Isolation and Study of Temperature-sensitive Mutants of Rabies Virus

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SUMMARY

One-hundred and seventeen temperature-sensitive (ts) mutants have been isolated from the challenge virus strain (CVS) of rabies virus (RV). The criterion used for this selection was the absence of plaque-forming units on CER cells under agarose medium at the non-permissive temperature (NPT) of 38.5 °C. Of these mutants, 102 were induced by 5-fluorouracil. This compound was much more effective as a mutagen than either nitrous acid or the alkylating agents ethyl methanesulphonate and methyl methanesulphonate. Seventy-six of the ts mutants exhibited residual multiplication at NPT which was less than 2% of that of the wild-type virus. Attempts to demonstrate complementation were unsuccessful, although three phenotypic groups have been established by biochemical and immunological tests. Possible reasons for the failure to obtain complementation are discussed.

Viruses of the family Rhabdoviridae are subdivided into two groups: vesiculoviruses, whose prototype is vesicular stomatitis virus (VSV), and lyssaviruses, whose prototype is rabies virus (RV). For a number of years, VSV has been intensively studied both genetically and biochemically (for review, see Wagner, 1975), while RV has been studied to a lesser extent. Isolation of temperature-sensitive (ts) mutants of RV was first described by Clark & Koprowski (1971). Five ts mutants were selected out of 161 clones of mutagen-treated virus. No complementation tests were reported but the pathogenicity of the mutants was analysed in mice. The present report describes the isolation of 117 ts mutants of RV out of 5421 clones. The parental virus was the challenge virus standard (CVS) strain, also used in Clark & Koprowski's work (1971). Our strain was kindly provided by Dr P. Atanasiu (Institut Pasteur, Paris, France). A preliminary report describing some of these ts mutants was presented at the conference on Negative Strand Viruses in Cambridge, 1977 (Bussereau & Flamand, 1978b). These ts mutants have been used subsequently for vaccination assay in animals (Aubert et al., 1980) and biochemical study (Saghi & Flamand, 1979).

The following procedures were used by Clark & Koprowski (1971) in their genetic work with RV. Virus cloning was performed by the agarose-suspended baby hamster kidney (BHK-21 clone 13S) cell plaque technique (Sedwick & Wiktor, 1967) at 35 °C. Virus stocks were prepared by growth in BHK-21 cells at 33 °C. Each clone was assayed on BHK-21 cells for virus growth in liquid medium at 33 °C and 40.5 °C, the permissive (PT) and non-permissive (NPT) temperatures respectively. Virus clones giving yields at NPT that were 10-fold less than at PT were considered as ts clones.

Our basic material and screening procedure differed in the following way: BHK-21 cells were used for virus multiplication since, of six cell types examined (Fig. 1), these cells furnished the best titre (8 x 10⁷ p.f.u./ml) after 32 to 40 h between 33 and 39.6 °C. Chick embryo-related (CER) cells (Smith et al., 1977) were used for virus titration. The cells were a generous gift from Dr T. J. Wiktor (Wistar Institute, Philadelphia, Pa., U.S.A.). The plaquing system for RV was perfected in our laboratory to obtain reproducible large plaque size at low and high temperatures. CER cells presented two advantages as a plaquing system for RV compared to BHK-21 clone 13S: cells could be used at any passage level, and cells could be used at high temperatures. A temperature of 33 °C was adopted as PT. For NPT 38.5 °C was adopted in solid medium and 38.5 °C and 39.6 °C in liquid medium. Regardless of the
Table 1. Characteristics of clones arising from mutated or wild-type rabies virus suspensions

<table>
<thead>
<tr>
<th>Mutagen</th>
<th>Dose</th>
<th>No. of plaques tested</th>
<th>Forming no plaque at 38.5 °C</th>
<th>Low residual growth at 39.5 °C*</th>
<th>ts mutant phenotype†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>600</td>
<td>2</td>
<td>0.3</td>
<td>2</td>
</tr>
<tr>
<td>HNO₂±</td>
<td>0.03 M-NaNO₂ (pH 4.5)</td>
<td>450</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.03 M-NaNO₂ (pH 4.2)</td>
<td>675</td>
<td>2</td>
<td>0.3</td>
<td>2</td>
</tr>
<tr>
<td>EMS§</td>
<td>10 μl/ml</td>
<td>850</td>
<td>6</td>
<td>0.7</td>
<td>2</td>
</tr>
<tr>
<td>MMS§</td>
<td>0.5 μl/ml</td>
<td>500</td>
<td>5</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>5-Fluorouracil‖</td>
<td>50 μg/ml</td>
<td></td>
<td>1</td>
<td>69</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>†Σ = 2,346</td>
<td>4.3</td>
<td>69</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>†Σ = 5,421</td>
<td></td>
<td>†Σ = 117</td>
<td>76</td>
</tr>
</tbody>
</table>

