Proc. Nat. Acad. Sci., USA
Vol. 71, No. 1, pp. 220–224, January 1974

Transformation of Cultured Mammalian Cells by Viable Herpes Simplex Virus Subtypes 1 and 2

(carcinoma/Rous sarcoma virus)

B. GARFINKLE AND B. R. McAUSLAN

Roche Institute of Molecular Biology, Nutley, New Jersey 07110

Communicated by Renato Dulbecco, September 4, 1973

ABSTRACT **A continuous line of rodent cells carrying the genome of the Rous sarcoma virus was stably transformed by exposure to viable herpes simplex virus of either subtype 1 or 2. Transformation was accompanied by alteration of morphology, growth characteristics, acquisition of a thymidine kinase activity (EC 2.7.1.21) resembling the corresponding specific activity of the enzyme in the herpes simplex virus, and capacity to express continuously some antigens specific for herpes simplex virus.**

A considerable body of evidence implicates herpesvirus as the etiological agent of certain forms of cancer (1, 2). Of particular current interest is the possible link between human cervical carcinoma and herpes simplex virus (HSV) subtype 2, but the evidence to date is largely circumstantial. Cells transformed in culture by HSV might provide a valuable system to explore the oncogenic potential of this virus. Biochemical transformation of thymidine-kinase-deficient (TK$^-$) mouse cells to the thymidine-kinase-positive phenotype has in fact been achieved using ultra-violet inactivated HSV (3). The molecular basis of the mode of selection of transformants and of the acquisition of new kinase function has yet to be rigorously established, although the available evidence is consistent with the conclusion that the cells stably inherit the capacity to synthesize thymidine kinase (TK) that is specific for HSV.

To determine which biochemical properties of HSV most closely reflect its putative oncogenic function, it would be useful to have a cell system which could be transformed by viable virus or conditional lethal mutants thereof. We describe here the transformation of cells by HSV, subtype 1 or 2, that had not been subjected to prior ultraviolet irradiation. These transformed cells carry new information for expression of viral-like TK and HSV-specific antigens. The basis of the transformation procedure was to use cells in which HSV infection was nonproductive and noncytopathic apparently as a result of prior modification of cells by an RNA tumor virus (4).

Abbreviations: HSV, herpes simplex virus; TK$^-$, thymidine-kinase deficient; TK, thymidine-kinase; XC, rat cells; F-11, Eagle's minimal essential medium; HAT, F-11 with 10% fetal calf serum, 5 mM glutamine, 0.0006 mM aminopterin, 0.05 mM quanosine, 0.05 mM adenosine, 0.016 mM thymidine, and 0.1 mM glycine; XCTK$^-$, TK$^-$, BrdU-resistant variants of XC cells; HSV$_1$, HSV subtype 1; HSV$_2$, HSV subtype 2; PBS, phosphate-buffered saline; XCHSV$_1$, HSV$_1$-transformed cells; XCHSV$_2$, HSV$_2$-transformed cells.

MATERIALS AND METHODS

Cells and Virus. XC cells (5, 6) were grown in Eagle's minimal essential medium (F-11), obtained from Grand Island Biological Co., New York, supplemented with 10% fetal-calf serum, 5 mM glutamine, and nonessential amino acids. Transformed cells were grown in a medium which consists of F-11 supplemented with 10% fetal-calf serum, 5 mM glutamine, 0.0006 mM aminopterin, 0.05 mM guanosine, 0.05 mM adenosine, 0.016 mM thymidine, and 0.1 mM glycine (HAT).

TK$^-$ cells were selected by repeated passage of cells in F-11 supplemented as above but containing 5-bromodeoxyuridine (BrdU) (Sigma Chemical Co., St. Louis, Mo.) (15). Initially a concentration of 15 μg of BrdU per ml was employed and later 50 μg/ml. Clones were selected that were resistant to this latter concentration. One such clone, a TK$^-$, BrdU-resistant variant of XC cells (XCTK$^-$), is now normally passaged in the presence of 50 μg/ml of BrdU but can tolerate much higher levels. XCTK$^-$ cells fail to grow in HAT and lack the major kinase activity found in XC cells. However, both XC and XCTK$^-$ cells carry the group-specific antigen (gs) for Rous sarcoma virus.

