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Genetics

Cell cycle-dependent regulation of thymidine kinase activity introduced into mouse LMTK$^-$ cells by DNA and chromatin-mediated gene transfer

(transgenome/gene expression/synchronized cells)

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ABSTRACT **We report on the expression of thymidine kinase (tk) activity in synchronized populations of mouse cells that have been transformed to the tk$^+$ phenotype with purified DNA from various sources or with metaphase chromosomes from human cells. The viral (herpes) tk gene is constitutively expressed but, in all other cases examined, the activity is regulated as in normal tk$^+$ mouse cells: There is a dramatic increase at the beginning of the S-phase. This regulation is observed whether the transgenome is stably integrated into the host genome or whether it is still in an unstable nonintegrated state.**

The feasibility of transferring genes into mammalian cells by using metaphase chromosomes or a calcium phosphate precipitate of DNA has been demonstrated in a number of laboratories in recent years (1–9). The transfer has been successful with selectable genes, such as those for thymidine kinase (tk) (3, 4, 7), hypoxanthine phosphoribosyl transferase (1, 2, 8, 9), adenine phosphoribosyl transferase (6, 9), and others, and a number of nonselectable genes have been introduced by cotransformation (3, 5). When there is a selection for a gene, such as tk, it must necessarily be expressed, but the expression of cotransferred genes—for example, the β-globin gene—is variable and often suppressed (5).

There is general agreement that the transgenome appears to exist in a nonintegrated state for some time, during which it can be lost by segregation in the absence of selective pressure, but eventually a stable association with the host genome is achieved by a random integration into a chromosome of the recipient (10, 11).

A gene transferred into a recipient by the procedures cited above faces several potential problems. (*i*) Because only a small fragment of the DNA is rescued and eventually integrated, regulatory sequences linked to the structural gene may be lost. (*ii*) The gene is integrated into a new environment, where it may be subject to different regulatory controls.

We have examined the expression of heterologous thymidine kinase activity in synchronized populations of transformed mouse cells. Normally, thymidine kinase is absent in resting cells and in cells in the G_1 phase of the cell cycle. A very sharp induction of activity is observed at the beginning of the S phase (12–14). One may ask, therefore, whether the tk gene on a transgenome is also regulated normally or whether there is evidence for altered and possibly constitutive expression.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Maintenance. The recipient cell in all cases was the mouse LMTK$^-$ line. Several independent clones of such cells transformed to the tk$^+$ phenotype by human metaphase chromosomes were made available to us by R. Baker and Tracy Gross of the Massachusetts Institute of Technology Cancer Center (Cambridge, MA), and we isolated additional tk$^+$ clones by the DNA-mediated gene transfer procedure of Wigler *et al.* (4). Both hamster and rat purified genomic DNAs were successfully used. A clone transformed with the herpes tk gene was isolated by using plasmid pBR322 tk as donor DNA (15). These clones were selected in HAT medium (see below) by standard methods, and their stability was tested as described below. Another clone transformed with the herpes tk gene dissociated from the pBR322 plasmid DNA was made available to us by D. Jolly of the Department of Pediatrics, University of California at San Diego.

The cells were routinely maintained in 37°C water-jacketed incubators under 90% air/10% CO_2 in Dulbecco's modified Eagle's medium/10% fetal calf serum (Irvine Scientific, Irvine, CA)/0.1 mM hypoxanthine/0.5 μM methotrexate/50 μM thymidine (HAT medium).

Cell Synchronization. Two procedures were used to obtain population of cells at or near the beginning of the G_1 phase. In the first, 5–10 × 10^6 cells per 10-cm plate were washed with TD buffer (16) and then allowed to incubate in medium without serum for 72 ± 2 hr. After this serum-starvation period, the cells were trypsinized, suspended in medium containing 12% fetal calf serum, and distributed to 3 10-cm plates and about 30 3.5-cm plates for each original 10-cm plate. At various times, about 5 × 10^6 cells were harvested, pelleted, and frozen for a later assay of tk activity. At the same times, the rate of DNA synthesis was measured by pulse labeling of duplicate aliquots of cells in 3.5-cm plates with [^{3}H]thymidine. The procedure has been described (16).

