Replication and Complementation of Human Adenoviruses and Simian Papovavirus at an Elevated Temperature

MARYANN JERKOFSKY AND FRED RAPP

Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas 77025

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The simian papovavirus SV40 replicated as well in simian cells incubated at 41 C as in cells incubated at 37 C, although the latent period was shortened at the elevated temperature. Human adenoviruses differed in their responses to the elevated temperature. Some serotypes, such as 3, 4, 5, 7, 8, 16, and 21, replicated as well, or almost as efficiently, in human cells incubated at 41 C as in cells incubated at 37 C, whereas with other serotypes, such as 1, 2, 6, 12, and 14, maximal yields in cultures incubated at 41 C were much lower than the yields from companion cultures incubated at 37 C. This difference was also detected in simian cells co-infected with SV40 and a human adenovirus; maximal complementation occurred with some serotypes at the elevated temperature but not with other serotypes. The degree of complementation observed in the simian cells at 41 C was directly correlated with the ability of the adenovirus to replicate at 41 C in human cells. Therefore, the capacity of SV40 to serve as a helper virus is not affected by the elevated temperature, showing that the complementation event supplied by the simian virus is heat-stable between 37 and 41 C. Maximal complementation appeared to depend upon a characteristic present in the adenovirus genome.

Human adenoviruses replicate poorly in simian cells unless the cells are co-infected with a simian helper virus. The helper viruses that have been used to enhance the replication of the human adenoviruses in simian cells have included the simian papovavirus SV40 (1, 4, 6, 7, 10, 11, 14, 16, 21), defective SV40 (3, 4, 20), a monkey cell-adapting component (5), and the simian adenoviruses SV15 and SA7 (12). However, the nature of the interaction of the helper viruses with the human adenoviruses is unknown.

A technique that has been successfully employed to study the functional interaction of bacterial viruses is the complementation test (9). The test requires two virus mutants having functional defects in different cistrons, each of which is required for the production of progeny; co-infection of a cell with the mutants then leads to the replication of both viruses. To perform this type of analysis, conditional lethal mutants must be available. There must be some condition (permissive) in which the mutant can replicate and another condition (nonpermissive) in which replication is blocked. We are attempting to use temperature-sensitive conditional lethal mutants in order to study the functional interaction of adenoviruses and SV40. Our approach has been to use 37 C as the permissive temperature and 41 C as the nonpermissive temperature. This report describes studies undertaken to determine the effect of the elevated temperature on the replication and interaction of the wild-type parental viruses.

MATERIALS AND METHODS

Cells. Primary African green monkey kidney (GMK) cells were grown in 1-oz prescription bottles and in 60 X 15 mm plastic petri dishes in Melnick's lactalbumin hydrolysate medium with 2% calf serum and 0.08% NaHCO3. All media contained 100 units of penicillin and 100 µg of streptomycin per ml. The cells were used after incubation at 37 C for 1 week.

Human embryonic kidney (HEK) cells were grown in 35 X 10 mm plastic petri dishes in Melnick's lactalbumin hydrolysate medium with 10% fetal calf serum and 0.23% NaHCO3. The cells were supplied by the Human Tissue Procurement Program, National Cancer Institute.

Viruses. Adenovirus type 2 and 7(H) were obtained from Matilda Benyesh-Melnick, who had isolated them from clinical specimens; they were identified with prototype antisera obtained from the Communicable Disease Center, Atlanta, Ga. The adenovirus 2 had been passed seven times in KB cells, and the adenovirus 7 had been passed three to five times in HEK cells. Adenovirus types 1, 4, 5, 6, 8, 12, 14, and 16 were the prototype virus strains obtained from the

1 American Cancer Society Professor of Virology.
Communicable Disease Center, Atlanta, Ga. Adenovirus type 21 was the prototype strain obtained from the American Type Culture Collection. These viruses were passed twice in KB cells and once in HEK cells. Adenovirus type 3 was obtained from Wyeth Laboratories (Philadelphia, Pa.) and was designated seed pool 8 by them. It was passed four times in KB cells, plaque purified twice in HEK cells, and then passed once in HEK cells.

SV40 was the Baylor reference strain (15, 18). It had been passed seven times in GMK cells, purified by centrifugation in a gradient of CsCl, plaque purified twice in CV-1 cells, and then passed three times in CV-1 cells.