The macroplaque strains of HSV subtype 1 (HSV$_1$) (courtesy of Dr. B. Roizman) or a primary isolate of HSV subtype 2 (HSV$_2$) were used throughout the study. Virus was grown in primary rabbit-kidney cells at 34°. Nonpurified virus from disrupted, infected cells (usually 100 million plaque-forming units/ml) was stored at −196°.

Enzyme. Cells were assayed for TK by the method of Munyon *et al.* (3). Electrophoretic migration of TK was determined by the method of Munyon *et al.* (7). Heat lability of TK was determined by the method of Ogino and Rapp (8).

Antibody. Antiserum to HSV$_1$ or to HSV$_2$ was produced by inoculating New Zealand white rabbits (4 = kg weight) subcutaneously with disrupted, virus-infected, rabbit-kidney cells in Freund's complete adjuvant. (These rabbits were of the same allotype as the rabbit-kidney cells used to grow virus.) Inoculations were carried out twice weekly for 4 weeks. The rabbits were then rested 2 weeks, boosted with an additional inoculation, and bled once each week beginning 1 week after the booster injection.

Antiserum to rabbit-γ-globulin was prepared by inoculating goats with purified rabbit-γ-globulin (Miles Laboratories, St. Louis, Mo.). Goats were inoculated twice intradermally

with rabbit-γ-globulin in Freund's complete adjuvant, allowing 4 weeks between each inoculation. Goats were rested 2 weeks after the final inoculation and then bled.

The γ-globulin fractions were prepared by ammonium sulphate precipitation and conjugated with fluorescein isothiocyanate according to published procedures (9). Prior to use, the conjugated fractions were absorbed twice with pulverized, dehydrated mouse-liver (1 hr, 4°) and once with uninfected XC cells (12 hr, 4°).

Fluorescent antibody technique

(*a*) Membrane fluorescence (10) was assayed directly using conjugated rabbit antiserum to HSV_1 or HSV_2 absorbed as described above. Infected cells were pelleted (500 × g, 5 min, 4°), washed twice in phosphate-buffered saline (PBS), and incubated (37°, 60 min) with the fluorescein conjugate. Following incubation, cells were washed twice with PBS, resuspended in an appropriate volume of the same buffer, and examined with a Zeiss microscope equipped for fluorescence studies.

(*b*) For fixed cell, indirect immunofluorescence (9), cells growing on coverslips were washed three times in PBS (4°) and air dried (37°, 30 min). The cultures were then fixed in acetone (3 min, 4°). The cultures were rehydrated with PBS, antiserum to HSV_1 or HSV_2 was added, the coverslips were incubated (37°, 60 min), the cultures were then washed three times with PBS, and the conjugated antiserum to rabbit-γ-globulin was added (37°, 60 min). Unreacted conjugate was removed by washing three times with PBS, and the cultures were mounted, using 1 part Tris buffered saline and 9 parts glycerol (9), for fluorescence examination.

Immunodiffusion. Cells about 50 million were harvested and washed twice with PBS. Cell pellets were resuspended in 0.2 ml of PBS and disrupted in an MSE sonicator (45 sec, full power, 0°). The disrupted cells were then applied to the wells in the diffusion plates (Cordis Labs, Miami, Fla.), and the plates were incubated for several days at 37°.

Saturation Density. Saturation densities were determined by seeding 60-mm petri dishes with 100,000 cells and allowing the cells to replicate at 37°. Every 24 hr duplicate cultures were harvested with trypsin-EDTA and the cells enumerated with a haemocytometer. When the cell number reached maximal values, the saturation density was calculated.

Growth in Semisolid Agar. Growth in semisolid agar was determined by the method of Macpherson and Montagnier (12). The average diameter of cell colonies was determined after 7 days by means of a calibrated ocular micrometer.

