Isolation of Temperature-sensitive Mutants of Adenovirus Type 5

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SUMMARY

Temperature-sensitive mutants of adenovirus type 5 have been isolated from
virus stocks mutagenized by nitrous acid, hydroxylamine and 5-bromodeoxy-
uridine. The frequency of such mutants in the surviving fractions of nitrous acid
and hydroxylamine-treated stocks was 8.4 and 9.6 %, respectively, while it was
around 0.55 % in stocks of virus grown in 5-bromodeoxyuridine. Most of the
mutants tested so far appear to be relatively stable and show little evidence of
excessive leakiness or back mutation, and will be suitable for genetic analysis.
Preliminary experiments show good complementation between four of these mutants,
and they have been assigned to four complementation groups.

INTRODUCTION

Temperature-sensitive (ts) mutants have been obtained for bacteriophages (Campbell,
1961; Edgar & Lielausis, 1964) and for a variety of animal viruses (Fenner, 1969). This class
of mutants has proved extremely useful in the study of bacteriophage genetics and develop-
ment (Epstein et al. 1963), and a similar approach is being applied to animal virus genetics.

So far, the genetic functions of human adenoviruses have not been examined in this way,
although cytocidal mutants of adenovirus type 12 have been isolated (Takemori, Riggs &
Aldrich, 1968) and there has been a recent report of the isolation of ts mutants of avian adeno-
virus (Ishibashi, 1970). A deterrent to isolation of ts mutants of human adenoviruses has
been the relatively very slow formation of plaques by these viruses at the low temperatures
required for selection of mutants. The development of an improved, more rapid plaque assay
on HeLa cells (Williams, 1970) has made it easier to obtain adenovirus plaques at 31 ° and
to test the isolated plaques at 31 ° and 38 °.

This communication describes the isolation and partial characterization of ts mutants
of adenovirus type 5 using this improved plaque assay, and presents preliminary data on
complementation between four of these mutants.

METHODS

Virus. Adenovirus type 5 was obtained from Drs H. G. Pereira and W. C. Russell,
National Institute for Medical Research, Mill Hill, London. This virus strain was chosen
because a great deal of work has already been done with it at Mill Hill (Russell et al. 1967).
The virus was plaque-purified on HeLa cells, and stocks were prepared on HeLa cell monolayer cultures. Virus titration was carried out on HeLa cell monolayers as recently described
(Williams, 1970).
Mutagens and preparation of mutagenized stocks. Three different mutagens were used.

1. Nitrous acid (HNO₂): virus was treated with 0.7 M-NaNO₂ in 1 M-acetate buffer, pH 4.6 (Fried, 1965). The reaction was stopped at various times by adding four volumes of cold 1 M-tris buffer, pH 7.9, and the mixture was diluted a further 50 times with Eagle's medium. Samples thus diluted were either first frozen and stored at -70° or were immediately assayed for plaques at 31°.

2. Hydroxylamine: virus was treated with 1 M-hydroxylamine in 0.05 M-sodium phosphate buffer pH 7.5 (Freese & Freese, 1965). To stop the reaction, the mixture was diluted 100-fold and dialysed for 24 hr against the above buffer. It was then either first frozen and stored at -70° or immediately assayed at 31°.

3. 5-Bromodeoxyuridine (BUDR): mutagenized stocks were prepared by growing the virus in HeLa cells for 5 days at 31° in the presence of either 30 or 15 µg./ml. of BUDR. These stocks were frozen and thawed to release virus from the cells and stored at -70° until assayed at 31°.

Complementation. Quantitative measurement of the ability to complement was carried out as follows. Monolayers of HeLa cells on 50 mm. plastic Petri-dishes were infected with pairs of mutants, each at an input multiplicity of 5 p.f.u./cell (i.e. a combined multiplicity of 10). Parallel cultures were also infected with single mutants at input multiplicities of 10 p.f.u./cell. After adsorption of virus for 1 hr at 37°, infected cultures were washed with 4 ml. of Eagle's medium and treated for 15 min. with 1 ml. of adenovirus type 5 antiserum diluted 1:150 (neutralizing titre 1:10,000) to neutralize excess unpenetrated virus. Antiserum was left in contact with the cells for 15 min, then removed, and cultures were again washed with 4 ml. of Eagle's medium. Finally, 5 ml. of Eagle's medium containing 2% foetal calf serum was added, and the cultures were incubated for 40 hr at 38°. Replicate cultures were also incubated for 5 days at 31°. After these incubation periods the cells were scraped from the dishes, suspended in tris + saline, frozen and thawed three times to release virus and assayed at 31° for infectivity.

