Replication and Interaction of Virus DNA and Cellular DNA in Mouse Cells Infected by a Human Adenovirus

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SUMMARY

C57 black mouse cells infected with human adenovirus type 5 (Ad5) produced large amounts of early virus proteins, small amounts of late virus proteins and less than 0.2 infectious units (i.u.)/cell of infectious virus. Many cells died but the cultures recovered. Virus DNA and cellular DNA were synthesized. Some Ad5 DNA sedimented with cell DNA in alkaline sucrose, but virus DNA was rapidly lost from the culture after recovery and none of 28 unselected cloned survivors contained detectable amounts of virus DNA or antigens.

Ad5 ts36 was temperature-sensitive for virus DNA replication in mouse cells, but ts125 was defective at 32.5 °C as well as at 39.9 °C. No difference was detected in the percentage of virus DNA that sedimented in alkali with cell DNA, in mouse cells infected by Ad5 ts+, ts36 or ts125 at 32.5 or 39.9 °C. All parts of the virus genome were equally represented in virus DNA that sedimented with cell DNA, in mouse cells infected by Ad5 ts+ or ts36 at either temperature.

INTRODUCTION

There are a number of differences in cellular responses to oncogenic and ‘non-oncogenic’ adenoviruses. Cells transformed by non-oncogenic adenoviruses are seldom tumourigenic (Gallimore, 1972). During abortive infection of hamster cells, the oncogenic human adenovirus type 12 (Ad12) makes early proteins and induces abnormal cell DNA replication, but produces no virus DNA, late antigens or infectious progeny (Doerfler, 1969; Strohl, 1969a, b). However, the ‘non-oncogenic’ adenovirus type 2 (Ad2) replicates virus DNA in rat or hamster cells and produces some late proteins and infectious virus (Gallimore, 1974; McDougall et al. 1974). All or most of the virus genome is represented in sequences integrated in Ad12-transformed hamster cells (Fanning & Doerfler, 1976; Green et al. 1976; Groneberg et al. 1977), while the only virus sequence common to all Ad2 and human adenovirus type 5 (Ad5)-transformed cells are restricted to a small region at the left end of the genetic map (Sambrook et al. 1974). It has been suggested that the virus single-stranded DNA binding protein may somehow restrict integration and that removal of this restriction is responsible for the higher transformation frequency observed with the mutant ts125 of Ad5 (Mayer & Ginsberg, 1977).

Ad12 is tumourigenic in the mouse (Rabson et al. 1964; Yabe et al. 1964; Allison et al. 1967). Ad12 produces no late proteins in mouse cells (Levinthal et al. 1966). The ‘non-oncogenic’ group C adenovirus type 5 (Ad5) appears to undergo limited replication in mice
in vivo (Postlethwaite, 1973; Mautner & Willcox, 1974), but detailed in vitro studies have not been reported. We have characterized the infection of mouse cells by Ad5 with respect to synthesis of early and late proteins, virus DNA and infectious virus. We also describe here experiments on the induction of mouse cell DNA replication by Ad5, and an apparently covalent association of some virus DNA with cell DNA. Experiments on mutants of Ad5 with altered transformation ability are described. In another paper we shall report that Ad5 induces anchorage independent clones of mouse cells that differ in many properties from adenovirus transformed cells and which are fully tumourigenic in immunocompetent syngeneic mice.

METHODS

Radioactive isotopes. $^{32}$P (carrier-free) was obtained from the Australian Atomic Energy Commission, (Lucas Heights, Sydney, Australia) and methyl-$^3$H-thymidine (48 Ci/mmol) from The Radiochemical Centre, Amersham, U.K. Measurement of radioactivity was done as described previously (Bellett & Younghusband, 1972).

Medium. Eagle's medium ('Autopow', Flow Laboratories) was supplemented with 10% calf serum.

Mice. The strain of C57 black mice used was C57Bl/6J Cn f, bred under specific pathogen-free conditions.

Cells. Most experiments were done with a line of cells (C57Bl J.M.) established on a 3T3 schedule from C57 black mouse embryos by Dr J. T. May in 1973 and maintained since as a continuous cell line. Some experiments were done with secondary cultures from C57 black mouse embryos.

Viruses. Ad5 and Ad2 were grown in KB cells and titrated in HEK cells by the fluorescent antibody method [Philipson, 1961; titres in infectious units (i.u.)/ml]. Ad5 ts36 and ts125 were obtained from Dr J. F. Williams and were grown and titrated at 32°C. In most experiments virus was purified by one or two cycles of sedimentation in CsCl density gradients and dialysed in and dialysed against medium.

Antiserum. Sera ('V') used for titration of virus and detection of virion antigens were from rabbits that had received repeated intravenous inoculations of purified virus or two intramuscular injections of purified virus in Freund's complete adjuvant. Ad5 anti P serum was a gift from Dr W. C. Russell and was prepared by the method of Russell et al. (1967).

