If azaC selected for TK$^+$ cells, then the ratio of TK$^+$ to TK$^-$ cells should have increased. However, the ratio of TK$^+$ to TK$^-$ cells remained the same as in the initial mixture, unaffected by exposure to azaC. Thus, azaC exposure appears to induce the reexpression of TK activity.

We showed earlier that the expression of the viral TK activity decays in the HSV-transformed lines LH-1 and LH-2 (*1, 5*). Therefore, experiments were carried out to determine whether azaC could alter the decay in the expression of the viral TK gene. LH-2 cells and two azaC-induced revertants of the HT-Y lines were grown in nonselective medium in the presence or absence of 3 μM azaC. At weekly intervals, the cells were plated in HAT medium to determine the proportion of TK$^+$ cells. When LH-2

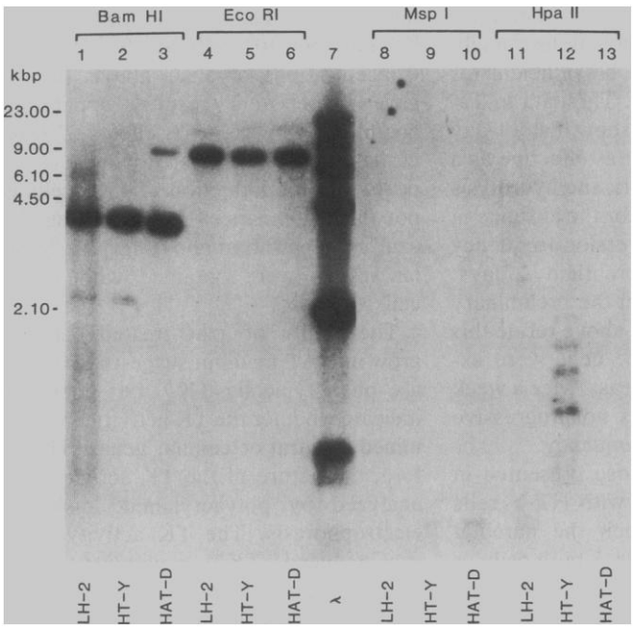

Fig. 1. Extent of methylation of TK genes in LH-2 cells (TK^+), HT-Y cells (TK^-), and HT-Y·1 azal·HAT clone D cells (azaC-induced TK^+). Restriction endonuclease reactions with Msp I and Hpa II were carried out as described by the manufacturer (New England Biolabs or Boehriger Mannheim). The final reaction was in the presence of lambda phage DNA to monitor completion of digestion. Restriction endonuclease digests from above were subjected to electrophoresis on horizontal agarose gels (0.7 or 0.8 percent agarose in 2 m*M* EDTA, 90 m*M* tris-borate, *p*H 8.0). The gels were blotted to nitrocellulose filters (*11*). The blots were hybridized overnight with a ^{32}P-labeled nick-translated (*13*) pBR322 probe containing the 3.4-kpb Bam HI fragment of HSV-1 DNA including the HSV-TK gene. The filters were washed, dried, and autoradiographed (Kodak X-omat film) against an intensifying screen at −80°C for 2 to 7 days. Restriction endonucleases and DNA size markers (*kbp*) are indicated. *HAT-D* indicates cell line HT-Y·azal·HAT clone D.

cells were grown in the absence of HAT, the proportion of cells expressing TK activity decayed rapidly, dropping from 50 to 12 percent within 26 generations. In contrast, LH-2 cells exposed to 3 μ*M* azaC while being cultured in the absence of HAT showed no evidence of decay after 23 generations. With one azaC-induced revertant line (HT-Y·1 azal·HAT clone D) grown in nonselective medium, the percentage of cells expressing TK activity decreased from 83 to 28 percent in 41 generations. When the cells were grown in the presence of 3 μ*M* azaC, however, the decay was slowed, and the percentage of TK^+ cells was still 54 percent after 41 generations in nonselective medium. With a second azaC-induced revertant cell line (HT-Y·1 azal·HAT clone B), the expression of TK activity remained stable whether or not azaC was present. These data show that azaC can either prevent or slow the rate of decay of TK activity that normally occurs when HSV-TK^+ cells are grown in the absence of selective pressure. There appear to be at least two classes of revertants that can be isolated after azaC induction: (i) those whose TK activity is unstable in the absence of selection but whose decay is inhibited by azaC and (ii) those whose TK activity is stable for more than 50 generations in the absence of selective pressure and azaC.