**Virus assay.** Adenovirus yields were determined by plaque assay in HEK cells growing in 35 × 10 mm plastic petri dishes (2). Tenfold dilutions of the virus were made in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (pH 7.4), and 0.1-ml amounts of the dilutions were placed onto cells in replicate cultures. The virus was allowed to adsorb for 1 to 2 hr at 37°C with occasional manual rotation. Then, 1.5 ml of an overlay consisting of 10% fetal calf serum, 0.23% NaHCO₃, and 1% agar in Eagle’s medium was added to the plates. One week later, a second overlay containing a 1:20,000 dilution of neutral red was added. Plaques were counted on the 10th day after inoculation.

SV40 yields were determined by plaque assay in GMK cells growing in 60 × 15 mm plastic petri dishes. Tris-buffered saline (0.3 ml) was added to each plate as carrier fluid. The plaque assay was the same as that described above except that the overlay was added in 5-ml amounts. Plaques were counted on the 12th day after inoculation. All virus assays were carried out with two plates per dilution.

**Analysis of virus replication.** Studies to determine viral replication were performed in HEK or GMK cells growing in 1-oz prescription bottles. The viruses were inoculated in 0.1-ml amounts and were allowed to adsorb for 1 hr at 37°C with occasional manual rotation. The cell sheet was then washed two times with warm Tris-buffered saline, and 5 ml of fluid consisting of 2% fetal calf serum and 0.08% NaHCO₃ in Eagle’s medium was added. Replicate cultures were incubated at 37 and 41°C in water-jacketed incubators. At each designated time, two cultures were removed and the cells were disrupted by two cycles of quick-freezing and thawing, the cell debris was removed by low-speed centrifugation, and the SV40 was assayed on GMK monolayers in 60-mm plastic petri dishes by the plaque technique. The results of a representative experiment are presented in Fig. 1.

At 37°C, the latent period for virus production was between 24 and 32 hr. The level of infectious virus then increased until 40 hr and remained relatively constant after that time. The growth curve was similar to that previously described. In cells incubated at 41°C, the latent period was shortened; an increase in infectious virus was detected at 24 hr. Again, maximal titers were obtained at 40 hr, and these were similar to those attained at 37°C. Similar results were obtained in many separate experiments. Each time, the latent period at 41°C was shorter than that observed at 37°C.

**RESULTS**

**Replication of SV40 in GMK cells at 37 and 41°C.** Initial experiments were concerned with analysis of the replication of SV40 in GMK cells incubated at 37 and 41°C. Replicate cultures of GMK cells in 1-oz bottles were inoculated at a multiplicity of about 5 plaque-forming units (PFU) per cell. After incubation at the appropriate temperature, virus from two bottles was harvested at the designated times. The cells were disrupted by two cycles of quick-freezing and thawing, the cell debris was removed by low-speed centrifugation, and the SV40 was assayed on GMK monolayers in 60-mm plastic petri dishes by the plaque technique. The results of a representative experiment are presented in Fig. 1.

![Fig. 1. Growth curves of simian papovavirus SV40 in green monkey kidney cells incubated at 37 and 41°C. Total virus yields were determined by plaque assay of disrupted cells on monolayers of green monkey kidney cells.](http://jvi.asm.org/)

**FIG. 1.** Growth curves of simian papovavirus SV40 in green monkey kidney cells incubated at 37 and 41°C. Total virus yields were determined by plaque assay of disrupted cells on monolayers of green monkey kidney cells.
Replication of adenoviruses in HEK cells at 37 and 41 C. Various human adenovirus serotypes were tested for their ability to replicate in human cells incubated at the elevated temperature. Human adenoviruses were classified into three groups based on the percentage of guanine plus cytosine (GC) present in their deoxyribonucleic acid (DNA) (13): those with high (56 to 60%) GC content, those with intermediate (50 to 53%) GC content, and those with low (47 to 49%) GC content. Representative serotypes of each group were selected for study, and growth analysis studies similar to those with SV40 were performed. A comparison of the titers obtained after incubation of the inoculated HEK cells for 48 hr at each temperature is presented in Table 1. This period of incubation was selected because previous experiments had revealed that most adenovirus serotypes reach maximal titers after this time.

