Transformation of Rat Embryo Cells by Temperature-sensitive Mutants of Herpes Simplex Virus

By JOAN C. M. MACNAB

Medical Research Council Virology Unit, Institute of Virology,
University of Glasgow, Glasgow, G11 5JR

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SUMMARY

Rat embryo cells have been transformed by temperature-sensitive mutants of herpes simplex type 2 and by wild-type virus of herpes simplex types 1 and 2. Transformation was scored using the morphological criterion of focus formation and the cell changes that lead to the final focus are described.

Herpes specified antigens can be detected in at least 50% of these transformed rat embryo cells by immunofluorescence studies on unfixed material.

Transformation experiments using hamster embryo cells and HSV-2 strain 333 are also described. These transformed cells are shown to be tumourigenic in newborn hamsters. Tumour cells in turn, as a single cell suspension, produce fresh tumours in animals up to 2 weeks old with almost 100% efficiency. Tumour cells also continue to retain the herpes specific antigens.

INTRODUCTION

Herpes simplex virus (HSV) has long been known to be able to exist in man in a latent state from which it can recurrently be activated to produce overt lesions from which infectious virus may be isolated. Recently Duff & Rapp (1970) reported that HSV type 2 virus, inactivated by u.v. radiation, had the potentiality to transform primary hamster embryo cells in vitro and that these transformed cells were oncogenic in hamsters. HSV-1 virus, similarly u.v. irradiated, was also reported to transform hamster cells (Duff & Rapp, 1973). Herpes virus genetic material has also been incorporated in L cells lacking thymidine kinase activity. These cells cannot grow in a selective medium which, by shutting off the de novo pathway of thymidilate synthesis, makes them dependent on metabolizing preformed thymidine. However, if such L cells are first infected by u.v. irradiated HSV type 1, then some clones can be isolated from the selective medium which can grow (Munyon et al. 1971). Davidson, Adelstein & Oxman (1973) have confirmed Munyon's findings and B. R. McAuslan & B. Garfinkle (personal communication), have also succeeded by using a line of cell not permissive to HSV-1 and HSV-2 and lacking thymidine kinase. A. Jamieson and J. C. M. Macnab (personal communication) have confirmed the LTK- finding and have demonstrated that both the new thymidine kinase and deoxycytidine kinase activities in the 'HSV transformed' L cells are herpes virus specific.

Darai & Munk (1973) have found that they can transform human embryo lung cells with HSV by incubating the infected cells at an elevated temperature which blocks the virus replication.

The present work confirms the transforming potential of u.v. inactivated HSV-2 in primary
hamster embryo cells and describes the successful transformation of rat embryo cells by the wild-type and several temperature-sensitive (ts) mutants of HSV-2 strain HSG-52, by HSV-1 strain 17 syn+, and by HSV-2 strain 333. The ts mutant transformation of rat embryo cells has been achieved without u.v. inactivation of the virus. Replication of the virus was prevented by keeping the ts mutant infected cultures at the non-permissive temperature of 38 °C for the initial period of the experiment.

METHODS

Viruses. HSV-1 strain 17 syn+ (Brown, Ritchie & Subak-Sharpe, 1973) was grown and titrated in BHK C13 cells. HSV-2 strain 333 (Duff & Rapp, 1971) was grown and titrated only in human embryo kidney or human embryo lung cells.

Wild-type virus and 13 ts mutants of HSV-2 strain HSG 52 were grown and titrated in BHK C13 cells. These mutants had been isolated (Timbury, 1971) and characterized in this laboratory (Halliburton & Timbury, 1973).

Cells. Rat embryo primary cells were prepared from approx. 20-day-old embryos (from especially inbred colonies of Buffalo or Hooded Lister rats which are being maintained in this Institute). Animals from a single litter were washed in phosphate-buffered saline (PBS), eviscerated, minced finely, washed again in PBS and finally dispersed into a single cell suspension by trypsinization (0.25 % Difco trypsin) at 37 °C. After sedimentation the cell pellet was resuspended in Eagle’s medium supplemented by 10 % (v/v) calf serum and 10 % (v/v) tryptose phosphate broth. Cells were grown in rotating Winchester bottles at a concentration of 4 x 10^8 cells per bottle.