Complementation was measured by comparing the yields of virus at 38° in these single and double infections.

RESULTS

Adenovirus plaque reproduction at 38° and 31°

As previously reported, 25 mM-MgCl₂ enhances adenovirus plaque production at 37° (Williams, 1970). MgCl₂ also greatly enhances plaque production at both 38° and 31°. At 38°, in the presence of MgCl₂, plaques first appear at 4 days and maximum plaque counts are attained at 5 to 6 days, about 1 to 2 days earlier than in the absence of MgCl₂. Adenovirus type 5 can also be assayed at 39° in the presence of MgCl₂, but although the plaque counts are comparable to those at 38°, the plaques are much smaller. At 31°, in the absence of MgCl₂, adenovirus type 5 plaques do not appear until around 14 days, and maximum counts are attained only after 3 weeks incubation; in the presence of 25 mM-MgCl₂ plaques appear at 8 to 9 days and maximum counts are obtained at 13 to 15 days.

Inactivation of adenovirus type 5 by mutagens

Treatment with 0.7 M-nitrous acid inactivated adenovirus type 5 with single hit kinetics. The results of two experiments are shown in Fig. 1. The inactivation rate of adenovirus 5 infectivity is about ten times more rapid than that of polyoma virus inactivated with 0.67 M-NaNO₂ (Fried, 1965), a result compatible with the size difference of the respective genomes. To obtain mutants, stocks inactivated to a level of 10⁻⁵ were used.
Treatment with 1 M-hydroxylamine for 5 hr reduced the surviving fraction of adenovirus type 5 to a value of about $10^{-5}$ and such stocks were used for isolation of mutants. We have not tried to obtain survival curves with hydroxylamine.

Treatment with 15 and 30 μg/ml. BUdR reduced the surviving fraction to $10^{-3}$, and such stocks were screened for mutants.

Isolation of mutants

Mutagenized stocks were appropriately diluted and plated for plaques on HeLa cell monolayers at 31°C. After some 15 to 18 days incubation, well-isolated plaques were picked at random from the cultures, frozen and thawed, and retitrated at 31°C and 38°C. Isolates showing a 50-fold or greater plaquing efficiency at 31°C than at 38°C were considered to be potential mutants, and from these a number of plaques were picked from dishes at 31°C seeded with highest dilutions. These were titrated again at 31°C and 38°C, and most showed at least a 1000-fold greater plaquing efficiency at 31°C than at 38°C. Representative results of the plaque-forming ability of 18 of these mutants are shown in Table 1. The yield of virus from single plaques titrated at 31°C ranges from $4.4 \times 10^5$ to $1.5 \times 10^6$ p.f.u./plaque. In all cases the infectivities at 38°C were less than $10^5$ p.f.u./plaque, and in most cases only the upper limits (e.g. 10 p.f.u./plaque) could be determined. It can be assumed that in most of these cases the real infectivity at 38°C was probably zero.

Table 1 shows that the ratio of the plaque-forming capacity at 38°C to that of 31°C ranged from $7.0 \times 10^{-4}$ to less than $8.3 \times 10^{-6}$, while the ratio for the parent (wild-type) virus was around 1.0.
The frequency of mutants in the surviving fractions of virus inactivated by the three different mutagens has been determined, and the values are given in Table 2. The incidence of $ts$ mutations in the wild-type stock has not been determined.

With $ts$ mutant frequencies approaching $10\%$ in the $\text{HNO}_2$ and hydroxylamine stocks it is quite likely that some of the isolates will be double mutants. However, it must be stressed that all of these mutants are independently isolated and cannot be clonally related.