The second method of synchronization involved mitotic selection by a cell cycle analyzer (CCA 2000, Talandic Research, Pasadena, CA). Cells (2–5 × 10^7) were plated in each of two roller bottles in medium containing 5% fetal calf serum. Mitotic cells were harvested at 60- or 120-min intervals by increasing the rate of rotation of the bottles from 0.5 rpm to 100 rpm for 3 min. The detached mitotic cells were removed and replated, and fresh medium was pumped into the roller bottles. After an overnight run, a series of populations of different ages were available for measuring the tk activity or the rate of DNA synthesis by pulse labeling with [^{3}H]thymidine.

tk Assay. A pellet of a few million cells was suspended in 0.3 ml of hypotonic buffer [0.01 M Tris/0.01 M KCl/1 mM $MgCl_2$/2 mercaptoethanol/50 μM thymidine (pH 8.0)]. This suspension was frozen and thawed three or four times in a dry ice/ethanol bath and then centrifuged for 20 min at 10,000 × *g*.

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Abbreviations: tk, thymidine kinase.

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Thirty microliters of the supernatant was mixed with 20 μl of reaction cocktail [0.06 M Tris, pH 8.0/0.015 M $MgCl_2$/0.015 M ATP/[^{3}H]thymidine at 0.08 mCi/ml (specific activity ≈ 50 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels)]. The mixture was incubated at 33°C for 20–60 min, and the reaction was stopped by addition of 30 μl of 50 mM EDTA and placing the tubes on ice. Finally, the mixture was spotted on DEAE filter discs (Whatman DE81); these were dried, washed three times with 0.01 M ammonium formate, once with water, and once with ethanol, and then dried and assayed in a Beckman scintillation counter. The scintillation fluid contained toluene, Triton X-100, and water (17).

Acrylamide Gel Electrophoresis. Acrylamide disc gel electrophoresis to separate human and mouse tk was performed by using a modification of the procedure of Kit *et al.* (18). Cell extract was prepared by adding an approximately equal volume of extract buffer (0.01 M Tris·HCl, pH 7.8/0.01 M dithiothreitol) to washed cell pellets, freezing and thawing three or four times, and centrifuging at 17,000 × *g* for 20 min. Supernatant (20–50 μl) was layered on top of a polyacrylamide gel composed of a 0.5-cm stacking gel and a 9.0-cm separating gel. Electrophoresis was performed at 1.5 mA per gel for 20 min and at 3 mA per gel for an additional 1.75 hr or until a bromophenol blue marker was just at the end of the gel. Gels were removed from the tubes, frozen on dry ice, and sliced into 1-mm slices. Each slice was assayed according to the procedure of Willecke *et al.* (19) by placing it in a well of a Falcon microtest II 96-well plate with 0.1 ml of reaction mixture and incubating for ≈ 40 hr at 37°C. The incubation mixture was then spotted on DE81 paper discs and washed and assayed as for tk.