Hamster embryo primary cells were similarly prepared from a non-inbred stock of Syrian hamsters by eviscerating approximately 15-day-old embryos.

BHK C13 cells (McPherson & Stoker, 1962) were used to prepare and titrate virus stocks except HSV-2 strain 333.

Human embryo kidney and human embryo lung cells were prepared from tissue of aborted normal foetuses. These cells were used for growing and titrating of HSV-2 strain 333, which has been maintained exclusively in human tissue.

Media. For transformation assays Eagle’s (Glasgow modification) growth medium was supplemented (v/v) by 10 % calf serum (Biocult) and 10 % tryptose phosphate broth. Eagle’s medium supplemented by 5 % (v/v) calf serum was used to titrate virus. One hundred μg/ml streptomycin, 100 units/ml penicillin and 0.2 μg/ml of n-butyl p-hydroxy benzoate were added to Eagle’s growth medium.

Phosphate-buffered saline (Dulbecco & Vogt, 1954) was used as a virus diluent and for washing the cell monolayers.

Immunofluorescence studies. Fluorescent antibody anti-rabbit immunoglobulin (sheep) was obtained from Wellcome Research Laboratories. Antiserum made against ts 1 by inoculation of RK13 cells infected with ts 1 at non-permissive temperature into rabbits was used. (This antiserum was prepared in this laboratory by Dr M. C. Timbury.) Heterologous antiserum (anti-rabbit) was obtained from Ital diagnostics.

U.v. inactivation of virus. High-titre stocks of HSV-1 strain 17 syn+ (2 x 10^9 p.f.u./ml in BHK C13), HSV-2 strain 333 (5 x 10^8 p.f.u./ml in human embryo lung) and HSV-2 strain HSG 52 (4 x 10^8 p.f.u./ml in BHK C13) were prepared.

The virus after clarification was diluted 1/10 in PBS for HSV-2 and 1/100 for HSV-1 to avoid problems arising through u.v. absorption by serum or certain amino acids.

0.6 ml of virus dilution was placed in a 25 mm plastic dish together with a small piece of
Table 1. Transformation of rat cells by HSV

<table>
<thead>
<tr>
<th>Virus</th>
<th>ts mutant</th>
<th>Transformation</th>
<th>DNA production at 38 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 strain 17 syn+</td>
<td><em>ts+</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HSV-2 strain 333</td>
<td><em>ts+</em></td>
<td>+</td>
<td>+*</td>
</tr>
<tr>
<td>HSV-2 strain HSG 52</td>
<td>ts+*</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>ts 1</td>
<td>+</td>
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<tr>
<td>ts 2</td>
<td>+</td>
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<td>ts 3</td>
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<td>ts 5</td>
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<tr>
<td>ts 6</td>
<td>+</td>
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<td>ts 7</td>
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<tr>
<td>ts 8</td>
<td>+</td>
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<td>ts 9</td>
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<td>ts 10</td>
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<tr>
<td>ts 11</td>
<td>+</td>
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<tr>
<td>ts 12</td>
<td>+</td>
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<tr>
<td>ts 13</td>
<td>+</td>
<td></td>
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</tr>
</tbody>
</table>

* Halliburton & Timbury (1973).
† Mechie (1974).

RESULTS

In rat embryo cells, positive transformation results were obtained from HSV-1 strain 17 syn+; HSV-2 strain 333; HSV-2 strain HSG 52 and without u.v. inactivation by HSV-2 strain HSG 52 ts mutants 1 and 7 (DNA negative at 38 °C) and 3, 4 and 5 (DNA positive at 38 °C). See Table 1.
Fig. 1. Transformed focus observed after 6 weeks. Rat embryo cells infected with HSV-2 strain HSG 52 ts 4. Unstained.

