Transformation of a Hamster Cell Line by Adenovirus Type 12

By R. M. McALLISTER* AND I. MACPHERSON

Medical Research Council Experimental Virus Research Unit, Institute of Virology, University of Glasgow, Scotland

(Accepted 28 July 1967)

SUMMARY

Four clones of the hamster cell line NIL-2 were transformed by adenovirus 12. The transformed cells formed foci of multilayered growth in monolayer cultures under agar medium and colonies when suspended in soft agar medium. A virus stock containing $5 \times 10^6$ particles and $1.1 \times 10^8$ p.f.u./ml. contained $2.8 \times 10^8$ focus-forming units (f.f.u.)/ml. in NIL-2 cells. The numbers of cell foci and of colonies induced by serial dilutions of virus were consistent with a linear dose response. Approximately $2 \times 10^7$ total virus particles or $4 \times 10^8$ infectious units were required to induce one focus of transformed cells. The highest transformation rate obtained was 0.002% for cells exposed to about 80 p.f.u. of virus per cell. For comparison, primary rat embryo cells were transformed by adenovirus 12. The results obtained were approximately the same as those with NIL-2 cells except that the rat cells did not form colonies when infected and suspended immediately in agar. However, rat cells transformed in cultures under liquid medium formed colonies when suspended in agar medium.

INTRODUCTION

A number of workers have described the in vitro transformation of embryonic hamster, rat and rabbit cells by adenovirus 12 (Freeman et al. 1967; Levinthal & Peterson, 1965; McBride & Wiener, 1964; Pope & Rowe, 1964; Reed, 1967; Strohl, Rouse & Schlesinger, 1966; Yamane & Kusano, 1967). The cells used were in primary or early tissue culture passage and therefore consisted of heterogeneous populations of cell types. The low rates of transformation observed (0.01 to 0.001 %) may have been due to a scarcity of susceptible cells (Yamane & Kusano, 1967). Also Reed (1967) has reported that batches of hamster embryo cells vary in their susceptibility to transformation.

Since previous studies have shown that in vitro transformation by polyoma and SV 40 viruses is most amenable to detailed study in established cell lines with a high degree of autonomy in vitro (Macpherson & Montagnier, 1964; Todaro & Green, 1966), we attempted to transform 2 cloned lines of hamster cells with adenovirus 12.

In agreement with others (Reed, personal communication; Yamane & Kusano, 1967), we were unable to transform the BHK 21 cell line. We did, however, successfully transform 4 clones of the NIL-2 cell line (Diamond, 1967). This report describes the transformation of these cells and compares it with transformation of primary rat embryo cells by the same virus.

* Present address: Children's Hospital of Los Angeles, California.
METHODS

**Virus.** Adenovirus type 12 (HuIE strain) was obtained from the American Type Culture collection and a stock prepared from the progeny of a single plaque on human embryonic kidney cells (HEK) (McAllister *et al.* 1966). The plaque-purified virus was identified as type 12 by a plaque neutralization test using antiserum from the Research Reference Reagents Branch (National Institute of Allergy and Infectious Diseases). The stock contained $5 \times 10^8$ virus particles per ml. when counted by electron microscopy (Watson, Russell & Wildy, 1963) and $1.1 \times 10^9$ p.f.u./ml. when assayed on HEK cells.

A dose of $2 \times 10^6$ p.f.u. of virus induced tumours in hamsters in 38 to 93 days (McAllister *et al.* 1966). The hamster tumours contained adenovirus 12 tumour antigen but no SV 40 tumour antigen, and the sera from the tumour-bearing hamsters contained antibodies to adenovirus 12 tumour and T antigens but not to SV 40 tumour or T antigens.

No adenovirus-associated virus was detected in the stock by electron microscopy.

**Cells.** The hamster cell line NIL-2 was obtained from Dr Leila Diamond (1967) in its 45th passage. A wide variety of different cell types was found when the line was plated to produce discrete colonies. A single colony with flattened fibroblastic cells and no piling up was selected for further study and single-cell clones were prepared by micromanipulation (Wildy & Stoker, 1958). These failed to produce colonies in agar suspension culture even when plated at high cell densities ($5 \times 10^6$ cells/5 cm. dish).

Rat embryo cells were isolated by trypsin dispersion of near-term embryos and used in primary tissue culture passage.

The methods of cell culture in liquid media and in soft agar media have been described (Macpherson & Montagnier, 1964; McAllister, Landing & Goodheart, 1964; McAllister *et al.* 1966; McAllister, Reed & Huebner, 1967).