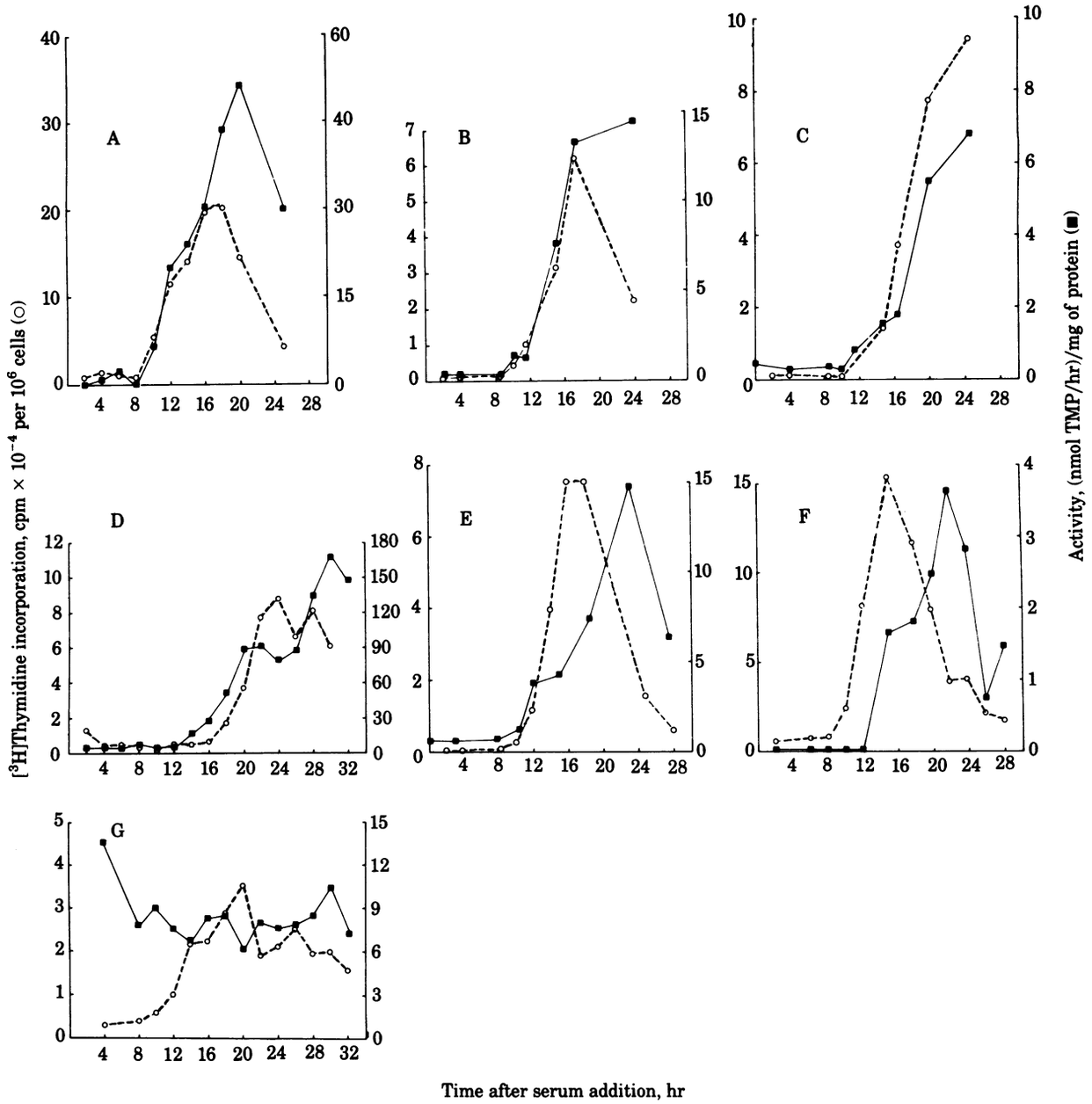

FIG. 1. tk activity in cells synchronized by serum manipulations. (*A*) LMTK$^+$-C1 (human), (*B*) LMTK$^+$-B4 (human), (*C*) L1A1 (hamster), (*D*) L60 (normal tk$^+$ mouse), (*E*) L3E1 (rat), (*F*) LMTK$^+$-A2 (human), and (*G*) L2C1 (herpes virus).