Fig. 2. Early initiation of focus of transformation in rat embryo cells by HSV-2 strain HSG 52 ts 4. Note several long large cells having banana-shaped appearance (arrowed). Unstained.
Fig. 3. Area at edge of focus between star-like arms of focus in Fig. 1 showing small epithelial (E) cells of focus and fibroblastic (F) normal monolayer. Unstained.

Fig. 4. Transformed focus as in Fig. 3, 4 weeks old, higher magnification showing long thick-walled cells (T) growing over monolayer cells (M) with epithelial cells (E) at focus centre. Unstained.
All the above viruses gave the same types of cellular changes which manifest the transformed focus in the rat embryo cultures (Fig. 1). Here the cell morphology change with HSV-1 was slightly less marked than with HSV-2 or its mutants. With HSV-2 the transformed focus appears to originate with one or two cells which become elongated and thick-walled and seem to bud smaller cells (Fig. 2). Frequently the focus takes up a star-like form and seems to invade the surrounding cell layer with the infiltrating arms of these stars (Fig. 1). Small epithelial cells were also found in the focus between the arms (Fig. 3). Some of the fibroblast-like cells seemed to fuse their cell walls and join together to form long rope-like structures and a few giant cells were also seen. The transformed focus split over the underlying primary or secondary cells and seemed to grow on top of them (Fig. 4). Foci became morphologically most pronounced if left to grow for up to 6 weeks (Fig. 1). Such foci were never seen in the numerous control cultures.

**Frequency of transformation in rat system**

The experiments to determine frequency of transformation were done in duplicate in Hooded Lister and Buffalo rat embryo cells on three separate occasions and using at least two different stocks of each virus. A mean of 2 to 3 transformed foci were seen per $3 \times 10^6$ cells; that is, a frequency of about 1 in $10^6$ cells. As the plates were split 2 days after infection into two new plates, this frequency estimate may be influenced by occasional division of a transformed or nascently transformed cell prior to the plate being split. The transformation frequency was, however, found to be consistent for different virus stocks and also for replicate experiments within a stock.

After focus formation some plates were split into two in order to establish if the focus formation is a heritable feature. After about 2 to 3 weeks transformed foci were again identified in the split plates but in much increased numbers. Up to 35 foci have been counted on plates after splitting. Thus the ability of the transformed cells to form foci appeared to be heritable.

Transformed lines were obtained in the rat embryo cell system by picking foci of morphologically transformed cells. The system was thus a restrictive one relying on morphology as the sole criterion of transformation. Foci were picked by removing and placing into a drop of 0.25% trypsin solution to disperse into a single cell suspension. They were thus not free of non-transformed cells. The transformed cell lines which subsequently form the monolayers may either be predominantly epithelial or fibroblastic in morphology. In some cases they continued to grow as epithelial or fibroblastic transformed cell lines but other cases gave rise on passage to mixed morphology transformed lines.

Hamster embryo fibroblast cultures were also used with HSV-2 strain 333 (obtained from Dr Rapp) to establish a system similar to that of Duff & Rapp (1971) as a comparison. The Syrian hamsters used were not of the same inbred strain as that used by Duff & Rapp (1971), and difficulty was experienced in picking out morphologically transformed foci from the monolayers made up of heterogeneous cell types. For this reason the infected cell sheets were passaged as whole cultures and long-lived lines of cells established from them. This system is thus non-restrictive.