**Liquid and agar media.** A liquid medium (liquid FM) described by Freeman *et al.* (1967) was used. It consisted of Eagle's medium without calcium and with twice the normal concentration of amino acids and vitamins, supplemented with $0.1$ mM calcium chloride, $5\%$ dialysed calf serum and $2\%$ foetal calf serum. Agar overlay medium (agar FM) contained the same components and $0.5\%$ agar.

Media for agar suspension cultures (Macpherson & Montagnier, 1964; McAllister *et al.* 1967) consisted of Eagle's medium supplemented with $20\%$ foetal calf serum and either $0.33\%$ or $0.5\%$ agar (EF 20 agar).

**Transformation assays in NIL-2 and rat embryo cells**

*In monolayer cultures.* Semi-confluent monolayers of NIL-2 cells (about $1.5 \times 10^6$ cells/culture) in 5 cm. plastic Petri dishes were rinsed with tris buffer solution, exposed to $0.1$ ml. of virus suspension for 3 hr at $37^\circ$ and overlaid with $2.5$ ml. of $0.5\%$ agar FM. The plates were incubated at $37^\circ$ in humidified atmosphere constantly gassed with approximately $10\%$ CO$_2$ in air. After 2 days, $2.5$ ml. of liquid FM were pipetted on top of the agar and replaced every 2 to 3 days for 25 to 50 days. Foci of transformed cells were counted after the agar was poured off and the culture stained with Giemsa stain.

Rat embryo cells were transformed by adenovirus 12 using the method described by Freeman *et al.* (1967). This was identical to that described for monolayers of
NIL-2 cells except that a confluent sheet (about $4 \times 10^6$ cells) was exposed to virus and no agar overlay was used.

*In agar suspension cultures.* Centrifuged pellets of trypsin-dispersed NIL-2 cells or rat embryo cells ($3 \times 10^6$ cells) were suspended in 1 ml. of virus suspension, agitated for 1 hr at room temperature and $5 \times 10^5$ cells in 1.5 ml. of $0.33\%$ agar medium (EF 20) were added to 5 cm. Petri dishes containing a preset base of $0.5\%$ agar medium (EF 20). After 1 week, 2 ml. of Eagle's medium containing 10% foetal calf serum was pipetted on top of the agar and replaced weekly.

**Serological methods and reagents**

Tests for complement-fixing antigens in the transformed cells were carried out as described previously (McAllister *et al.* 1966; McAllister *et al.* 1967).

**RESULTS**

**Transformation of NIL-2 cells**

In a series of preliminary experiments NIL-2 cells, clone E, were transformed by variations of the two methods described above. The transformed cells detected by both methods contained adenovirus 12 tumour antigen and resembled the cells cultured in liquid medium or in agar from hamster tumours induced by adenovirus 12 (Kitamura *et al.* 1964; McAllister *et al.* 1967; Strohl *et al.* 1963), and also the hamster, rat and rabbit cells transformed *in vitro* by adenovirus 12 (Freeman *et al.* 1967; Levinthal & Petersen, 1965; McBride & Wiener, 1964; Pope & Rowe, 1964; Reed, personal communication; Strohl *et al.* 1963; Yamane & Kusano, 1967).

Three clones of adenovirus-transformed NIL-2 cells were isolated. These were cultured individually and with the parent NIL-2 cells in liquid and agar media containing different concentrations (2 to 20%) of postnatal and foetal calf serum and of calcium ($0.1$ mm and $1.8$ mm) to determine the most suitable conditions for the detection and propagation of transformed cells. The two concentrations of calcium were used because of the reported calcium sensitivity of adenovirus-transformed cells (Freeman *et al.* 1966). The transformed NIL-2 cells grew best in liquid and agar medium ($0.33\%$ agar) consisting of Eagle's medium ($1.8$ mm calcium) and $20\%$ foetal calf serum (plating efficiencies of $80\%$ in liquid and $20\%$ in agar medium). However, when the transformed cells were added to confluent monolayers of untransformed NIL-2 cells, the foci of transformed cells were most easily distinguished from the untransformed cells when cultured in liquid FM and under $0.5\%$ agar FM. These studies provided the basis for the methods of carrying out the transformation assays described in Methods and were used for further experiments.