RESULTS

Selection of Cells for Transformation. Exposure of XC cells to either HSV_1 or HSV_2 results in a nonproductive infection in which some HSV functions, including induction of TK, are expressed only transiently, but the cells remain viable and continue to multiply (4). It was thought that at least some infected cells might become stably transformed by HSV, and so a system was chosen to select such transformants.

$XCTK^-$ cells were selected (*Materials and Methods*) and infected with either HSV_1 or HSV_2; F-11 was replaced with HAT 12 hr after infection. Colonies that survived and grew—the average yield per culture was routinely 0.1%—were isolated and subsequently maintained as lines by subculturing

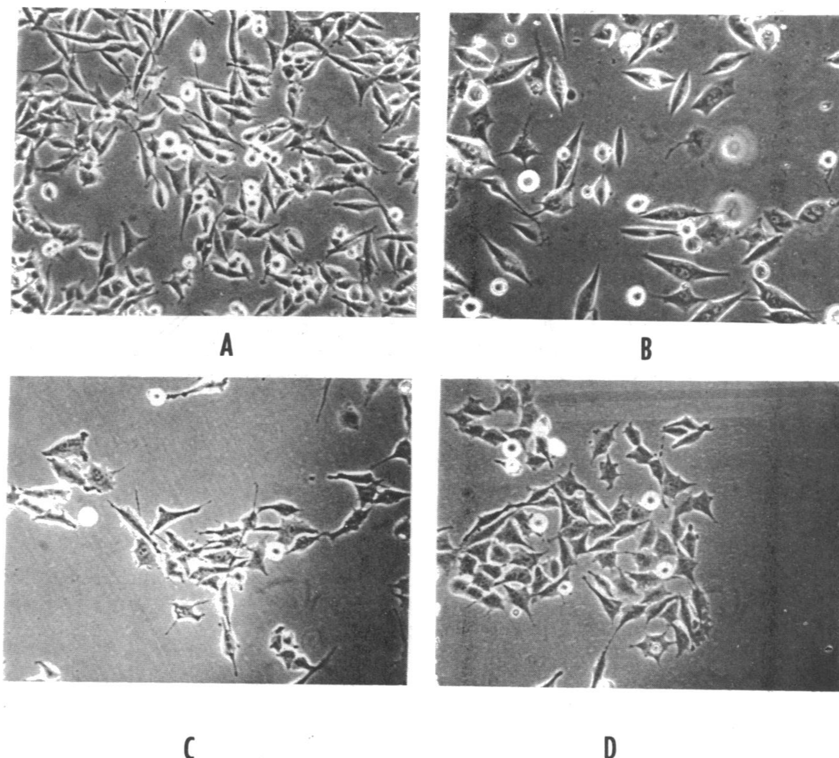

FIG. 1. General morphology of cells. Cells were seeded lightly into plastic culture dishes and after 3 days photographed under phase contrast. (A) XC cells. (B) $XCTK^-$ transformed by HSV_2 ($XCHSV_2$). (C) $XCTK^-$ cells. (D) $XCTK^-$ transformed by HSV_1 ($XCHSV_1$).

in HAT. These "transformed" lines are now in their 25th passage and show no sign of spontaneous formation of HSV. A line each of HSV_1-transformed cells ($XCHSV_1$) or of HSV_2-transformed cells ($XCHSV_2$) will be described.

Properties of HSV-Transformed Cells. Photographs of XC, $XCTK^-$, and the corresponding transformed lines are presented for comparison of general morphology (Fig. 1). Typically, $XCHSV_2$ cells appear considerably larger than XC or $XCTK^-$ cells and are more uniformly spindle shaped. When dispersed and subcultured in soft agar (12), the HSV-transformed cells grew as colonies (Table 1) whereas $XCTK^-$ colonies were so small as to be barely visible under the same conditions. When seeded onto $XCTK^-$ monolayers, the transformed lines and the parental XC grew as prominent colonies whereas, the $XCTK^-$ cells grew poorly under such conditions. The saturation densities of the HSV-transformed lines are significantly higher than of $XCTK^-$ cells (Table 1). However, the saturation densities of the HSV-transformed lines are about the same as for the original XC lines.