In our transformation experiments, the rat as well as the hamster lines always went through a period of cell crisis as described for the latter by Duff & Rapp (1971). At crisis large numbers of cells in a culture showed a c.p.e. In the case of the transformed hamster embryo cells this crisis occurred at passage number 22, but in the case of rat embryo cells it occurred usually between the 6 and 8 passages though sometimes as late as passages 11 to 12. This
Rat embryo cell transformation by ts HSV

difference in time was probably because the rat lines were established from foci, while the
hamster lines arose from continued passage of all cells in the infected cultures. Considerable
efforts were made to establish whether an infectious virus was the cause of the cytopatho-
genicity. The affected cells were treated in a sonicator, and tested with a variety of cells
e.g. HeLa, BHK, C13, M.K., Vero, human embryo lung) in order to try to obtain evi-
dence of infectious virus, but none was isolated. Complement fixation tests were made on
cells in crisis, using herpes sera, but they all proved negative. No RNA virus was demon-
strated at crisis or at other times by overlaying with XC cells and looking for syncytial
foci. Assay for reverse transcriptase activity was negative. Negative staining on a pellet of
cells at crisis revealed no herpes particles and sections of cells also examined under the
electron microscope showed no herpes particles although particular attention was given
to the nucleus of the cell.

Several attempts were made to demonstrate infectious herpes virus by co-cultivating the
transformed lines with susceptible host cells. Lines of established transformed cells were so
tested and also transformed cells undergoing crisis phase. No infectious virus was detected
by the criterion of c.p.e. in our BHK C13 cells, which are known to be very sensitive to
infection with HSV-1 and HSV-2.

Following the crisis, stable long-lived cells were nearly always established, but their
morphology varied from one isolated rat embryo line to another. For example, rat embryo
cells transformed by mutant ts 7 have given rise to an epithelial line and also a fibroblastic
line. Further passage of these lines can lead to further changes in the lines morphology,
e.g. an epithelial cell line may become one of mixed morphology.

Tests for contamination by mycoplasma were negative in the rat transformed lines at
passage 24.

In each set of experiments non-infected control cultures were treated in exactly the same
way as the HSV infected cultures: the growth potential of controls usually petered out
after about 12 to 15 passages. Efforts to produce cell lines from control cells have been
occasionally successful and we isolated some lines which still grow quite well at passage
20. The growth of these cell lines is not as vigorous as that of comparable HSV trans-
formed lines. The ease with which such lines can be established is considerably less than
that of lines of HSV transformed cells. Of course, these cell lines do not contain herpes
antigens as detected by immunofluorescence while the HSV transformed cell lines do, nor
do they go through the crisis phase, specific for herpes transformed lines.

Herpes specified antigens

The transformed cell lines were tested for HSV specific antigens by immunofluorescence.
Two methods of detection were used. The first was the indirect method using unfixed
transformed cells on coverslips and specific antiserum made in rabbits against the trans-
forming virus. Commercial sheep anti-rabbit fluorescein (Wellcome) was used to detect
specifically the presence on the cells of herpes antigen binding the rabbit antibodies. Washing
was kept short and gentle and only 15 min adsorption periods were used for the antiserum
and fluorescein reactions, with PBS and glycerol or liquid paraffin as mountant.

With a specific antiserum made by Dr Timbury against RK13 cells infected for 24 h
with HSV-2 ts 1 at 38 °C about 50 % or more of the cells in the rat embryo line transformed
by HSV-2 ts 1 gave good fluorescence (see Fig. 5). Rat embryo cells transformed by ts 7
or with HSV-2 strain 333 also gave fluorescence with this antiserum but only about 20 %
of cells fluoresced. Similarly about 20 % of hamster embryo cells transformed by HSV-2
strain 333 checked at passage 44, fluoresced as did tumour cells from both primary tumours
Fig. 5. Immunofluorescence of rat embryo cells transformed by HSV-2 strain HSG 52 ts 1. Cells adsorbed by specific antiserum against ts 1 and photographed. Unfixed.

and from later tumours after passage in animals. All the controls which included tests of normal cells with the herpes specific and pre-immune antiserum and of transformed cells with pre-immune antiserum were totally negative. In this system I have never obtained non-specific fluorescence. The amount of specific fluorescence does vary somewhat in different preparations.