Table 1. Cell lines used in this study

Designation	Origin of tk gene	Method of transformation	Stability of tk^+ phenotype
$LMTK^+$-A2	Human cells	Metaphase chromosomes	Unstable
$LMTK^+$-B4	Human cells	Metaphase chromosomes	Stable
$LMTK^+$-C1	Human cells	Metaphase chromosomes	Stable
L1-A1	Hamster cells	Ca phosphate precipitate of genomic DNA	Unstable
L2-C1	Herpes virus	Ca phosphate precipitate of pBR322tk DNA	Not tested
L3-E1	Rat liver	Ca phosphate precipitate of genomic DNA	Unstable

RESULTS

tk Activity in Synchronized Cells. Several different clones of mouse $LMTK^-$ cells transformed to tk^+ by metaphase chromosomes or purified DNA were investigated. Their origins, some of their characteristics, and their designation in this paper are given in Table 1. Synchronous cultures were obtained either by serum starvation and refeeding or by mitotic selection. The degree of synchrony was monitored by measuring the rate of incorporation of [^{3}H]thymidine into DNA in aliquots of the cultures. Aliquots were withdrawn at various times to measure the specific activity of thymidine kinase. The results are shown in Figs. 1 *A–G* and 2 *A–B*. The use of serum manipulations in the first series caused the cells to have an abnormally long G_1 phase (8–18 hr). However, the synchrony was good, as judged by the sharp DNA synthesis peak or rise. All the clones tested except the one expressing the viral tk gene (see Fig. 1*G*) regulated their tk activity in a manner very similar to that of wild-type cells. Not too surprisingly, the herpes tk gene appears to be constitutively expressed. The same observation was made with another, independently derived tk^+ clone transformed with the herpes tk gene. The absolute specific activities of tk varied from clone to clone, and there were small variations in repeat experiments. Variations in the extact timing of the increase in tk activity relative to that of DNA synthesis were also seen in different clones and in repeat experiments with the same clone, but the overall pattern of regulation was consistent.

In the experiment in which the cells were synchronized by mitotic selection the results were similar (see Fig. 2). The G_1 period was shorter, because the cell cycle was less perturbed. tk activity again increased dramatically during the S phase; in this respect, the transformant behaved like normal mouse cells. In this particular transformant, the induction of tk activity appeared to be multiphasic, with a reproducible second increase in activity in the G_2 phase. The physiological basis for this behavior is not clear; the same observation is not made with wild-type cells. Thus, there may be some variation in the details of the regulation of tk activity in different transformed clones.

Stability of the tk^+ Phenotype. The cells were routinely carried in HAT medium until the beginning of the synchronization procedure (start of serum starvation or plating in roller bottles for mitotic selection), when they were placed in regular Dulbecco's modified Eagle's medium. We also monitored the stability and possible integration of the transgenome in the host chromosome by growing the cells for longer periods of time in regular medium—i.e., in the absence of selective pressure. Periodically, 1000 cells were plated in medium containing bromodeoxyuridine at 30 μg/ml; the number of colonies arising under those conditions is a measure of the loss of the tk gene. Clones $LMTK^+$-B4 and $LMTK^+$-C1 transformed with human metaphase chromosomes yielded no or very few tk^- segregants, confirming the information provided by the laboratory of origin. On the other hand, several of the clones derived from DNA-mediated gene transfer were unstable, suggesting that the transgenome was probably in an unintegrated state (Fig. 3). We did not note any consistent differences in the regulation of tk activity between stable and unstable transformants.

Characterization of tk in the Transformants. The $LMTK^-$ cell line has never been observed to revert to tk^+. Moreover,