*Less than 2% of the virus growth of the wild-type strain or the same mutant at PT.
†ts mutants were classified by Saghi & Flamand (1979). Group F⁻ consists of mutants which perform primary transcription, but no secondary transcription or replication. Mutants from group F⁻/+ perform protein synthesis which increases with incubation time. Mutants from group F⁺ synthesize proteins to about the same extent as does strain ts O⁺.
‡A 1 ml virus suspension of ts O⁺ containing 10⁷ p.f.u. was incubated at ambient temperature in 9 ml of a HNO₂ solution (NaNO₂ added to 0.25 M-citrate-phosphate buffer pH 4.2 or 4.5; final NaNO₂ concn. 0.1 M or 0.03 M). Controls were untreated and incubated with buffer alone. After 10, 20, 40 or 60 min incubation, 1 ml of each mixture was removed and immediately diluted 100 times to interrupt mutagenesis. These samples were titrated and stored at −70 °C. Doses which gave a 75% (pH 4.5) and 99% (pH 4.2) virus inhibition were chosen to screen for ts mutants.
§A 10 ml suspension of ts O⁺ containing 10⁸ p.f.u. was added at ambient temperature to an equal volume of a mutagen solution (EMS or MMS) at final concentrations of respectively, 10 and 5 μg/ml. At 10 min intervals during 1 h, 1 ml of each mixture was removed and diluted in 0.2 M-phosphate buffer pH 7.2, containing 1% thiosulphate in order to stop mutagenesis. After 2 h at 4 °C, the samples were titrated and after 6 h at the same temperature calf serum was added to a final concentration of 5% and samples were stored at −70 °C. Titration demonstrated a 90% inhibition with doses of 10 μl/ml EMS and 0.5 μl/ml MMS and these samples were chosen to screen for ts mutants.
‖BHK-21 cell monolayers were infected with ts O⁺ virus at an m.o.i. of 5 and covered with production medium containing different doses of 5-fluorouracil (from 10 to 50 μg/ml). After 48 h at 33 °C, titration of virus yield demonstrated a 90% inhibition with the dose of 50 μg/ml and this dose was chosen to screen for ts mutants. In order to verify the persistence of the mutation, a new multiplication cycle in the absence of 5-fluorouracil was performed with an aliquot of the virus suspension obtained with 50 μg of 5-fluorouracil/ml and at an m.o.i. of 1. Virus production after 48 h at 33 °C reached 2 × 10⁷ p.f.u./ml. The virus suspension was stored at −70 °C. (The same protocol was used for the three mutageneses.)

Modifications of our plaquing system, no lytic plaques of rabies virus could be obtained at a temperature greater than 38.5 °C. Nevertheless, CER cells survive in solid medium at 40-2 °C (Bussereau & Flamand, 1978 a) and RV multiplies on these cells in liquid medium at 39-6 °C. The search for ts clones was therefore based on their ability to plaque at 38-5 °C. This criterion is very reproducible and was chosen by different workers for VSV (Flamand, 1969; Pringle, 1970). The same experimental protocol was used with RV regardless of whether the virus suspension had been mutagenized or not (Table 1). Each plaque was recovered with a Pasteur pipette and suspended in liquid medium. Each suspension was used to infect two confluent monolayers of CER cells which were then covered with Glasgow's modified Eagle's MEM-agarose overlay. One Petri dish was placed at PT and the other at NPT. If no plaque appeared in the dish incubated at NPT, the virus was presumed to be ts.
Fig. 1. Virus production by rabies virus CVS (ts O+). Every year, a fraction of hamster BHK-21 (●) and CER (○) cells was thawed and regularly transferred twice weekly in Eagle's medium supplemented with 10% calf serum. Two glial cell lines NN (also shown by ○) and C6 (●), and NS20 (○) neuroblastoma cells, were used. Dulbecco's modified medium supplemented either with 8% calf serum, 1% glutamine for glial cells, or 5% foetal calf serum for neuroblastoma cells was used. Chick embryo fibroblast (CEF) (△) were prepared from first explants in Eagle's medium supplemented with 10% calf serum. The results obtained at 33, 38.5 and 39.5 °C were comparable to those obtained at 37 °C. (b) Influence of incubation temperature on the multiplication of ts O+ (—) and mutants ts O 2 (—•—) and 6 (—•—). BHK cells were used for all experiments at confluence on the day following their transfer. The cell monolayer was infected with an m.o.i. up to 5. After 30 min of adsorption at ambient temperature, the inoculum was removed and the cells were washed twice with saline medium and then covered with production medium. Time zero corresponds to the beginning of incubation (between 33 and 39.5 °C) in incubators containing a 5% CO2 atmosphere. After 1 h, the medium was replaced by fresh medium to eliminate desorbed virions. Supernatants of infected cells were sampled and titrated at 33 °C on CER cells with the plaque-forming technique. Growth at the incubation temperatures 33 °C (●), 38.5 °C (○) and 39.5 °C (●) are indicated.