The other fluorescent antibody test used was the indirect method of Margalith, Volk-Fuchs & Goldblum (1969) using fixed material. After acetone fixation cells were adsorbed with commercially obtained anti HSV rabbit antiserum (Ital Diagnostics) and stained with commercial anti-rabbit fluorescein. Fluorescence was less intense and appeared to be mainly cytoplasmic by this method and moreover non-specific fluorescence seen in the control cells suggested that this approach was less discriminating and reliable.

Tumourigenicity of the transformed hamster line

Hamster embryo cells transformed by HSV-2 strain 333 (HE/HSV-2/333) were injected at passages 9, 12, 16, 18, 22, 24, 28 and 34 into a newborn litter of hamsters both subcutaneously and intraperitoneally, at amounts varying from $1.5 \times 10^6$ to $4 \times 10^6$ cells per newborn hamster. No tumours were obtained until passage 34 gave after 13 weeks a total of two tumours in ten intraperitoneally injected and one tumour in seven subcutaneously injected animals. These tumours were excised, trypsinized and injected immediately in single cell suspensions of $5 \times 10^6$ and $1 \times 10^6$ cells into newborn, 1-week and 2-week-old hamsters, where they produced almost 100% tumours within 1 week (Table 2).

The tumours were excised and the animals examined for metastases, but no metastases
Rat embryo cell transformation by ts HSV

Table 2. Tumour formation with hamster embryo cells transformed by HSV-2 strain 333 (HE/HSV-2-333)

<table>
<thead>
<tr>
<th>Cells inoculated</th>
<th>Age of animals at inoculation</th>
<th>Time for tumour to develop</th>
<th>No. of tumours</th>
<th>Total animals in litter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ no. of cells</td>
<td></td>
<td>i.p.*, s.c.†</td>
<td></td>
</tr>
<tr>
<td>HE/HSV-2-333</td>
<td></td>
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<td></td>
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<tr>
<td>passage 34</td>
<td>Newborn</td>
<td>13 weeks</td>
<td>2/10</td>
<td>1/7</td>
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<tr>
<td>Primary tumour</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>single cell suspension</td>
<td>Newborn</td>
<td>1 week</td>
<td>—</td>
<td>20/21</td>
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<tr>
<td>Primary tumour</td>
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<td>single cell suspension</td>
<td>1 week</td>
<td>1 week</td>
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<td>5/5</td>
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<tr>
<td>Primary tumour</td>
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<tr>
<td>single cell suspension</td>
<td>2 weeks</td>
<td>1 week</td>
<td>2/2</td>
<td>—</td>
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</table>

* Intraperitoneal injection.
† Subcutaneous injection.

were detected. Histologically the tumours were classified as fibrosarcomas by Dr A. Cochran of the Pathology Department, University of Glasgow.

Control cell lines inoculated up to passage 12 have not given rise to any tumours.

The possible tumourigenicity of the HSV transformed rat cell lines is at present being investigated.

**Superinfectability of the transformed cell lines**

HE/HSV-2/333 was superinfectable by wild type HSV-1 at an input multiplicity of 5 p.f.u./cell and HSV-2 at 37 °C and also by all the temperature-sensitive mutants of HSG 52 at 31 °C, when tested at passages 22 and 44. This was tested by exposing monolayers to the virus at 31 °C, harvesting after 72 h and then testing the sonicate in C13 cells for plaque production. Similarly the transformed rat embryo cell lines were superinfectable at passage 15. Experiments using differing input multiplicities and quantitating the results as has been done by Doller, Duff & Rapp (1973) are at present in progress.

Experiments to demonstrate possible marker rescue are being carried out at the non-permissive temperature of 38 °C with the ts mutants of HSG 52. So far no recombinant wild-type virus has been isolated from progeny virus obtained in the HE/HSV-2/333 transformed cell line or from the transformed rat embryo cell lines.