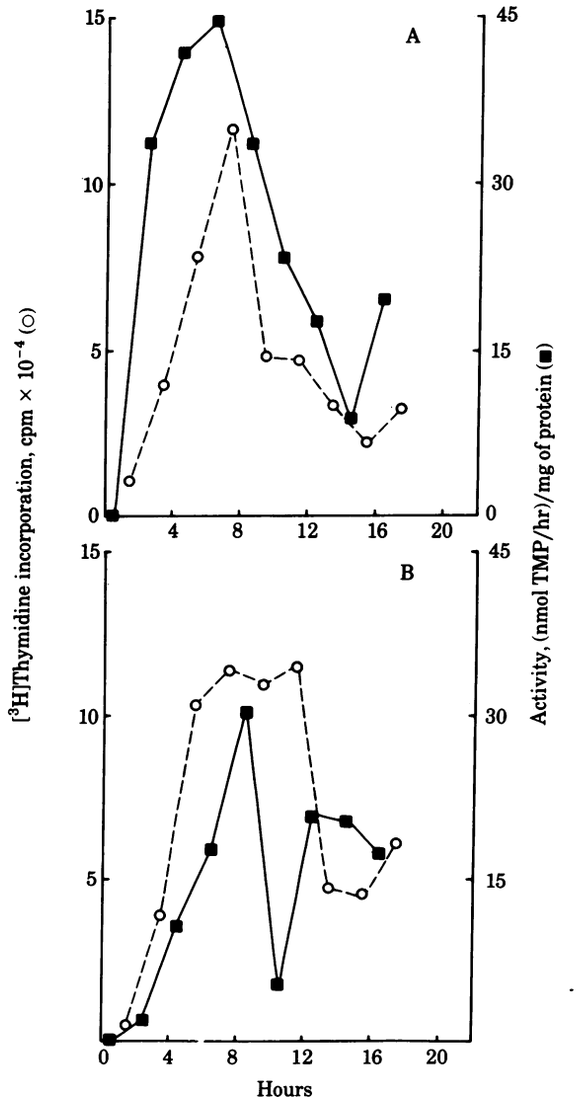

FIG. 2. tk activity in cells synchronized by mitotic selection. (*A*) L60 (normal tk^+ mouse), (*B*) $LMTK^+$-C1 (human).

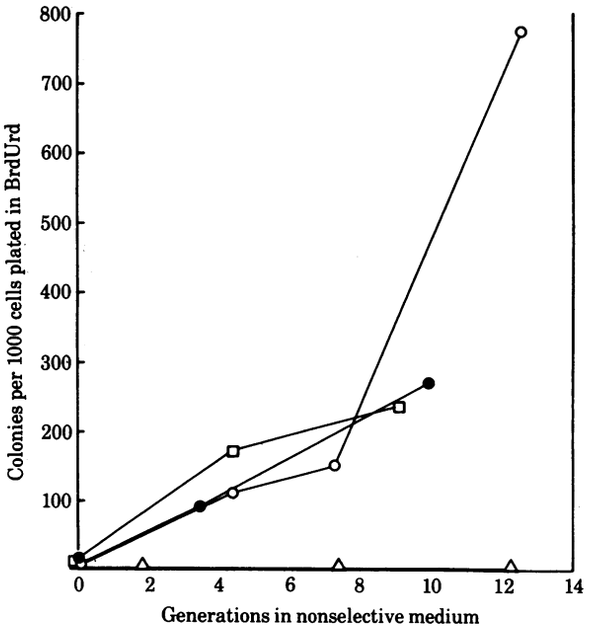

FIG. 3. Stability of tk^+ phenotype of transformed mouse cell lines. ○, L3E1 (rat); ●, L1A1 (hamster); □, $LMTK^+$-A2 (human); △, $LMTK^+$-B4 (human).

many of our transformants are unstable and readily yield tk^- segregants. Therefore, it is unlikely that any of our transformants are revertants of the mouse tk gene. Nonetheless, we did attempt to verify that the tk activity in the transformants was not of mouse origin.

Polyacrylamide disc gel electrophoresis was used to verify the presence of human tk in the clones derived from human metaphase chromosome transfer (Fig. 4). The peaks of tk activity in extracts of clones $LMTK^+$-C1 and $LMTK^+$-A2 corresponded to the peak of tk activity in an extract of HeLa cells and were clearly different from the tk peak in an extract of the tk^+ mouse line L60. In a mixture of HeLa and L60 extracts, the two enzymes could also be clearly resolved (results not shown).

The tk activity of transformants derived from rat or hamster genomic DNA (L3E1 and L1A1, respectively) could not be verified by electrophoresis to be of nonmouse origin, because the rodent enzymes are not separable. We attempted to make the distinction by measuring the stability of the enzymes to heating at 65°C. In both cases, the activity from the transformants was more stable than the activity in wild-type mouse cells, but these results were not definitive. The instability of these two clones under nonselective conditions (see Fig. 3) provides the strongest evidence that transformation by rat or hamster DNA has taken place. There have been no reports of the original tk^- mouse cells reverting to the tk^+ phenotype.