$^{32}$P-labelled Ad5 DNA. Purified virus DNA was prepared as described previously (Laver et al. 1971). For preparation of high specific activity Ad5 $^{32}$P-DNA, 5 mCi of $^{32}$P was added to each 75 cm$^2$ Falcon bottle of infected KB cells. The specific activity of Ad5 $^{32}$P-DNA ranged between $1 \times 10^6$ and $1.5 \times 10^6$ Cerenkov ct/min/μg.

Total virus DNA in infected cells. Infected cells from two dishes were trypsinized, washed in PBS and resuspended in a small volume of PBS. The cells were then lysed by the addition of 4 vol. of a solution containing 0.5% SDS, 0.1 M-NaCl, 0.01 M-tris pH 7.9, 0.005 M-EDTA and sonicated for 1 min with a Branson sonicator at maximum setting. Pronase was added to the lysate to a final concentration of 1 mg/ml and the mixture incubated at 37°C for 2 h.

The DNA was extracted twice with buffer-saturated phenol, dialysed against SSC, (0.15 M-NaCl, 0.015 M-sodium citrate), digested with RNase (100 μg/ml, 37°C for 1 h) followed by Pronase at 500 μg/ml at 37°C for 1 h. The DNA was then extracted twice with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1) and dialysed against STE (0.1 M-NaCl, 0.05 M-tris, 0.001 M-EDTA, pH 7.2). The amount of virus DNA present was determined by reannealing kinetics as described previously (Bellett, 1975; Tyndall et al. 1978).

Analysis of free and cell DNA-associated virus DNA in Ad5, Ad5 ts125 and Ad5 ts36-infected C57Bl J.M. mouse cells. C57Bl J.M. mouse cell cultures were pre-labelled for 18 to 24 h...
with $^3$H-dThd (0.5 to 1 $\mu$Ci/ml) prior to infection. Semi-confluent cell monolayers (10$^6$ to $2 \times 10^6$ cells/60 mm Petri dish) were then inoculated with Ad5 virus or its mutants ts125 or ts136 at a m.o.i. of 10 when virus was grown at 32.5 or 37°C and at a m.o.i. of 50 when virus was grown at 39.9°C. The virus inoculum was allowed to adsorb to the cells for 1 h at room temperature; medium pre-warmed at the appropriate temperature was then added to the cultures and incubation continued for 23 to 24 h at 39.9°C or for 70 to 72 h at 32.5°C. The cells were then washed and lysed in 0.3 M-NaOH, 0.5% SDS, 0.3 M-NaCl, 3 mM-EDTA. The lysate was centrifuged on alkaline sucrose gradients as described previously (Tyndall et al. 1978). After centrifugation, the gradients were separated into cell DNA and free virus DNA pools, the latter being detected by the sedimentation position of $^{32}$P-labelled Ad5 DNA of high specific activity added to the infected cells at the time of lysis (Tyndall et al. 1978) and the amount of virus DNA in each was determined by reassociation kinetics, using $^{32}$P-labelled Ad5 DNA, as described previously (Bellett, 1975; Tyndall et al. 1978). In control experiments, from 4 to 10 $\mu$g of Ad5 DNA was added to each 60 mm dish of mock-infected, $^3$H-pre-labelled mouse cells. The cells were then lysed and processed as described.

The amount of virus DNA specifically associated with the cell DNA in infected cells ($V_s$) was corrected for contamination with free virus DNA by the method of Tyndall et al. (1978) as follows:

$$V_s = V_{ci} - V_{cu} (V_{ti}/V_{tu}),$$

where $V_{ci}$ = virus DNA in the cell peak from infected cells; $V_{ti}$ = total virus DNA in the gradient from infected cells; $V_{tu}$ = total virus DNA in the gradient from uninfected cells.

When the sequences of virus DNA associated with cell DNA were investigated using restriction fragments of virus DNA as probes, $V_{cu}$ was measured using whole virus DNA. It was assumed that sequences corresponding to different restriction fragments of virus DNA contaminated the cell DNA in equimolar amounts.

**Digestion of $^{32}$P-labelled Ad5 DNA with restriction endonucleases HpaI or HindIII.**

Restriction endonuclease HpaI was kindly donated by Dr K. D. Brown, restriction endonuclease HindIII was purchased from New England Biolabs. The HpaI enzyme reaction mixture contained: 10 mM-tris-HCl, pH 7.9, 10 mM-MgCl$_2$, 10 mM-2-mercaptoethanol and the HindIII reaction mixture contained: 7 mM-tris-HCl, pH 7.9, 7 mM-MgCl$_2$, 60 mM-NaCl.