Adenovirus types 1, 2, 4, 5, 6, and 8 were selected as representatives of adenoviruses with a high GC content. With adenovirus types 1 and 6, the titers obtained at 41 C were only 11 to 14% of those obtained at 37 C; the most pronounced difference was with adenovirus type 2 where only 3% of the yield at 37 C was obtained at the elevated temperature. Adenovirus types 5 and 8 yielded titers at 41 C which were only 26 to 38% of those obtained at 37 C; with type 4, the titer obtained at the elevated temperature was higher than that attained at 37 C.

Adenovirus types 3, 7, 14, 16, and 21 were selected as members of the adenovirus group with an intermediate GC content. Although the titer obtained with type 14 were significantly lower at 41 than at 37 C, the titers obtained with all of the other serotypes were higher or within 53 to 80% of the titer at 37 C.

Adenovirus type 12 was selected from the adenoviruses with a low GC content. The titers obtained at 41 C were significantly lower than the titers obtained at 37 C.

Replication of adenoviruses in GMK cells at 37 and 41 C. Although human adenoviruses replicate poorly in simian cells, a small increase in titer can be detected with certain serotypes (17). Experiments were therefore performed to determine whether the elevated temperature influences the abortive cycle of human adenoviruses in the simian cells.

Typical results are presented in Fig. 2. Adenoviruses with a high GC content (types 1, 2, and 6) showed no increase in virus titer at 41 C. Although a slight increase in titer was noted with adenovirus type 1 at 37 C, this increase was abolished at 41 C, and adenovirus types 2 and 6 showed no increase at either temperature. Adenovirus types 3, 7, and 21 (intermediate GC content) showed a small increase in infectious virus in cells incubated at 37 C. This increase was detected more rapidly and was of greater magnitude in cells incubated at 41 C. However, the total amount of virus present in the cultures never exceeded the amount of virus inoculated.

Table 2 summarizes the results obtained with other adenovirus serotypes after 48 hr of incubation at each temperature. Members of the adenovirus group with a high GC content (types 4, 5, and 8) had the same or a higher titer at the elevated temperature. Although adenovirus type 16 followed the pattern of other adenoviruses with an

<table>
<thead>
<tr>
<th>Adenovirus group</th>
<th>Adenovirus serotype</th>
<th>Titer of adenovirus in PFU&lt;sup&gt;a&lt;/sup&gt; per culture</th>
<th>Y yield of at 41 C/ yield of at 37 C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Incubated at 37 C</td>
<td>Incubated at 41 C</td>
</tr>
<tr>
<td>High GC&lt;sup&gt;a&lt;/sup&gt; in DNA</td>
<td>1</td>
<td>4.5 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>6.5 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
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<td></td>
<td>2</td>
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<td>8</td>
<td>2.5 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>6.5 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
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<tr>
<td>Intermediate GC&lt;sup&gt;a&lt;/sup&gt; in DNA</td>
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<td>16</td>
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<td></td>
<td>21</td>
<td>1.5 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.2 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Low GC&lt;sup&gt;a&lt;/sup&gt; in DNA</td>
<td>12</td>
<td>3.0 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.5 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Plaque-forming units.

<sup>b</sup> Guanine + cytosine.
intermediate GC content by showing a greater increase in titer at 41 C, adenovirus type 14 replicated less effectively at 41 than at 37 C. Adenovirus 12 (low GC content) did not increase in titer at 41 C. The elevated temperature did not allow any of the adenovirus serotypes to replicate autonomously in the simian cells.

Complementation of adenoviruses by SV40 in GMK cells at 37 and 41 C. Since the reaction of both adenoviruses and SV40 to the elevated

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**TABLE 2. Replication of human adenoviruses in green monkey kidney cells incubated at 37 or 41 C**

<table>
<thead>
<tr>
<th>Adenovirus group</th>
<th>Adenovirus serotype</th>
<th>Titer of adenovirus in PFU a per culture</th>
<th>Yield at 41 C/ yield at 37 C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Incubated at 37 C</td>
<td>Incubated at 41 C</td>
</tr>
<tr>
<td>High GC b in DNA</td>
<td>4</td>
<td>$4.0 \times 10^3$</td>
<td>$6.5 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>$4.0 \times 10^3$</td>
<td>$4.0 \times 10^2$</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>$4.0 \times 10^4$</td>
<td>$9.0 \times 10^4$</td>
</tr>
<tr>
<td>Intermediate GC c in DNA</td>
<td>14</td>
<td>$3.0 \times 10^4$</td>
<td>$6.5 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>$8.0 \times 10^3$</td>
<td>$5.5 \times 10^3$</td>
</tr>
<tr>
<td>Low GC c in DNA</td>
<td>12</td>
<td>$6.0 \times 10^3$</td>
<td>$7.5 \times 10^3$</td>
</tr>
</tbody>
</table>

a Plaque-forming units.

b Guanine + cytosine.

c Guanine + cytosine.