DISCUSSION

Our major conclusion is that the regulation of expression of transgenomic tk in transformed mouse cells appears to be normal. In a synchronized cell population, there is little or no enzyme activity present in cells in the G_1 phase of the cell cycle and a sharp induction of enzyme activity at the beginning of the S phase. There are variations in the amount of enzyme produced and in the exact timing of its appearance relative to the onset of DNA synthesis, but the overall pattern of normal regulation of the enzyme is intact. This is true in cells synchronized by serum starvation or by mitotic selection and is independent of whether the transgenome is in a stable or an unstable association with the mouse genome. These results suggest that the regulatory apparatus for the expression of cellular tk is intact in the transformants. Any necessary regulatory DNA sequences that may be linked to the structural gene are present on the transgenome, and the signals produced by the mouse cells are recognized and functional for control of the tk genes from human, rat, or hamster sources.

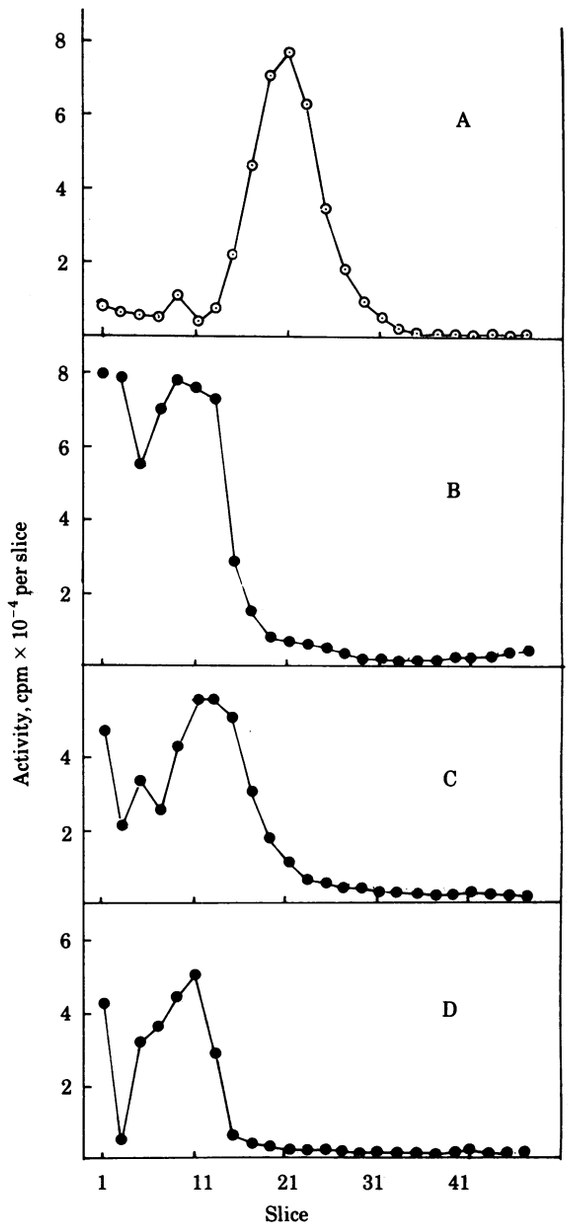

FIG. 4. Polyacrylamide disc gel electrophoresis of tk from mouse cells transformed with human metaphase chromosomes. (*A*) Activity from a wild-type mouse cell line (L60); (*B*) activity from human HeLa cells; (*C* and *D*) activities from transformed lines $LMTK^+$-A2 and $LMTK^+$-C1, respectively.