The amount of enzyme required for complete digestion was determined in trial reaction mixtures containing unlabelled Ad5 DNA and twice this amount was used. Digestion was usually done in a vol. of 50 to 60 $\mu$l at 37°C for 1 to 2 h and was stopped by cooling in ice.

**Gel electrophoresis and purification of $^{32}$P-labelled Ad5 DNA restriction fragments.** Gel electrophoresis was done in horizontal slab gels of 0.8% agarose (Sigma; electrophoresis grade). The electrophoresis buffer contained 5 mM-sodium acetate, 40 mM-tris-HCl, pH 8.2, 1 mM-EDTA. After electrophoresis, the DNA was visualized within the gel by staining with ethidium bromide (0.5 $\mu$g/ml in electrophoresis buffer) for 0.5 h and photographed through a Kodak no. 23 filter on to Polaroid film using u.v. illumination. Each of the $^{32}$P-Ad5 DNA fragments was cut out of the agarose gel, dissolved in 5 M-NaClO$_4$ at 60°C for 0.5 h and purified by chromatography on hydroxylapatite (Daniell, 1976). The purity of the $^{32}$P fragment was checked by electrophoresis followed by autoradiography of the gel on Kodak RP-X-Omat film.

**Cellular and virus DNA replication in serum-starved cells.** C57Bl J.M. cells were plated at 5 x 10$^5$ cells/50 mm dish in medium containing 10% calf serum. One day later the medium was replaced by Autopow plus 0.2% serum and this was repeated at 3 days. On the fourth day the cultures were infected with 20 i.u./cell Ad5 in 0.2% serum. At daily intervals one culture was labelled for 16 h with 0.2 $\mu$Ci/ml of $^3$H-dThd in medium containing 0.2% serum.
Fig. 1. Incomplete replication cycle of Ad5 in C57 black mouse cells. C57Bl J.M. cells were inoculated when half confluent with 10 i.u. of Ad5/cell. The cultures were subcultured on day 2. At the times shown, one culture was trypsinized and the cells counted (■—■). Other cells were stained by the indirect fluorescent antibody method with Ad5 early (P) antiserum (○—○) or late virion (V) antiserum (●—●). Cell-associated virus was titrated in another culture (□—□).

This was followed by unlabelled medium (0·2 % serum) for 2 h. The cells were then lysed and total DNA was prepared as described previously (Tyndall et al. 1978). DNA samples were mixed with 32P-labelled Ad5 DNA, sarkosyl (80 µl of a 10 % solution) and CsCl (final vol. 8 ml, density 1·715 g/ml) and centrifuged for 66 h at 33000 rev/min and 20 °C in the Ti50 rotor. Acid-insoluble radioactivity was determined on 0·2 ml fractions of the gradients. The bimodal curves were analysed into virus DNA and cell DNA components taking the shape of the virus DNA peak from the 32P-labelled Ad5 DNA in the same gradient and the shape of the cell DNA peak from a parallel gradient of DNA from uninfected cells labelled with 3H-dThd during growth in 10 % serum.

RESULTS

Human adenoviruses undergo an incomplete replication cycle in C57 black mouse cells

Cells from C57 black mice (C57Bl J.M.) showed c.p.e. from 2 days to about 7 days p.i. with 10 i.u. of Ad5/cell and their plating efficiency was reduced to 10 to 50 % of control cells, but the cultures then recovered. Cell death was minimal in actively growing cultures that were subcultured soon after infection, but was extensive in confluent monolayers that were not subcultured. An experiment of the first type is shown in Fig. 1. The cell number increased more slowly than usual and showed a slight drop after subculture on the second day. Staining of cells with early (P) antiserum was maximal at 43 % on day 2, and then decreased slowly. Cells that reacted with late (V) antiserum appeared later, were lost from the culture more rapidly, and were always less numerous than those that reacted with P antiserum. The amount of cell-associated infectious virus increased 10-fold from 1 day to 3 days p.i. and then decreased. This indicated a limited degree of replication in some cells,
but the maximum yield was 0.1 i.u./cell on day 3, two to three orders of magnitude less than the yield in semi-permissive rat cells (Gallimore, 1974) and four to five orders of magnitude less than the yield in human cells (our own results).

Replication of virus DNA in mouse cells infected by adenoviruses

Virus DNA (density 1.715 g/ml) was detected in Ad2 and Ad5-infected mouse cell DNA prepared 2 days after infection and examined by analytical CsCl gradient centrifugation. The virus DNA content of cultures was also determined by DNA reassociation kinetics at various times after infection by Ad5. Figure 2 shows the amount of Ad5 DNA per diploid amount of mouse cell DNA at various times p.i. The sharp drop in virus DNA content of the cultures between 7 and 10 days probably represents cell death and an overgrowth of infected cells by cells which had escaped a lethal event during infection. The cells were subcultured at days 3, 5 and 7 and the plating efficiency of these cells was 10% of uninfected controls. After 40 days virus DNA was undetectable in the cultures.