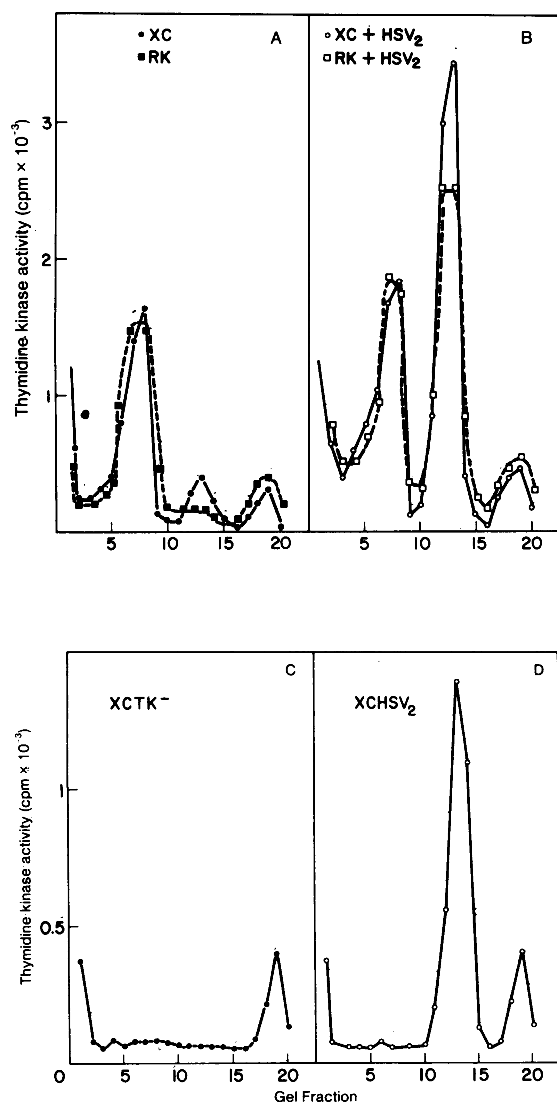

FIG. 2. Thymidine kinase activity. Aliquots of cell extracts containing approximately 100 μg of protein were applied to 5% acrylamide gels and electrophoresed (2 V/cm, 90 min, 4°) according to published procedures (7). Gels were mechanically fractionated into 2.5-mm fractions and each slide was assayed for TK activity. (*A*) TK activity from XC cells (●) and rabbit-kidney cells (RK) (■). (*B*) TK activity from XC cells infected with HSV_2 (○) and RK cells infected with HSV_2 (□). Similar results were obtained with either cell infected with HSV_1. (*C*) TK activity from $XCTK^-$ cells. (*D*) TK activity from XC cells transformed by HSV_2. Identical results were obtained with XC cells transformed by HSV_1. (The numbers on the ordinates have been multiplied by 10^{-3}.)

Acquisition of TK Activity. The basis for the selection of transformed cells on HAT is that those cells in which the information for HSV-specific TK synthesis is stably incorporated will be able to utilize thymidine and multiply, whereas the $XCTK^-$ cells will not (13). Alternative pathways that would enable cells to bypass the aminopterin block are conceivable, so we tested the transformed lines for TK activity and determined the migration rate of TK activities by gel electrophoresis as described by Munyon *et al.* (7). Total TK activity in extracts of HSV_1 or HSV_2-transformed cells was usually 6–15 times greater than that in extracts of $XCTK^-$ cells. The low TK activity of the $XCTK^-$ cells probably corresponds to mitochondrial TK (14). The major TK activity of the tranformed lines migrated in gels with an R_F approximately that of the corresponding activity induced in cells nonproductively infected (Table 1 and Fig. 2) or productively infected by HSV_1 or HSV_2 (cf. ref. 7).