Clones of transformed mouse cells produced by DNA transfer with the plasmid pBR322 tk that carries the herpes virus tk gene are an exception to the general observation of normal cell cycle regulation of tk (15). Expression of tk in these clones is constitutive, with no significant variation in enzyme activity during the cell cycle. This is not unexpected for the herpes tk gene, which is under different regulatory control from cellular tk in its usual context in an infected cell. Therefore, one might

expect that the herpes virus tk gene would not respond to the mouse cell regulatory signals in the same way that another cellular tk gene would respond. These results are consistent with those reported by Lin and Munyon (20), who studied the expression of thymidine kinase activity in normal and herpes simplex virus-transformed L cells. They also found that the viral gene was not placed under the control of the cellular mechanism.

Although these experiments suggest that the regulation of tk in transformants is similar to that in normal tk^+ mouse cells, they do not indicate how that regulation is achieved. We have shown with the cell lines used here that protein synthesis takes place normally throughout the long G_1 period and that the appearance of tk activity is not due to a change in the rate of overall protein synthesis. Previous work with synchronized Chinese hamster cells and various metabolic inhibitors showed that mRNA synthesis just before the appearance of tk activity is required for normal induction of tk (21). Some preliminary experiments with the mouse transformants and the inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (22) suggest that new mRNA synthesis during the G_1 phase is necessary for induction of tk in the transformants (unpublished observations), but detailed information about the mechanism for the regulation of thymidine kinase is not yet available. The demonstration that the regulation of the gene is intact on transgenomic pieces of DNA should aid further investigations into these mechanisms.

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1. McBride, O. W. & Ozer, H. L. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 1258–1262.
2. Willecke, K. & Ruddle, F. H. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1792–1796.
3. McBride, O. W., Burch, J. W. & Ruddle, F. H. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 914–918.
4. Wigler, M., Pellicer, A., Silverstein, S. & Axel, R. (1978) *Cell* **14**, 725–731.
5. Wigler, M., Sweet, R., Sim, G. K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S. & Axel, R. (1978) *Cell* **16**, 777–785.
6. Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G. & Chasin, L. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1373–1376.
7. Lewis, W. H., Srinivasan, F. R., Stokoc, N. & Siminovitch, L. (1980) *Somatic Cell Genet.* **6**, 333–347.
8. Willecke, K., Klomfass, M., Mirau, R. & Dohmer, J. (1979) *Mol. Gen. Genet.* **170**, 179–185.
9. Lester, S. C., LeVan, S. K., Steglich, C. & DeMars, R. (1980) *Somatic Cell Genet.* **6**, 241–256.
10. Klobutcher, L. A. & Ruddle, F. H. (1979) *Nature (London)* **280**, 657–660.
11. Klobutcher, L. A., Miller, C. L. & Ruddle, F. H. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3610–3614.
12. Brent, T. P., Butler, J. A. V. & Crathorn, A. R. (1965) *Nature (London)* **207**, 176–177.
13. Stubblefield, R. & Murphree, S. (1967) *Exp. Cell Res.* **48**, 652–656.
14. Kit, S. & Jorgenson, G. H. (1976) *J. Cell. Physiol.* **88**, 57–64.
15. Enquist, L. W., Vande Woude, G. F., Wagner, M., Smiley, J. R. & Summers, W. C. (1979) *Gene* **7**, 335–342.
16. Scheffler, I. E. & Buttin, G. (1973) *J. Cell. Physiol.* **81**, 199–216.
17. Ihler, G. & Rupp, W. D. (1969) *Proc. Natl. Acad. Sci. USA* **66**, 138–143.
18. Kit, S., Leung, W.-C. & Trkula, D. (1973) *Arch. Biochem. Biophys.* **158**, 503–513.
19. Willecke, K., Reber, T., Kucherlapati, R. S. & Ruddle, F. H. (1977) *Somatic Cell Genet.* **3**, 237–245.
20. Lin, S.-S. & Munyon, W. (1974) *J. Virol.* **14**, 1199–1208.
21. Landy-Otsuka, F. & Scheffler, I. E. (1980) *J. Cell. Physiol.*, in press.
22. Schgal, P. B., Derma, E., Molloy, G. R., Tamin, I. & Darnell, J. E. (1976) *Science* **295**, 431–433.