Cell and virus replication in serum-starved mouse cells infected by Ad5

C57Bl J.M. cells were incubated for several days in medium containing 0.2% serum, infected with Ad5 in 0.2% serum and labelled with 3H-dThd at various times. Mock-infected controls were labelled after identical treatment without virus. One uninfected culture was also labelled after stimulation by addition of 10% serum. DNA was prepared from the samples, mixed with 32P-labelled Ad5 DNA and centrifuged in CsCl density gradients. 3H-DNA from infected cells gave a bimodal peak (Fig. 3) and could be readily analysed into virus and cell DNA components. The rate of cell DNA replication was 1.6 times that in the uninfected control in 0.2% serum by 21 h p.i. and continued to rise to more than three times the control by the third day. Incorporation of 3H into Ad5 DNA was less than that into cell DNA at all times and could not be detected until the second day (Fig. 3). The virus-induced cell DNA replication was apparently followed by mitosis, since the cell number in the infected culture doubled in 0.2% serum by 2 days p.i. compared with a 6% increase in mock-infected cells in 0.2% serum over the same period. However, on the third day the infected culture showed c.p.e. and the cell number had fallen below the control value.
Fig. 3. Ad5-induced cell and virus DNA replication in G1-arrested, abortively infected mouse cells. C57 black mouse cells were incubated in 0.2 % serum for 3 days and then infected by Ad5 or mock-infected, also in 0.2 % serum. (a) CsCl gradient centrifugation of DNA from cells labelled with 3H-thymidine from 5 to 21 h after mock-infection in 0.2 % serum (O--O) and 32P-labelled Ad5 DNA (○—○); the bottom of the gradient is to the left. (b) A similar gradient of DNA from cells labelled with 3H-thymidine from 53 to 69 h p.i. with Ad5 in 0.2 % serum (O--O); the bimodal curve analysed into virus (V--V) and cell (V--V) DNA components. (c) Analysis of CsCl gradients similar to those above was used to calculate the average rates of DNA synthesis in 0.2 % serum at various times: V--V, cell DNA replication in mock-infected cells; O--O, cell DNA replication in Ad5-infected cells; n--n, virus DNA replication in infected cells. (d) Total cell numbers in mock-infected cells (V—V) and Ad5-infected cells (O—O) in 0.2 % serum, and the percentage of infected cells reacting in the indirect fluorescent antibody test with Ad5 early (P) antiserum (V—V).

Assocation of virus DNA with cell DNA in Ad5-infected mouse cells

C57Bl J.M. cells were pre-labelled with 3H-dThd, infected with Ad5 at a multiplicity of 10 i.u./cell and incubated at 32.5 or 39.9 °C. The cells were lysed in alkaline SDS and the cell and virus DNA separated on alkaline sucrose gradients as described previously by Tyndall et al. (1978) [Fig. 4]. The gradient was divided into cellular DNA and free virus DNA pools, each of which was tested for virus DNA by reannealing kinetics using 32P-labelled Ad5 DNA (Tyndall et al. 1978). In parallel experiments mock-infected mouse cells, to which a known amount of Ad5 DNA had been added, were processed and analysed in the same way.

Virus DNA corresponding to 614 to 727 copies per diploid amount of cell DNA was found specifically associated with cellular DNA in infected cells (Table 1). This represents 8 to 10% of the total virus DNA after correction for contamination by free virus DNA (see Methods), which in different control experiments ranged from 1.8 to 3%. No significant difference was found between the amount of virus DNA produced and associated with
DNA in adenovirus-infected mouse cells

Fig. 4. Alkaline sucrose gradients of mouse cell DNA from (a) infected and (b) uninfected cells. Sedimentation is from right to left. Mouse cells were pre-labelled with $^3$H-dThd prior to infection

- - - - , $^3$H cell DNA; ■—■, $^{32}$P-Ad5 DNA added as a sedimentation marker.