### Table 1. The plaque-forming ability of wild type and $ts$ mutants of human adenovirus type 5 at $31^\circ$ and $38^\circ$.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mutagen</th>
<th>Infectivity (p.f.u./ml)</th>
<th>Ratio of plaque-forming capacity, $38^\circ/31^\circ$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$38^\circ$</td>
<td>$31^\circ$</td>
</tr>
<tr>
<td>$5\ wild$-type None</td>
<td>$9.5 \times 10^5$</td>
<td>$8.2 \times 10^5$</td>
<td>1.2</td>
</tr>
<tr>
<td>$5\ wild$-type None</td>
<td>$4.0 \times 10^6$</td>
<td>$4.2 \times 10^6$</td>
<td>1.0</td>
</tr>
<tr>
<td>$5ts1a^*$ Hydroxylamine</td>
<td>$10$</td>
<td>$3.7 \times 10^6$</td>
<td>$2.7 \times 10^{-5}$</td>
</tr>
<tr>
<td>$5ts2a$ HNO$_2$</td>
<td>$&lt; 10^5$</td>
<td>$4.0 \times 10^5$</td>
<td>$&lt; 2.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5ts3a$ HNO$_2$</td>
<td>$&lt; 10$</td>
<td>$1.0 \times 10^5$</td>
<td>$&lt; 1.0 \times 10^{-2}$</td>
</tr>
<tr>
<td>$5ts4a$ HNO$_2$</td>
<td>$&lt; 10^5$</td>
<td>$1.5 \times 10^5$</td>
<td>$&lt; 6.6 \times 10^{-5}$</td>
</tr>
<tr>
<td>$5ts5a$ HNO$_2$</td>
<td>$&lt; 10$</td>
<td>$5.5 \times 10^5$</td>
<td>$&lt; 1.8 \times 10^{-5}$</td>
</tr>
<tr>
<td>$5ts6b$ HNO$_2$</td>
<td>$&lt; 10$</td>
<td>$4.4 \times 10^5$</td>
<td>$&lt; 2.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5ts7c$ HNO$_2$</td>
<td>$&lt; 10$</td>
<td>$1.2 \times 10^5$</td>
<td>$&lt; 8.3 \times 10^{-6}$</td>
</tr>
<tr>
<td>$5ts8b$ HNO$_2$</td>
<td>$&lt; 10$</td>
<td>$1.0 \times 10^5$</td>
<td>$&lt; 1.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5ts9a$ HNO$_2$</td>
<td>$&lt; 10$</td>
<td>$6.5 \times 10^5$</td>
<td>$&lt; 1.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>$5ts12c$ Hydroxylamine</td>
<td>$1 \times 10^6$</td>
<td>$2.0 \times 10^5$</td>
<td>$5.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5ts13e$ Hydroxylamine</td>
<td>$3 \times 10^6$</td>
<td>$3.7 \times 10^5$</td>
<td>$&lt; 8.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5ts14a$ Hydroxylamine</td>
<td>$1 \times 10^6$</td>
<td>$3.2 \times 10^5$</td>
<td>$3.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5ts17a$ Hydroxylamine</td>
<td>$&lt; 10^5$</td>
<td>$8.0 \times 10^5$</td>
<td>$&lt; 1.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5ts18a$ Hydroxylamine</td>
<td>$&lt; 10^5$</td>
<td>$7.0 \times 10^5$</td>
<td>$&lt; 1.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5ts19a$ Hydroxylamine</td>
<td>$&lt; 10^5$</td>
<td>$1.2 \times 10^5$</td>
<td>$&lt; 8.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5ts21a$ Hydroxylamine</td>
<td>$1 \times 10^5$</td>
<td>$1.4 \times 10^5$</td>
<td>$7.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5ts22a$ BUdR</td>
<td>$&lt; 10$</td>
<td>$1.0 \times 10^5$</td>
<td>$&lt; 1.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5ts23a$ BUdR</td>
<td>$&lt; 10$</td>
<td>$6.0 \times 10^5$</td>
<td>$&lt; 1.0 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

* The number 5 prefixed to the $ts$ notation indicates that these are mutants of type 5 adenovirus. We propose that, in future, $ts$ mutants of other types should receive the appropriate numbers prefixed to the notation.