Because azaC appears to cause gene activation by decreasing the extent of methylation in DNA (*8*), we determined the degree of methylation for DNA's from cells with an active HSV-TK gene (LH-2), an inactive TK gene (HT-Y), and an azaC-induced active gene (HT-Y·1 azal·HAT clone D). The DNA's were purified (*10*) and digested to completion with several restriction endonucleases to assess both the structure and the methylation patterns of the TK genes. The digested DNA's were subjected to electrophoresis and analyzed by Southern blot hybridization (*11, 12*). Hybridization was carried out with a nick-translated (*13*) plasmid containing the viral TK gene in a 3.4-kbp Bam HI fragment of HSV-1 DNA. All three cell lines contained a prominent 3.4-kbp Bam HI fragment, consistent with the presence of HSV-1 TK genes that are structurally similar in the three lines (lanes 1 to 3 in Fig. 1). Digestion with Eco RI also suggested that the TK genes were structurally similar in the three lines (lanes 4 to 6 in Fig. 1). Restriction endonucleases Hpa II and Msp I have identical restriction sites (CCGG) except that Hpa II will not cut if the internal cytosine residue at this site is methylated (*14*). The DNA's of all three cell lines were digested by Msp I to molecular weights of less than 0.57 kbp (lanes 8 to 10 in Fig. 1). The DNA's from LH-2 cells (TK^+) and from HT-Y·1 azal·HAT clone D cells (azaC-induced TK^+) were digested to the same extent by Hpa II (lanes 11 and 13) as by Msp I. However, digestion of DNA from HT-Y cells (TK^-) with Hpa II yielded several bands of higher molecular weight (greater than 1.8 kbp) (lane 12 in Fig. 1). Thus, there appear to be restriction sites in the viral gene that are methylated (restriction-resistant) in the TK^- cells, but unmethylated in the TK^+ cells. These results suggest an association between DNA methylation and the expression of the viral TK genes in these cells.

Our results show that azaC can induce the reexpression of the viral TK activity in cells containing an inactive HSV-TK gene. Two days of exposure to azaC seems to be sufficient for maximal induction of TK expression; this suggests that all the primary events involved in the induction of TK activity occur within the first two generations of exposure to azaC and that further propagation in the presence of azaC does not result in further induction. However, in the absence of azaC, an additional 2 days seems to be necessary to allow for maximal expression.

5-Azacytidine causes hypomethylation of DNA, presumably by substituting for cytosine residues that can be methylated (*8*). Our results with Hpa II and Msp I digestion suggest that azaC affects the induction and disappearance of HSV-TK expression by altering methylation patterns, and that the changes in HSV gene expression in the transformed cells are regulated by DNA methylation. The effect of azaC on both reexpression and decay also suggests that hypomethylation of the inactive TK gene leads to reexpression, but that the gene can be methylated again—even in the continued presence of azaC—leading again to the loss of TK activity. The expression of transferred genes in other systems may also be regulated by DNA methylation. These suggestions are consistent with the observations that the HSV-TK gene is hypermethylated in a line of transformed cells which have stably lost the viral TK activity (*15*) and that the reexpression of the TK gene in these

cells can be induced by azaC (*16*). Further evidence of a role for DNA methylation in the regulation of exogenous viral genes in eukaryotic cells is provided by studies with adenovirus type 12 (*17*) and herpes saimirii (*18*), in which correlations between methylation and gene expression have been observed. The system we have described should allow a specific and quantifiable assessment of the role of DNA methylation in the regulation of gene expression in eukaryotic cells.