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**FIG. 2. Growth curves of human adenovirus types 1, 2, 3, 6, 7, and 21 in green monkey kidney cells incubated at 37 and 41 C. Total virus yields were determined by plaque assay of disrupted cells on monolayers of human embryonic kidney cells.**
temperature had been determined, their complementation at the elevated temperature was then investigated. SV40 and one of the adenoviruses were inoculated simultaneously onto monolayers of GMK cells, and growth analysis studies similar to the ones described above were performed. The multiplicity of infection varied somewhat but was generally about 2 PFU of SV40 per cell and 1 to 3 PFU of the adenovirus per cell. The titers of both viruses were determined by plaque assay of the harvested material on GMK and HEK monolayers, respectively. Since SV40 forms plaques on GMK cells but not on HEK cells and the human adenoviruses form no plaques on GMK cells but plaque well on HEK cells, the titer of each virus could be determined independently.

Complementation of human adenoviruses of high GC content by SV40. Growth analysis studies with types 1, 2, and 6 are presented in Fig. 3. At 37°C, the latent period was 24 to 32 hr and maximal titers were usually obtained at 48 hr. At 41°C, the latent period was again shortened but the maximal titers were much lower than in the companion cultures incubated at 37°C.

The results obtained with types 4, 5, and 8 are presented in Table 3. This table compares the titers obtained from the 48-hr harvest of cells incubated at the two temperatures. Adenovirus types 5 and 8 yielded titers at 41°C that were very close to the titers obtained at 37°C, and adenovirus type 4 showed an increased titer at 41°C.

Complementation of human adenoviruses of intermediate GC content by SV40. The results obtained with adenovirus types 3, 7, and 21 are also presented in Fig. 3. In cells incubated at 37°C, the latent period was 24 to 32 hr and maximal titers were obtained at 40 hr. In cells incubated at 41°C, the latent period was shortened so that an initial rise in titer could be detected at 24 hr. However, maximal titers at 40 to 48 hr were very similar to those obtained at 37°C.

The results obtained with adenovirus types 14

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**Fig. 3.** Growth curves of human adenovirus types 1, 2, 3, 6, 7, and 21 in green monkey kidney cells co-infected with the simian papovavirus SV40 and incubated at 37 and 41°C. Total adenovirus yields were determined by plaque assay of disrupted cells on monolayers of human embryonic kidney cells.
and 16 are presented in Table 3. Adenovirus 16 followed other members of this group in that the titer was slightly higher at 41 than at 37 C. However, with adenovirus 14, titers obtained in cells incubated at 41 C were markedly lower than those from cultures incubated at 37 C.

**Complementation of human adenoviruses of low GC content by SV40.** Adenovirus type 12 was again selected as the representative of this group. The results of growth analysis studies are presented in Fig. 4. At 37 C, the first increase in virus titer was detected at 40 hr postinoculation and the titer increased up to 96 hr. However, no increase over the base level of unclipped virus could be detected in cells incubated at 41 C.

**SV40 yields.** SV40 yields were also determined in the presence of the co-infecting adenoviruses. The results obtained in the presence of various adenoviruses were similar; the results obtained with adenovirus 12 are presented in Fig. 5. The growth curve of SV40 virus at 37 C is very similar to the one in the absence of the adenovirus. The latent period was between 24 and 32 hr and maximal titers were obtained at 72 hr. At 41 C, there was a decrease in the latent period, which was now 20 to 24 hr. Maximal titers were obtained at 72 hr and were almost identical to the titers obtained at 37 C.