### Table 2. Proportions of $ts$ mutants of adenovirus type 5 in mutagenized stocks

<table>
<thead>
<tr>
<th>Mutagen</th>
<th>Concentration</th>
<th>No. of plaques tested</th>
<th>No. of $ts$ mutants</th>
<th>$ts$ mutants (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNO$_2$</td>
<td>0.70 M</td>
<td>95</td>
<td>8</td>
<td>8.42</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>1.00 M</td>
<td>146</td>
<td>14</td>
<td>9.60</td>
</tr>
<tr>
<td>BUdR</td>
<td>30 μg./ml</td>
<td>180</td>
<td>1</td>
<td>0.55</td>
</tr>
<tr>
<td>BUdR</td>
<td>15 μg./ml</td>
<td>175</td>
<td>1</td>
<td>0.57</td>
</tr>
</tbody>
</table>

### Stability of mutants

Several mutants have been passaged twice at $31^\circ$ in order to obtain high titre stocks. The infectivities of these range from $7.5 \times 10^8$ to $4.0 \times 10^9$ p.f.u./ml when titrated at $31^\circ$, while at $38^\circ$ the infectivities are in most cases $< 10^9$ p.f.u./ml. However, the infectivity at $38^\circ$ cannot be accurately determined, since high concentrations of many of the mutants cause a cyto-toxic effect upon the cells after a few days. The cytotoxicity of individual mutants varies in degree; for instance, in the case of $ts$ 2, the cytotoxic effect is produced with an input multiplicity of around 0.2 p.f.u./cell. This effect at the non-permissive temperature might result from accumulation of virus subunits within cells and suggests that the impaired function of this mutant gene might be a late one.
ts mutants of adenovirus type 5

A better measure of mutant stability is obtained from comparisons of the yields from single growth cycles at 31° and 38° in cells infected at a multiplicity of 10 p.f.u./cell. The virus yields and their ratios for four mutants are shown in Table 3. All four mutants show very little leakiness or signs of a high back mutation rate. Many of the other mutants listed in Table 1 show very low infectivity ratios at 38° and 31°, though at the time of writing the yields for these have not been measured.

Table 3. ts mutant yields at 31° (5 days) and 38° (2 days)

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Virus yield (p.f.u.)</th>
<th>Ratio of yields, 38°/31°</th>
</tr>
</thead>
<tbody>
<tr>
<td>5ts 1a</td>
<td>2.8x10⁹</td>
<td>1.0x10²</td>
</tr>
<tr>
<td>5ts 2a</td>
<td>1.8x10⁹</td>
<td>2.0x10²</td>
</tr>
<tr>
<td>5ts 3a</td>
<td>1.5x10⁹</td>
<td>4.0x10²</td>
</tr>
<tr>
<td>5ts 4a</td>
<td>1.8x10⁹</td>
<td>1.0x10²</td>
</tr>
</tbody>
</table>

Cells were infected at an input multiplicity of 10 p.f.u./cell and were incubated at 31° for 5 days and 38° for 2 days. The virus yields at both temperatures were assayed at 31°.

Table 4. Complementation among ts mutants of adenovirus type 5

<table>
<thead>
<tr>
<th>Mutant</th>
<th>5ts 1</th>
<th>5ts 2</th>
<th>5ts 3</th>
<th>5ts 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>5ts 1</td>
<td>1.0x10⁵</td>
<td>2.6x10⁶</td>
<td>3.6x10⁴</td>
<td>2.4x10⁵</td>
</tr>
<tr>
<td>5ts 2</td>
<td>---</td>
<td>2.0x10⁵</td>
<td>9.8x10⁴</td>
<td>9.4x10⁵</td>
</tr>
<tr>
<td>5ts 3</td>
<td>---</td>
<td>---</td>
<td>4.0x10⁵</td>
<td>5.0x10⁵</td>
</tr>
<tr>
<td>5ts 4</td>
<td>---</td>
<td>---</td>
<td>1.8x10⁴</td>
<td>---</td>
</tr>
</tbody>
</table>

Cells were infected either with pairs of mutants each at input multiplicities of 5 p.f.u./cell, or with single mutants at input multiplicities of 10 p.f.u./cell. Infected cultures were incubated for 40 hr at 38°, after which time virus yields were assayed at 31°. In this table the yields are expressed as p.f.u./ml.