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Transplantation of the Cockroach Circadian Pacemaker

Abstract. *Surgical removal of the optic lobes of the cockroach* Leucophaea maderae *followed by transplantation of the optic lobes from another individual led to a restoration of the circadian activity rhythm in 4 to 8 weeks. The free-running period of the restored rhythm was determined by the period of the donor rhythm before surgery. The results suggest that the transplanted optic lobe contains a circadian clock that regenerates those neural connections with the host brain that are necessary to drive the circadian rhythm of activity.*

A generally accepted criterion for the localization of a circadian pacemaker that controls a specific behavioral rhythm is to demonstrate that transplantation of the putative pacemaker tissue from one animal to another (in which the tissue has been removed) both restores a free-running rhythm and confers either the phase or the period of the donor's rhythm on the rhythm of the host. This criterion has been successfully met in three organisms: the silk moth (*1*), the fruit fly (*2*), and the sparrow (*3*). In each of these cases, the transplanted tissue appears to control rhythmicity by release of a hormone, and neural connections between the pacemaker tissue and the nervous system of the host are not required.

In the cockroach *Leucophaea maderae*, there is substantial evidence to suggest that the circadian pacemaker driving the rhythm of locomotor activity is composed of two bilaterally paired and mutually coupled oscillators—one in each optic lobe of the protocerebrum—that are independently able to drive rhythmicity (*4–6*). However, the ability of the optic lobe to sustain a rhythm of activity depends on intact neural connections between optic lobe and the midbrain (*4, 5*). This requirement seemed to preclude critical transplantation experiments. I have discovered, however, that although bilateral section of the optic tracts invariably abolishes the free-running rhythm of locomotor activity, if the optic lobes are left in situ, the rhythm consistently reappears in 3 to 5 weeks, with a free-running period near that of the rhythm before surgery (*7*). The return of rhythmicity likely depends on regeneration of neural connections between the optic lobe and the midbrain since (i) if the optic lobes are removed from the animal, arrhythmicity persists indefinitely (*4, 7, 8*); (ii) histological (*7, 9*), electrophysiological (*7*), and behavioral (*9*) data show that regeneration between neurons of the optic lobe and midbrain does occur; and (iii) the time course of regeneration is similar to that of the return of rhythmicity (*7*).

These results prompted an effort to transplant the optic lobes from one animal into another whose own optic lobes had been removed. In these experiments, adult males that had been raised from birth in LD 11:11 or LD 13:13 were used (*10*). The periods (τ) of the free-running activity rhythms of animals that have been reared in these two conditions are substantially different (*11*). The average τ in constant darkness for males raised in LD 11:11 is 22.7 ± 0.27 (standard deviation) hours (N = 21), and for males raised in LD 13:13 is 24.2 ± 0.26 hours (N = 15). A period difference of more than 1 hour is maintained between the two groups for at least 5 months after the animals are transferred to constant darkness (*11*) and probably persists throughout the life of the cockroach (*12*).

Two separate series of transplantation experiments were performed with a total of 18 animals (nine from each light cycle). Individuals were placed in activity monitors in constant darkness for approximately 4 weeks to determine the period of the free-running activity (*13*). Optic lobes were then exchanged between individuals of the two groups, and the animals were returned to the activity monitors (*14*). Of the 18 animals, 12 survived for 40 or more days after surgery.

Activity was invariably disrupted and apparently aperiodic for several weeks after transplantation; however, in 10 of the 12 animals that survived surgery, a clear circadian rhythm of activity reappeared between 26 and 56 days postoperatively (*15*) (Fig. 1). The remaining two animals were a pair between which the optic lobes had been exchanged. Activity patterns of these two animals, recorded for 75 days after surgery, showed no convincing evidence of periodicity.

In every case in which a rhythm was reestablished, the free-running period was near the period of donor animal's rhythm before transplantation of the optic lobes (Fig. 2). In most cases, there was either an increase or decrease of more than 1 hour in the period of the host rhythm—a major change in τ for adult *Leucophaea* in which the free-running period is normally stable (*11*). It is substantially larger than any period changes occurring spontaneously (*11, 16*), as a result of aging or aftereffects of entrain-

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