**DISCUSSION**

It has been demonstrated that human adenovirus serotypes differ in their replicative cycle at the elevated temperature of 41 C when compared to replication at 37 C. The response appears to be related to the elevated temperature and not to the cell type, for an adenovirus serotype will give the same type of response in a productive infection of human cells, an abortive infection of monkey cells, and a productive infection of monkey cells co-infected with SV40. Adenovirus types 3, 4, 5, 7, 8, 16, and 21 replicated as well in human cells and in monkey cells co-infected with SV40 at 41 C as in cells incubated at 37 C, and there was a slight increase in the amount of virus produced in the abortive infection of monkey cells incubated at the elevated temperature with types 3, 4, 7, 16, and 21. In the simian cells, the latent period was shortened at 41 C. With adenovirus types 1, 2, 6, 12, and 14, the maximal yields produced in a productive infection of human cells or

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**Table 3. Replication of human adenoviruses in green monkey kidney cells co-infected with SV40 and incubated at 37 and 41 C**

<table>
<thead>
<tr>
<th>Adenovirus group</th>
<th>Adenovirus serotype</th>
<th>Titer of adenoviruses in PFU* per culture</th>
<th>Yield at 41 C/ yield at 37 C</th>
</tr>
</thead>
<tbody>
<tr>
<td>High GCb in DNA</td>
<td>4</td>
<td>8.5 \times 10^4</td>
<td>2.5 \times 10^6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.0 \times 10^6</td>
<td>7.5 \times 10^6</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>6.0 \times 10^5</td>
<td>1.5 \times 10^6</td>
</tr>
<tr>
<td>Intermediate GCb in DNA</td>
<td>14</td>
<td>7.5 \times 10^7</td>
<td>2.5 \times 10^6</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>2.5 \times 10^5</td>
<td>1.7 \times 10^6</td>
</tr>
</tbody>
</table>

* Plaque-forming units.

b Guanine + cytosine.
in monkey cells co-infected with SV40 and incubated at 41°C were much lower than the titers obtained from cells incubated at 37°C. However, the latent period was shortened at the elevated temperature. No increase in titer in the abortive cycle in monkey cells was detected.

Human adenoviruses are classified by several properties. In addition to serological distinctness, Rosen (19) classified adenoviruses into four groups, based on the ability of the adenovirus serotypes to hemagglutinate rat and rhesus red blood cells. Piña and Green (13) classified the adenoviruses on the basis of the percentage of GC present in their DNA. This is the classification that has been followed in the grouping of adenoviruses in this paper. Adenoviruses used in this study with a high GC content (56 to 60%) include types 1, 2, 4, 5, 6, and 8; those with an intermediate GC content (50 to 53%) include types 3, 7, 14, 16, and 21; type 12 is the only representative of the adenoviruses with a low GC content (47 to 49%). Freeman et al. (8) have recently classified the adenoviruses into three groups and an unclassified group based on their oncogenic potential and their cross-reacting tumor antigens. Group A contains the highly oncogenic adenovirus type 12; group B contains the weakly oncogenic types 3, 7, 14, 16, and 21; and group C contains the nononcogenic types 1, 2, and 5. The other serotypes belong in the unclassified group. The results described in this report suggested that, with a few exceptions, the ability of adenoviruses to replicate at 41°C is correlated with previous groupings of adenoviruses based on GC content in the DNA.

The defect in the adenovirus that prevents its maximal replication at the elevated temperature is not known. Samaille and Warocquier also reported that the maximal titer in KB cells incubated with adenovirus type 5 and incubated at 40.8°C did not approach titers possible at 37°C (22). The first stage of virus replication did not appear to be affected and the authors postulated a defect in the synthesis of viral capsid antigen.

Complementation of the replication of human adenoviruses in GMK cells by simian papovavirus SV40 at 41°C appears to be affected by differences in the genotype of the adenovirus serotypes. Simian papovavirus SV40 replicates as efficiently in monkey cells incubated at 41°C as in cells incubated at 37°C. Maximal titers obtained are similar but the latent period is reduced at the elevated temperature. The function that SV40 gains supplies to the human adenovirus to permit replication in simian cells is operative at the elevated temperature because complementation can be detected with certain serotypes. Therefore, the complementation of human adenoviruses by SV40 in simian cells appears to be a heat stable event and is independent of the temperature of incubation between 37 and 41°C. However, less than maximal complementation is obtained with other serotypes. Therefore, the adenovirus must have the potential for replication at the elevated temperature before it can be complemented by SV40; mere replication of SV40 in the cell is not enough. We hope that the isolation of temperature-sensitive conditional lethal mutants of SV40 will enable us to delineate this interaction.

ACKNOWLEDGMENTS

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