Heat Lability of TK Activity in HSV-Transformed Cells. HSV_2-induced TK activity has been reported to be more heat labile than HSV_1-induced TK activity (8); we have confirmed this (Fig. 3). We compared also the heat lability of TK activity in extracts of XC, $XCTK^-$, $XCHSV_1$, and $XCHSV_2$ cells. We found that the heat lability of TK activity in extracts of transformed cells corresponded to that of the activity elicited by the corresponding HSV subtype in nonproductively or productively infected cells (Fig. 3, ref. 8). Together with the electrophoretic studies, these data suggest that the TK activity of HSV-transformed cells is HSV-specific.

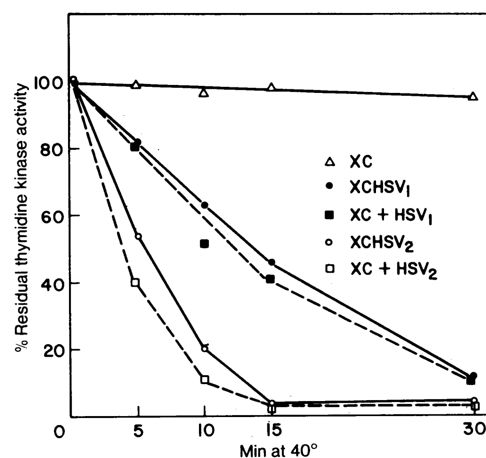

FIG. 3. Residual TK activity after heat inactivation. Cell extracts (500 μg of protein per ml) were incubated at 40° and aliquots were removed after 5, 10, 15, and 30 min and placed in an ice bath. The extracts were then assayed for TK activity. The heat lability of TK induced in rabbit-kidney cells infected with herpes virus (a permissive system) was practically identical to the corresponding activity induced in XC cells.

TABLE 1. *Characteristics of HSV-transformed XC cells*

Cell type	Colony Size in soft agar (mm)	Saturation density (cells·cm^{-2})†	RF of the major TdR* kinase activity	Colony growth on XCTK$^-$
XC	0.2	5.4×10^5	0.25	+++
XCTK$^-$	0.02	2.2×10^5	None	+
XCHSV$_2$*	0.80	4.0×10^5	0.45	++++
XCHSV$_1$*	0.77	4.0×10^5	0.45	++++

* All cells show a small peak of kinase activity with an RF of about 0.75. This probably corresponds to the mitochondrial activity (14).

† The values of XCHSV$_1$ and XCHSV$_2$ are probably underestimated as the cells tended to float off the medium after forming multilayers.

Expression of HSV antigens by transformed cells

(*a*) *Immunofluorescence:* The presence of HSV-specific antigens in transformed cells was detected by either direct immunofluorescence assay of unfixed cells for HSV-specific, membrane-associated antigen as described recently (10) or by indirect immunofluorescence assay using fixed cells (*Materials and Methods*).

The percentage of cells clearly exhibiting intense positive fluorescence was estimated using the direct assay with unfixed cells (Table 2). These values probably underestimate the number of cells actually synthesizing HSV antigens since, in addition, some of the transformants exhibited a "beaded" membrane fluorescence around the cell periphery (Table 2) similar to that described by others (10). These values remained constant with continued subculture. When samples of transformed cells were first fixed and then examined for antigens by the indirect assay, fluorescence was detected only in the cytoplasm of either XCHSV$_1$ or XCHSV$_2$ cells (Fig. 4).

(*b*) *Immunodiffusion:* HSV-specific antigens in extracts of HSV-transformed cells were detected by immunodiffusion in thin-layer gels (11). About seven distinct immunoprecipitin lines could be detected in extracts of productively infected rabbit-kidney cells but only two diffuse lines were detectable in HSV$_1$-transformed or HSV$_2$-transformed cells (Fig. 5).

DISCUSSION

Although XC cells are in one sense already transformed by an RNA virus, they have certain characteristics that make them suitable for transformation by HSV; they allow only partial expression of HSV functions, their growth is not impaired by

TABLE 2. *Immunofluorescence of HSV-transformed cells*

Cell type	Fluorescent (%)	"Beaded" fluorescent (%)
XC	0	0
XCTK$^-$	0	0
XCHSV$_2$	16	12
XCHSV$_1$	12	12

For each cell type, five fields each with approximately 200 cells were viewed.