Table 1. Quantitative analysis of adenovirus DNA associated with mouse cell DNA after alkaline sucrose gradient sedimentation*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Conditions</th>
<th>Virus DNA in virus peak (μg)</th>
<th>Virus DNA in cell peak (μg)</th>
<th>Copies/cell†</th>
<th>% Virus DNA in cell peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5 WT</td>
<td>10 i.u./cell, 72 h.p.i. 32.5 °C</td>
<td>1.75</td>
<td>0.140</td>
<td>727</td>
<td>8</td>
</tr>
<tr>
<td>Ad5 WT</td>
<td>50 i.u./cell, 24 h.p.i. 39.9 °C</td>
<td>1.80</td>
<td>0.180</td>
<td>614</td>
<td>9</td>
</tr>
<tr>
<td>Ad5 ts36</td>
<td>10 i.u./cell, 70 h.p.i. 32.5 °C</td>
<td>2.30</td>
<td>0.160</td>
<td>588</td>
<td>7</td>
</tr>
<tr>
<td>Ad5 ts36</td>
<td>50 i.u./cell, 24 h.p.i. 39.9 °C</td>
<td>0.030</td>
<td>0.004</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Ad5 ts125</td>
<td>10 i.u./cell, 72 h.p.i. 32.5 °C</td>
<td>0.050</td>
<td>0.004</td>
<td>40</td>
<td>7</td>
</tr>
<tr>
<td>Ad5 ts125</td>
<td>50 i.u./cell, 24 h.p.i. 39.9 °C</td>
<td>0.013</td>
<td>0.0013</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

* Mouse cells pre-labelled with $^3$H-dThd were infected with wild-type (WT) or mutant virus under the indicated conditions. Cells were then lysed, centrifuged on alkaline sucrose gradients and divided into cell and virus pools which were tested for virus DNA by renaturing kinetics (Tyndall et al. 1978). The amount of virus DNA in the cell peak was corrected for contamination by free virus DNA (see Methods).
† The number of copies/cell represents the number of virus DNA equivalents per diploid amount of cell DNA, assuming that Ad5 DNA has a mol. wt. of $23 \times 10^6$ and the diploid amount of DNA in mouse cells is $6 \times 10^{-9}$ μg.
cell DNA in mouse cells infected by Ad5 at 32.5 or 39.9 °C under the conditions of infection used.

Ad5 mutants ts36 and ts125 have different defects in virus DNA replication in mouse cells, but resemble Ad5 ts+ in association of virus DNA with cell DNA

Ad5 mutants ts36 and ts125 are temperature-sensitive in virus DNA replication in human cells. Ad5 ts36 transforms rat cells with the same frequency as Ad5 ts+ at the permissive temperature, but is transformation negative at the non-permissive temperature (Williams et al. 1974). Ad5 ts125 transforms rat cells with higher frequency than Ad5 ts+ at both temperatures (Ginsberg et al. 1974; Williams et al. 1974).

It was of interest to determine whether the differences in the frequencies of transformation by Ad5 ts+, ts36 and ts125 were correlated with differences in the association of virus DNA with cell DNA. 3H-dThd-labelled mouse cell monolayers were inoculated with 10 i.u./cell of Ad5 ts36 or ts125 at 32.5 °C or with 50 i.u./cell at 39.9 °C in order to have sufficient virus DNA for analysis. Cells were incubated for 70 to 72 h at 32.5 °C or for 24 h at 39.9 °C to allow for different rates of DNA replication at the two temperatures. The cells were then lysed, sedimented on alkaline sucrose gradients and divided into cell DNA and free virus DNA pools, in which the amount of virus DNA was determined as before.

Surprisingly, Ad5 ts125 appeared to be defective in DNA replication in mouse cells at 32.5 °C, while Ad5 ts36 DNA was produced in approximately the same amount as Ad5 wild-type DNA (Table I). Ad5 ts125 grew to very high titres in human HEK cells at 32.5 °C, and Ad5 ts125 DNA was efficiently replicated (about 4 μg/2 × 10⁸ cells at 24 h p.i.) in human KB cells at the same temperature.

The percentage of virus DNA specifically associated with mouse cell DNA in alkaline sucrose gradients ranged from 7 % at 32.5 °C to 12 % at 39.9 °C for ts36 and from 7 % at 32.5 °C to 9 % at 39.9 °C for ts125. These figures are very similar to those obtained with Ad5 ts+ (Table I). The absolute number of copies of virus DNA/cell specifically associated with cell DNA was lower for both mutants at the non-permissive temperature and for ts125 at 32.5 °C since the virus DNA was not replicated.

Restriction endonuclease analysis of virus DNA sequences associated with cell DNA in mouse cells infected by Ad5 ts+ and ts36

The above experiments show that the percentage of virus DNA associated with the DNA of mouse cells infected by the Ad5 ts mutants at the non-permissive temperature is not very different from that found at the permissive temperature or that found for wild-type Ad5 at either temperature. However, it is still possible that the left hand 7 % of the Ad5 DNA, which contains the sequences necessary for cell transformation by Ad5 (Graham et al. 1974), might be under-represented or absent from the virus DNA sequences associated with the cell DNA at the non-permissive temperature in cells infected by the transformation defective mutant ts36. We therefore determined whether all parts of Ad5 DNA were equally represented among the sequences associated with cell DNA in mouse cells infected with Ad5 ts36 at the permissive and non-permissive temperatures. Similar experiments were done with wild-type Ad5 as a control for the results with ts36 and to test the suggestion of Mayer & Ginsberg (1977) that the Ad5 sequences integrated are restricted in cells infected by wild-type virus. 32P-labelled Ad5 DNA of high specific activity was cleaved with restriction endonuclease HpaI. The DNA fragments were separated by electrophoresis on agarose gels and purified as described in Methods. Each 32P-labelled Ad5 DNA HpaI fragment was reannealed in the presence of cell DNA purified by alkaline sucrose gradient sedimentation from mouse cells infected by Ad5 ts36 at 32.5 °C and the number of copies/cell of each virus DNA fragment associated with cell DNA was calculated. A similar analysis using
DNA in adenovirus-infected mouse cells