Three new clones of NIL-2 cells growing as semi-confluent monolayers were exposed to virus and overlaid with agar. Suspensions of cells of each clone were also exposed to virus and plated in agar suspension cultures. Foci of transformed cells appeared after 17 days in the monolayer cultures and were assayed after staining at 25 days (Pl. 1, fig. 1; Table 1). Colonies, $0.1$ to $0.2$ mm. in diameter, were present in the agar suspension cultures after 21 days incubation and were counted with the aid of a low-power microscope after 25 days (Pl. 1, fig. 2; Table 2). These colonies, when transplanted to liquid FM, yielded transformed cells. The highest transformation rate, $0.002\%$ of cells, was obtained by both methods using clone 3.
Transformation of rat embryo cells

When, as in Freeman's method, confluent monolayers of rat embryo cells were exposed to virus, foci of transformed cells were visible 21 days after infection. Pl. 1, fig. 3, shows foci of transformed cells 65 days after infection. These transformed cells contained adenovirus 12 tumour antigen and formed colonies when plated in soft agar. The results of these experiments are summarized in Table 3. Agar overlays were apparently not required to prevent the formation of secondary foci. Some dishes contained only 1 or 2 large foci from the 42nd to the 65th day of culture. The failure of adenovirus-12-induced foci to induce secondary foci was also noted by Freeman et al. (1967).

Table 1. Transformation of three NIL-2 cell clones by adenovirus 12

<table>
<thead>
<tr>
<th>Virus input</th>
<th>No. of foci per monolayer culture*</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.f.u./cell</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clone 1</td>
</tr>
<tr>
<td>73</td>
<td>2, 2</td>
</tr>
<tr>
<td>18</td>
<td>0, 2, 2</td>
</tr>
<tr>
<td>7:3</td>
<td>0, 0, 0</td>
</tr>
<tr>
<td>2:4</td>
<td>0, 0, 0</td>
</tr>
<tr>
<td>Control</td>
<td>0, 0</td>
</tr>
</tbody>
</table>

* 1.5 x 10⁶ cells per culture exposed to virus.

Table 2. Transformation of three NIL-2 cell clones by adenovirus 12

<table>
<thead>
<tr>
<th>Virus input</th>
<th>No. of colonies per agar suspension culture*</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.f.u./cell</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clone 1</td>
</tr>
<tr>
<td>84</td>
<td>3, 2, 2</td>
</tr>
<tr>
<td>42</td>
<td>1, 1, 0</td>
</tr>
<tr>
<td>Control</td>
<td>0, 0, 0</td>
</tr>
</tbody>
</table>

* Initially 5 x 10⁶ cells per culture.

Comparison of the transformation of NIL-2 cells and rat embryo cells by adenovirus 12

In monolayer cultures of both NIL-2 cells and rat embryo cells, cells transformed by adenovirus 12 formed, after 17 to 21 days, multilayered foci easily recognized against a monolayer background of untransformed cells. Transformed cells of both cell types had the same characteristic epithelioid morphology and contained adenovirus-12-specific tumour complement-fixing antigen (Huebner, personal communication). In contrast to transformed NIL-2 cells, virus-infected rat cells did not form colonies when seeded directly in agar. However, when the rat cells were transformed in cultures under liquid medium and transplanted into agar medium, they did form colonies with a plating efficiency of 5%. Table 4 compares further the transformation of the two cell types by one stock of adenovirus 12.
Hamster cell transformation by adenovirus 12

Table 3. Transformation of rat embryo cells by adenovirus 12

<table>
<thead>
<tr>
<th>Virus input p.f.u./cell</th>
<th>No. of foci per monolayer culture*</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>54, 38, 17</td>
</tr>
<tr>
<td>5</td>
<td>5, 5</td>
</tr>
<tr>
<td>0.5</td>
<td>3, 2, 2, 1</td>
</tr>
<tr>
<td>0.05</td>
<td>0, 0, 0, 0</td>
</tr>
<tr>
<td>Control</td>
<td>0, 0, 0</td>
</tr>
</tbody>
</table>

* 4 × 10⁶ cells per culture exposed to virus.

Table 4. Transformation of NIL-2 cells and rat embryo cells by adenovirus 12

<table>
<thead>
<tr>
<th>Focus-forming units (f.f.u.)/ml.</th>
<th>NIL-2 cells</th>
<th>Rat embryo cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus stock</td>
<td>2.8 × 10⁶</td>
<td>1 × 10⁶</td>
</tr>
<tr>
<td>VP/f.f.u.</td>
<td>1.7 × 10⁷</td>
<td>5 × 10⁶</td>
</tr>
<tr>
<td>p.f.u./f.f.u.</td>
<td>3.9 × 10⁶</td>
<td>1.1 × 10⁶</td>
</tr>
<tr>
<td>Percentage of cells transformed (50 to 80 p.f.u./cell)</td>
<td>0.002</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* 5 × 10⁶ total virus particle (VP)/ml. and 1.1 × 10⁶ p.f.u./ml.