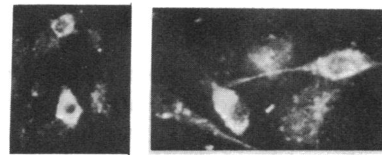

FIG. 4. Immunofluorescence of HSV$_2$-transformed XC cells. XC cells transformed by HSV$_2$ were fixed and examined for HSV$_2$-specific antigens by indirect immunofluorescence. Fluorescence was detected only in the cytoplasm.

HSV infection, and TK-deficient variants retaining the RSV group-specific antigen can be readily selected.

Cell lines arising from XCTK$^-$ cells nonproductively infected with HSV$_1$ or HSV$_2$ are capable of continued growth in HAT and appeared to be stably transformed by HSV according to the following criteria.

(*a*) The cells had acquired TK activity with some characteristics of that activity elicited by HSV during productive infection (Figs. 2 and 3). Presumably this is the means by which the HSV infected cells survive or grow in HAT.

(*b*) The growth characteristics and morphology of the HSV-transformed lines were different from those of the parental XCTK$^-$ cells. For example, XCHSV$_1$ or XCHSV$_2$ grew as colonies in soft agar but XCTK$^-$ did not. They also grew profusely as multilayers when seeded on XCTK$^-$ monolayers and reached saturation densities at least twice that of XCTK$^-$.

(*c*) The transformed cells expressed at least some HSV antigens detectable by immunofluorescence and immunodiffusion. The antigens detectable by fluorescence assay were located exclusively in the cytoplasm and peripheral membrane.

Since some characteristics (electrophoretic mobility and heat lability) of the major TK activity in extracts of HSV-transformed cells correspond to those of HSV-elicited TK in productively or nonproductively infected cells (cf, ref. 7), it is likely that synthesis of TK is due to the continued presence of the HSV genome. However, we have always been able to detect a very small peak of TK activity in XC cells (or in uninfected rabbit-kidney cells) corresponding in R_F to that elicited by HSV infection (see Fig. 2). Therefore, more detailed studies will be necessary to establish that the TK activity elicited by HSV is entirely coded by HSV rather than

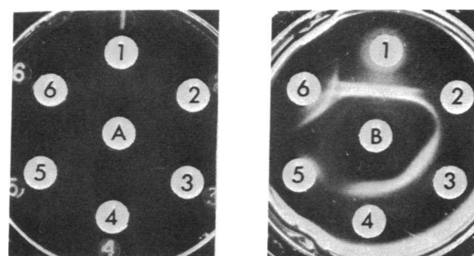

FIG. 5. Immunodiffusion tests. Cell extracts were prepared as described in *Materials and Methods*. Samples (50-μl) were placed in the wells of this layer immunodiffusion plates and incubated at 37° for 4 days. (*A*) Control. Center well contained serum from nonimmunized rabbit. Test well: *1*, HSV$_1$-infected rabbit-kidney cells; *2*, XC cells; *3*, HSV$_1$-transformed XCTK$^-$ cells; *4*, HSV$_2$-transformed XCTK$^-$ cells; *5*, XCTK$^-$ cells; *6*, HSV$_2$-infected rabbit-kidney cells. (*B*) Experimental. Center well contained rabbit antiserum to HSV$_1$. Contents of test wells same as for *A*.

representing a viral modification or derepression of a minor host activity.

We have not been able to detect spontaneous release of viable HSV from the transformed lines by infectivity assays. The absence of HSV antigens detectable by immunofluorescence in the cell nuclei and the absence of some major HSV antigens detectable by immunodiffusion supports this findings. While it is possible that all HSV antigens are expressed in the transformed cells but in amounts too low to be detected, our data suggest that the herpesvirus genome is only partially expressed in $XCHSV_1$ or $XCHSV_2$ cells. The percentage of cells in the lines described or in subclones of these HSV antigens detectable by immunofluorescence remained constant with repeated passage.