Table 2. Quantitative analysis of HpaI restriction endonuclease fragments of Ad5 DNA associated with infected mouse cell DNA following alkaline sucrose gradient sedimentation*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Conditions</th>
<th>No. of equivalents of each HpaI restriction fragment of Ad5 DNA/cell</th>
<th>No. of equivalents of Ad5 DNA/cell†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5 ts36</td>
<td>32.5 °C, 70 h p.i.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(cell peak)</td>
<td>10 i.u./cell</td>
<td>E  4  24  26  A  58  86  90  100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C  576  792  604  650  743</td>
<td>588</td>
</tr>
<tr>
<td>Ad5 ts36</td>
<td>32.5 °C, 70 h p.i.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(virus peak)‡</td>
<td>10 i.u./cell</td>
<td>F  950  850  870  550  900</td>
<td>600†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus</td>
<td>Conditions</td>
<td>No. of equivalents of each group of HpaI restriction fragments of Ad5 DNA/cell</td>
<td></td>
</tr>
<tr>
<td>Ad5 WT</td>
<td>32.5 °C, 70 h p.i.</td>
<td>L  900</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 i.u./cell</td>
<td>M  650</td>
<td></td>
</tr>
<tr>
<td>Ad5 WT</td>
<td>39.9 °C, 24 h p.i.</td>
<td>R  1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 i.u./cell</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mouse cells, pre-labelled with ³H-dThd, were infected by wild-type (WT) or mutant virus under the indicated conditions. Cells were then lysed, centrifuged on alkaline sucrose and processed as described. Each ³P-labelled HpaI restriction fragment or a combination (L, M and R) of equimolar amounts of fragments was reannealed in the presence of the cellular and virus DNA pools from infected cells. The results in section (b) of the table were obtained from reannealing kinetics in the presence of the cell DNA peak only. The results were corrected for contamination of the cell DNA peak by free virus DNA (see Methods). The table includes a map of the HpaI restriction fragments of Ad5 DNA (Mulder et al. 1974). The numbers on the map are percentages of the distance from the left-hand end (0).
† The number of equivalents of Ad5 DNA were determined by reannealing kinetics with whole ³²P-Ad5 DNA.
‡ Total number of equivalents (× 10⁻⁹) of virus DNA fragments.

DNA from cells infected by ts36 at 39.9 °C was not possible in this experiment because insufficient virus DNA was obtained, but was done later using endonuclease HindIII.

All the HpaI fragments of Ad5 DNA were represented in approx. equimolar amounts in cell DNA from mouse cells infected by Ad5 ts36 at 32 °C (Table 2a). The average number of copies of each fragment per cell (after correction for contamination by free virus DNA) was 670, which was not significantly different from the number of virus DNA equivalents (588) detected in the same cell DNA by reannealing kinetics with the whole Ad5 DNA. The amount of each Ad5 DNA HpaI fragment present in the free virus DNA peak was also estimated by reannealing kinetics. Since fragments of virus DNA should be represented in equimolar amounts in virion size DNA, this serves as an internal control. As expected, all fragments of virus DNA were present in approximately equal amounts (Table 2a).

Combinations of equimolar amounts of ³²P-labelled Ad5 DNA HpaI fragments, representing the left 24 % (L), the middle 60 % (M) and the right 14 % (R) portions, respectively, of the Ad5 DNA genome, were used as probes for reannealing kinetics in the presence of cell DNA purified by alkaline sucrose sedimentation from mouse cells infected by Ad5 wild-type at either 32.5 or 39.9 °C (Table 2b). All parts of the virus genome were present in the cell DNA from mouse cells infected by Ad5 at either temperature.