DISCUSSION

The assay method for adenovirus-transformed cells by counting foci formed on semiconfluent monolayers of NIL-2 cells under an agar overlayer is similar to the assay method for Rous-sarcoma-virus-transformed chick cells developed by Temin & Rubin (1958). The agar suspension culture method for the assay of adenovirus-transformed cells was the same as that described by Macpherson & Montagnier (1964) for the assay of polyoma-transformed cells.

The characteristic morphology of cultured adenovirus-induced tumour cells and transformed cells has led to the suggestion that the tumours and foci are derived from special and probably rare target cells (Yamane & Kusano, 1967). Our results indicate that several clones of hamster fibroblasts were transformed by adenovirus 12 with about the same low efficiency as a heterogenous population of rat embryo tissues. This suggests that the efficiency of transformation is primarily a function of the virus and not due to a dearth of susceptible cells in these cultures.

In our studies 1 to 4 × 10⁶ infectious units of adenovirus 12 were required per transformation compared with 10⁴ and 10⁵ infectious units for polyoma and SV40 viruses respectively (Macpherson & Montagnier, 1964; Todaro & Green, 1966). In view of the comparatively low transformation efficiency of adenovirus 12, it is of interest that in preliminary studies we found that the presence of SV40 genetic material in adenovirus 7 (the hybrid virus E 46+, type 7/SV40) greatly enhanced the transformation efficiency (2.7 × 10⁴ p.f.u./transformation) of NIL-2 cells when compared to adenovirus 12 or to adenovirus 7 (E 46−) without SV40 genetic material (4.3 × 10⁷ p.f.u./transformation), despite the fact that neither Diamond (1967) nor ourselves were able to transform NIL-2 cells with SV40 virus.

Recent studies suggest that the hitherto non-oncogenic types 1, 8, 24 can induce
tumours in hamsters (Trentin, van Hoosier & Samper, 1967). These observations suggest that the entire adenovirus group will have to be reinvestigated for oncogenic potential. Both the NIL-2 cell and the rat cell adenovirus transformation assay systems may be useful in such studies. For instance Freeman et al. (personal communication) have observed that the weakly oncogenic virus, type 3, can transform rat cells, and preliminary studies by us suggest that the type 1, MONT strain (McAllister et al. 1964) can also transform rat cells in vitro.

The authors wish to thank Dr E. A. C. Follett for the electron microscopy, Drs R. J. Huebner and M. C. Timbury for the serological studies, Miss Joan Beveridge for technical assistance, and Professor M. G. P. Stoker for helpful discussions.

One of us (R. M. M.) was aided by a Scholar Grant in Cancer Research from the American Cancer Society. This investigation was supported by Public Health Service research grant CA-04865 from the National Cancer Institute.

REFERENCES


Freeman, A. E., Black, P. H., Wolford, R. & Huebner, R. J. (1967). The adenovirus type 12—


Levinthal, J. D. & Petersen, W. (1965). In vitro transformation and immunofluorescence with

neoplastic and non-neoplastic tissues of children. Lab. Invest. 13, 894.

McAllister, R. M., Reed, G. & Huebner, R. J. (1967). Colonial growth in agar of cells derived from

McAllister, R. M., Goodheart, C. R., Mirabal, V. Q. & Huebner, R. J. (1966). Human adeno-
viruses: tumor production in hamsters by types 12 and 18 grown from single plaques. Proc.


Macpherson, I. & Montagnier, L. (1964). Agar suspension culture for the selective assay of cells

transformed or infected by adenoviruses. J. exp. Med. 120, 577.


derived from adenovirus-induced hamster tumors. Virology 21, 513.


sarcoma cells in tissue culture. Virology 6, 669.

Virology 28, 756.
Hamster cell transformation by adenovirus


(Received 28 June 1967)
EXPLANATION OF PLATE

Fig. 1. Focus of transformed NIL-2 cells 25 days after infection. Unstained.
Fig. 2. Colonies of transformed NIL-2 cells after 25 days incubation in agar medium. Unstained.
Fig. 3. Culture of rat embryo cells showing about 50 foci of transformed cells 65 days after infection on Giemsa stain about twice natural size.
R. M. McALLISTER AND I. MACPHERSON

(Facing p. 106)