Complementation

As the four mutants 5ts 1, 5ts 2, 5ts 3, and 5ts 4 show very little leakiness, even at high multiplicities of infection, they are well suited for complementation analysis. Complementation was measured by comparing the yields of virus at 38° in single and double infections. The infectivities of the yields were measured at 31°. The results are given in Table 4.

At present, in this communication, we will assume that complementation is positive when the yield of the mixed infection at 38° exceeds by more than 10 times the higher of the two yields of the single infections at 38°. If this is accepted, then all four mutants must be assigned to different complementation groups. All four mutants complement each other in all combinations, though some combinations complement much better than others. The most efficient complementation was obtained for the crosses 5ts 2 x 5ts 3 and 5ts 3 x 5ts 4, with increased yields at 38° of 2.4x10⁵-fold and 4.5x10⁵-fold, respectively. The best complementation yields amounted to approximately 5% of the yields at 31° (1.0 to 2.0x10⁴ p.f.u./ml), which is very efficient complementation. Because of the low leakiness of the mutants, the system allows for greater sensitivity in determining complementation.

DISCUSSION

Using an improved plaque assay, ts mutants of human adenovirus type 5 have been isolated from mutagenized stocks of virus. There was a striking difference in the proportions of mutants in HNO₂ and hydroxylamine stocks and those in BUdR stocks. BUdR at concentrations lower than those used here is an efficient mutagen for other DNA viruses; for
instance, rabbitpox (Sambrook, Padgett & Tomkins, 1966) and herpes simplex virus (J. H. Subak-Sharpe, personal communication). At present we do not know why BUdR appeared to be less effective in the adenovirus system compared with HNO₂ and hydroxylamine, but it may be relevant that the mutants were isolated from stocks inactivated to 10⁻⁵ of the control level of infectivity in the case of HNO₂ and hydroxylamine, whereas the yield of adenovirus grown in the presence of BUdR was only reduced to 10⁻³ of the control virus. This problem is under further investigation. Experience with certain other DNA viruses (Sambrook et al. 1966; Eckhart, 1969; Di Mayorca & Callender, 1970) indicates that a fairly high degree of leakiness or reversion can be expected in ts mutants. This is not apparent with adenovirus type 5 so far, where there is no evidence of leakiness or a high rate of back mutation, but it remains to be seen whether this generally applies to all adenovirus ts mutants. In this respect, it is encouraging that ts mutants of avian adenovirus (Ishibashi, 1970) do not appear to show a high degree of leakiness.

The results to date indicate that the adenovirus mutants will prove extremely useful for genetic analysis of virus functions. This belief is supported by the results of preliminary complementation analysis for four mutants. All four mutants can be assigned to different complementation groups, and the efficiency of complementation with some of the mutants is very high. It is not unexpected that the first four mutants tested fall into four complementation groups. The adenovirus genome has a molecular weight of some 20 to 25 x 10⁶ (Green et al. 1967), which means that there is sufficient information to code for 20 to 50 average-size proteins. At least nine polypeptides have been resolved by acrylamide gel electrophoresis of purified virus particles (Maizel, White & Scharff, 1968a). The total molecular weight of these structural proteins accounts for about one-third of the genetic information (Maizel, White & Scharff, 1968b), so that two-thirds of the genome codes for non-structural proteins presumably involved in virus multiplication. Temperature-sensitive mutations are theoretically expected to alter most indispensable proteins, whether structural or not. For this reason, ts mutants will be extremely valuable in investigating adenovirus-specified proteins.

The fact that four complementation groups have been found so readily suggests that there is no highly mutable cistron, as is the case with vesicular stomatitis virus (Pringle, 1970), although further analysis with other mutants will be required to substantiate this view.

Further genetic analysis with other mutants is now in progress, along with experiments to elucidate the functions controlled by the mutant genes. In this context, the possibility that some of these mutants may be able to transform mammalian cells more effectively than the wild-type is receiving attention, as transformation with adenovirus 5 and other group C adenoviruses occurs with very low efficiency and low frequency (Freeman et al. 1967; Williams, unpublished observations).

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