In subsequent experiments ³²P-labelled Ad5 DNA was digested with HindIII restriction
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Table 3. Quantitative analysis of HindIII restriction endonuclease fragments of Ad5 DNA associated with infected mouse cell DNA following alkaline sucrose gradient sedimentation*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Conditions</th>
<th>HindIII</th>
<th>L</th>
<th>M</th>
<th>R</th>
<th>No. of equivalents of each group of HindIII restriction fragments of Ad5 DNA/cell</th>
<th>No. of equivalents of Ad5 DNA/cell†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5 ts36</td>
<td>39.9 °C</td>
<td>Expt. 1</td>
<td>6</td>
<td>9.7</td>
<td>11.4</td>
<td>9.8</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>24 h p.i.</td>
<td></td>
<td>7.3</td>
<td>12.3</td>
<td>9.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 i.u./cell</td>
<td></td>
<td>591</td>
<td>640</td>
<td>620</td>
<td></td>
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<tr>
<td>Ad5 WT</td>
<td>32.5 °C</td>
<td>Expt. 2</td>
<td>7</td>
<td>12.3</td>
<td>9.2</td>
<td>7.3</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>70 h p.i.</td>
<td></td>
<td>591</td>
<td>640</td>
<td>620</td>
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<tr>
<td></td>
<td>10 i.u./cell</td>
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* Mouse cells, pre-labelled with 3H-dThd, were infected by wild-type (WT) or mutant virus under the indicated conditions. Cells were then lysed, centrifuged on alkaline sucrose gradients and processed as usual. Combinations (L, M and R) of equimolar amounts of HindIII fragments of 32P-labelled Ad5 DNA were reannealed in the presence of DNA from the cell peak. The same control as that described previously was used to estimate and correct for contamination of the cell DNA peak by free virus DNA. HindIII restriction fragments of Ad5 DNA (J. F. Sambrook & J. Sussenbach, reported by Mayer & Ginsberg, 1977). The numbers on the map are percentages of the distance from the left-hand end (o).

† The number of equivalents of Ad5 DNA/cell were calculated by reannealing kinetics using whole 32P-labelled Ad5 DNA.

endonuclease and the DNA fragments were fractionated by electrophoresis on agarose gels. Combinations of equimolar amounts of HindIII restriction fragments of Ad5 DNA representing the left 32% (L), the middle 38% (M) and the right 27% (R) of the Ad5 DNA molecule, were used as probes in reannealing kinetics with the cell DNA (Table 3). At 39.9 °C the portions of the virus genome were associated with the DNA of mouse cells infected with Ad5 ts36 in approximately equimolar amounts. The same pattern was also observed in Ad5 wild-type infected mouse cells (Tables 2b and 3).

Absence of virus DNA in cloned survivors of abortive infection

C57Bl J.M. cells were infected with 50 or 20 i.u./cell of Ad5 and plated 2 or 3 days p.i. at 100 to 200 cells per 60 mm dish in medium containing 10% bovine serum. Colonies of more than 20 cells were picked after 10 to 20 days of incubation (dishes contained three or less colonies). The clones were established in plastic dishes and then grown to approximately 10⁸ cells in plastic flasks.

Total cellular DNA was prepared (see Methods) and tested for Ad5 DNA by reannealing kinetics using conditions which could detect less than one virus equivalent/cell (Tyndall et al. 1978). Of 28 clones tested at 5 to 8 weeks p.i. only one appeared to contain any virus DNA (2 copies per cell). However, on re-testing this clone 3 weeks later, it too was negative. These data indicate that less than 3.5% of the surviving cells contain stably integrated virus sequences detectable with whole virus DNA as probe. The cloned cells also failed to stain with virus early (P) antiserum. Some clones were re-tested for virus sequences using as probe restriction endonuclease HpaI fragments C and E of 32P-labelled Ad5 DNA. These fragments represent the left-hand 24% of the virus genome (Table 2) and all transformed cells contain several copies of all or part of this portion of virus DNA (Sambrook et al. 1974). However, the results with DNA from clones of mouse cells that survived infection by Ad5 were again negative.
DISCUSSION

Our results show that when C57 black mouse cells are infected by Ad5, early virus antigens (DNA-binding protein and T antigen) are made in most cells, but fewer cells make late (virion) antigens and the yield of infectious virus is much less than that in semi-permissive rat cells (Gallimore, 1974). Both virus and cellular DNA replication are induced in the infected mouse cells and continue for several days. In serum-starved cells, induction of cell DNA replication is followed by abnormal mitosis and cell death. Under all conditions, cells in mitosis 1 to 3 days p.i. show frequent chromosome aberrations (A. J. D. Bellett & A. Tandy, in preparation).

Some Ad5 DNA became associated with mouse cell DNA by bonds that appeared to be covalent as judged by sedimentation in alkali. We and others have interpreted such experiments as evidence for integration of adenovirus DNA in both productive and abortive infection (Doerfler, 1970; Fanning & Doerfler, 1976, 1977; Tyndall et al. 1978). We examined virus DNA sequences in unselected, cloned survivors of Ad5-infected mouse cells to see whether the preponderance of virus DNA sequences from the left end of the map found integrated in transformed rat cells (Sambrook et al. 1974) was also found immediately after recovery from abortive infection. One clone apparently contained about 2 copies/cell of virus DNA when first tested, but this could no longer be detected when the clone was re-tested 3 weeks later. The other 27 clones were all negative when first tested. Some clones were also tested using as probe DNA from the left end of the Ad5 map, again with negative results. Similarly, none of the surviving clones reacted with virus P antiserum at 5 to 8 weeks. Uncloned survivors of abortive infection lacked detectable virus DNA 60 days p.i.