**DISCUSSION**

We have shown that several ts mutants of HSV-2 (HSG 52) are able to transform rat embryo cells without the need of u.v. irradiation of the virus. A reliable method of producing foci of morphologically transformed rat embryo cells is described, and evidence provided that the ability to form foci is heritable. U.v. irradiated wild-type HSV-2 strain HSG 52 and also HSV-2 strain 333 (the original virus used by Rapp), produce morphologically similar foci. Five out of 13 ts mutants of HSV-2 strain HSG 52 also produced transformed foci which did not differ morphologically from those produced by HSV-2 strain 333. Thus the transformed focus we recognize is typically produced by at least two different strains of HSV-2 and five ts mutants of one of these, and probably represents a general feature of HSV transformation of rat embryo cells.

So far only five ts mutants have been shown to transform, although experiments were
performed in duplicate using different stocks of mutants and at least two different embryo cultures. One possible reason for this low rate of success may be that some mutants were only available in low-titre stocks (e.g. $2 \times 10^7$ p.f.u./ml) so that the input multiplicity of the virus was never greater than 1 p.f.u./cell. This low input may not have been sufficient to transform. Of the transforming mutants, three – ts 3, ts 4 and ts 5 – made virus DNA at the non-permissive temperature and two – ts 7 and ts 1 – did not. Of course, transformation in this system depended on detecting a focus during the first 6 weeks of observation. Had the apparently negative cultures been split and carried on with the positive cultures, it is quite possible that more of the ts mutants would have proven transformation positive. This possibility is being further investigated.

The particle to infectivity ratios of HSV-2 $ts^+$ virus and the ts mutants were always about 100 to 1000 EM particles to 1 infectious p.f.u. In the case of HSV-1 this ratio was about 10 to 1 but we did not detect any significant difference in the initial efficiency of transformed focus formation. Thus the non-infectious particles in these experiments did not seem able to transform.

The characteristic crisis phase through which all HSV infected cell lines pass is unique for transformation with a DNA virus. The fluorescent antibody test convincingly detected herpes specific antigens in the unfixed transformed and also tumour cells on the two occasions tested by me. This specific fluorescence has since been independently confirmed by Professor K. B. Fraser and Dr P. Shirodaria in Queens University, Belfast, to whom the lines were sent. At no time was it possible to isolate or detect the presence of any RNA tumour virus in these transformed cell lines.

The only criterion used to determine whether or not a particular mutant had transforming potential was formation of a detectable, characteristic focus. It is quite possible that further experiments and/or extension to use of other criteria might produce transformed lines with several more mutants.

The transformed hamster cells, HE/HSV-2/333, although subtly distinguishable do not differ greatly in morphology from the original embryo cells. In contrast the morphology differences seen with the transformed rat cell lines are much more pronounced although the cells morphological characteristics seem to retain the potential to vary somewhat on further subculture. This makes morphology a not entirely satisfactory sole criterion of successful transformation.

We are now extending the studies aimed at demonstrating specificity and survival of HSV genetic information to hybridization experiments, using either labelled herpes DNA or labelled cRNA made on herpes DNA. Experiments have also been started to determine the transforming potential in the rat embryo cell system of the ts mutants of HSV-1 strain 17 isolated by Subak-Sharpe (Brown et al. 1973). Lastly it should be pointed out that, while we have evidence that HSV transformed hamster embryo cells are tumourigenic, oncogenicity has not yet been shown to be a property of the HSV transformed rat embryo cells.

I wish to thank Professor J. H. Subak-Sharpe for helpful discussion; Dr M. C. Timbury for supplying the ts mutants of HSV-2 strain HSG 52 and also the specific antisera against ts mutant 1; Dr F. Rapp for his HSV-2 strain 333 virus grown in human cells; Dr E. A. C. Follett for electron microscopy and particle counts of virus; Mr N. Hallam for reverse transcriptase assays in transformed cells; Dr A. Cochran, Pathology Department, Glasgow University, for looking at histology of tumours; Professor K. B. Fraser and Dr A. Shirodaria of Queens University, Belfast, for confirming the fluorescent antibody results; Professor
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M. C. McNaughton, Royal Infirmary, Glasgow, for abortis material; Mrs Alice Birrell for skilful and expert technical assistance.

REFERENCES


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