It would of course be of great interest to determine the nature of the association between the HSV and host genomes in these transformed lines and to use conditional lethal mutants of HSV to determine the minimum set of functions necessary to achieve transformation. However, pertinent to the more general question of tumor formation is whether the presence of an integrated RNA virus genome in a cell is the key requirement for that cell to be transformable by herpesvirus (see ref. 4).

The expert assistance of Donna Testa contributed greatly to this project. XC cells were generously provided by Dr. Ruy Soeiro. We thank Dr. H. Hanafusa for assaying the Rous sarcoma virus gs antigen in XC and $XCTK^-$ cells. B.G. is a recipient of a Roche Postdoctoral Research Fellowship.

1. Klein, G. (1972) "Herpesviruses and oncogenesis," *Proc. Nat. Acad. Sci. USA* **69**, 1056–1064.
2. Aurelian, L., Standboy, J., Melendez, L. & Johnson, L. (1971) "Herpesvirus type 2 isolated from cervical tumor cells grown in tissue culture," *Science* **174**, 704–707.
3. Munyon, W., Kraiselburd, E., Davis, D. & Mann, J. (1971) "Transfer of thymidine kinase to thymidine kinaseless L cells by infection with ultraviolet-irradiated Herpes simplex virus," *J. Virol.* **7**, 813–820.
4. Garfinkle, B. & McAuslan, B. (1973) "Non-cytopathic, non-productive infection by Herpes simplex viruses types I & II," *Intervirology*, in press.
5. Svoboda, J. (1963) "Presence of chicken tumor virus in the sarcoma of the adult rat inoculated after birth with Rous sarcoma tissue," *Nature* **186**, 980–981.
6. Klement, V., Row, W. P., Hartley, J. W. & Pugh, W. E. (1969) "Mixed culture cytopathogenicity: A new test for growth of murine leukemia viruses in tissue culture," *Proc. Nat. Acad. Sci. USA* **63**, 753–757.
7. Munyon, W., Buchsbaum, R., Paoletti, E., Mann, J., Kraiselburd, E. & Davis, D. (1972) "Electrophoresis of thymidine kinase activity synthesized by cells transformed by Herpes simplex virus," *Virology* **49**, 683–689.
8. Ogino, T. & Rapp, F. (1969) "Differences in thermal stability of deoxythymidine kinase activity in extracts from cell infected with Herpes simplex virus type 1 or type 2," *Virology* **46**, 953–955.
9. Vogt, P. (1969) "Immunofluorescent detection of viral antigens" in *Fundamental Techniques in Virology*, eds. Habel, K. & Salzman, N. (Academic Press, New York), pp. 316–326.
10. Nahmias, A. J., Del Buono, I., Scheweiss, K. E., Gordon, D. S. & Thies, D. (1971) "Type specific surface antigens of cells infected with Herpes simplex virus (1 and 2)," *Proc. Soc. Exp. Biol. Med.* **138**, 21–27.
11. Wadsworth, C. (1957) "A slide technique for the analysis of immune precipitates in gel," *Int. Arch. Allerg. Appl. Immunol.* **10**, 355–360.
12. Macpherson, I. & Montagnier, L. (1964) "Agar suspension culture for the selective assay of cells transformed by Polyoma virus," *Virology* **23**, 291–294.
13. Littlefield, J. (1963) "The inosinic acid pyrophosphorylase activity of mouse fibroblasts partially resistant to 8-azoguanine," *Proc. Nat. Acad. Sci. USA* **50**, 568–573.
14. Attardi, B. & Attardi, G. (1972) "Persistence of thymidine kinase activity in mitochondria of a thymidine kinase-deficient derivative of mouse L cells," *Proc. Nat. Acad. Sci. USA* **69**, 2874–2878.
15. Kit, S., Dubbs, D., Piekarski, L. & Hsu, T. (1963) "Detection of thymidine kinase activity from L-cells resistant to Bromodeoxyuridine," *Exp. Cell Res.* **31**, 297–312.