Fanning & Doerfler (1976) showed a rapid loss of virus DNA from hamster cells infected with a thousand or more p.f.u./cell of Ad12, but the amount of virus DNA appeared to stabilize at less than 5 copies/cell 2 to 3 days p.i. However, the cultures were not examined at later times and we know of no other study of virus DNA in unselected cloned survivors of abortive adenovirus infection in rodent cells.

There are three possible explanations for the apparent integration of virus DNA in abortively-infected mouse cells and its absence from unselected cloned survivors. One is that the association of virus and cell DNA detected in abortively-infected cells is not covalent and its persistence in alkali is due to some artefact. This cannot be discounted completely. However, the qualitative and quantitative agreement between measurements of association of virus DNA with cell DNA by velocity sedimentation in alkali, by buoyant density sedimentation in neutral and alkaline CsCl and by the network method indicates that these methods probably measure real integration of adenovirus DNA, providing appropriate controls are used (Doerfler, 1970; Fanning & Doerfler, 1977; Tyndall et al. 1978). Long concatemers of virus DNA such as those reported late in productive infection by SV40 (Rigby & Berg 1978) would not have fractionated with cell DNA in CsCl density gradient or network experiments with adenovirus. Covalently closed concatemers or circles of adenovirus DNA have not been found in spite of many attempts to demonstrate them (see discussion by Tyndall et al. 1978) and the structure of adenovirus DNA suggests that their formation by known mechanisms is impossible. The adenovirus terminal protein can join the ends of DNA molecules, but this is due to protein–protein interactions and the joints are not alkali stable. Hence, although we cannot exclude an unknown artefact as the basis of sedimentation of some adenovirus DNA with cell DNA, there are no grounds at present for believing that this is the explanation.

It is possible, but unlikely, that integration of virus DNA is followed by efficient excision. The third explanation for the absence of virus DNA from cloned survivors and the one that we favour, is that massive integration of virus DNA is incompatible with cell survival. The surviving cell population would then consist mainly of cells that for some reason had...
not undergone the integration of large amounts of virus DNA and extensive cytogenetic damage that killed most of the cells. A similar explanation for the loss of integrated virus DNA from an acutely infected cell population has been proposed for reticuloendotheliosis virus (Keshet & Temin, 1978).

Providing sedimentation of virus DNA with cell DNA in alkali is a true indication of integration after allowance is made for contamination by free virus DNA, our experiments suggest that about the same percentage of virus DNA is initially integrated in mouse cells infected by wild-type Ad5, ts36 or ts125 at 32.5 or 39.9 °C. Similar results were obtained previously in human cells by the network method (Tyndall et al. 1978).

All parts of the virus genome were apparently associated with cell DNA with equal frequency in mouse cells infected by Ad5 or by ts36 at either temperature. Fanning & Doerfler (1977) reported that the right molecular end of Ad2 DNA was over-represented in virus DNA associated with KB cell DNA that sedimented at 50S to 90S in alkali, whereas the left end of Ad2 DNA was more abundant in association with KB cell DNA that sedimented at > 100S. Since we combined species of cell DNA with different apparent sedimentation coefficients we might not have detected such an effect in mouse cells, although the nominal sedimentation coefficient of our main cell DNA peak was > 100S.

Our results suggest that virus DNA replication may not be required for initial integration and that the difference in transformation frequency between Ad5 wild-type, ts36 and ts125 at 32.5 and 39.9 °C may not be due to differences in the initial frequency of integration. There also appears to be no specific defect in integration of the transforming region of the virus DNA in cells infected by ts36 at 39.9 °C. However, it is possible that events in the small proportion of cells that integrate virus DNA and survive are different from those in the majority of cells. Since these cell populations cannot be distinguished experimentally, conclusions about integration cannot be regarded as definitive.

We found that ts125 was almost as defective in virus DNA replication in mouse cells at 32.5 °C as at 39.9 °C. This shows that the ts125 mutation causes a host range defect as well as a ts defect in DNA replication. The most likely explanation is that the virus DNA-binding protein has to interact with a host protein to function in virus DNA replication. A host-range defect may help to explain why this mutant transforms rodent cells more frequently than does wild-type virus at the permissive as well as the non-permissive temperature.

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REFERENCES


DNA in adenovirus-infected mouse cells


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