The isolates were then passaged by three cycles of multiplication in BHK-21 cells at PT: the first in a 96-well plate, and the next two in 6 cm and 10 cm diam. Petri dishes respectively. It was necessary to perform these steps to obtain an enrichment in p.f.u., i.e. a titre of about 10^6 to 10^7 p.f.u./ml from the low number of p.f.u. (10^2 to 10^3) contained in a single lytic plaque. If, after these passages, the ts character of the virus was confirmed, the 10 ml of virus stock was distributed in aliquots and stored at -70 °C. All mutant clones thus recognized to be temperature-sensitive received the symbol ts, followed by the first letter of the laboratory of origin (e.g. O for Orsay) and by a number. All the presently described experiments were performed with the original stock of mutants.

Initially, an attempt was made to select spontaneous mutants with RV, as described for VSV ts mutants (Flamand, 1969). Only two mutant clones (ts O 103 and 104) were obtained out of 600 plaques (Table 1). We attempted, therefore, to increase this mutant frequency (0.3%) by using four chemical mutagens. Relatively low doses of mutagen were used in order to obtain mutants in which only one base was changed. Three of the mutagens act in vitro: nitrous acid (HNO2) produces deaminations, ethyl methanesulphonate (EMS; Sigma) and methyl methanesulphonate (MMS; K and K laboratories) are alkylating agents, and the fourth mutagen, 5-fluorouracil is a base analogue which acts in vivo. Three mutagenizations
with 5-fluorouracil were performed to obtain independent mutants. The treatments with HNO$_2$ (1125 tested plaques), EMS (850 tested plaques) or with MMS (500 tested plaques) gave respectively two ($ts$ O 106, 107), six (including $ts$ O 116, 117) and five (including $ts$ O 108) mutants. These compounds did not increase greatly the mutation frequency in comparison to the spontaneous frequency. After treatment with 5-fluorouracil, however, there were considerably more mutants. A total of 102 $ts$ mutants were obtained from 2346 plaques. The mutant frequency was 4.3% versus 0.3% for the spontaneous frequency. In all, 117 $ts$ mutants were obtained by these methods. In Clark & Koprowski's work (1971) no search for spontaneous mutants was reported but three mutants were obtained from similar mutagenic treatments: one $ts$ mutant (44 tested plaques) from HNO$_2$ treatment and two $ts$ mutants (26 tested plaques) from 5-fluorouracil treatment. The other $ts$ mutants were obtained from 5-azacytidine treatment (91 tested plaques). The frequencies obtained by them were higher than ours: 2.3% versus 0.3% for HNO$_2$ and 7.7% versus 4.3% for 5-fluorouracil. A comparable depression in virus yield after mutagenesis was obtained in each case.

The multiplication of our $ts$ mutants at different temperatures was studied in vitro. At PT, they behaved identically to the wild-type strain; the first new virions appeared within 8 h post-infection and maximal production was observed within 32 h. Virus production by certain mutants, however, was lower than normal and inhibition reached 90% in some cases (Fig. 1). At the NPT of 38.5 °C, the inhibition manifested by mutants varied between $10^{-1}$ and $10^{-4}$ of the yield at PT at 40 h post-infection (Fig. 1). All mutants were also assayed at 39.6 °C and the yield obtained was either similar (11 mutants: $ts$ O 18, 22, 35, 36, 42, 44, 64, 67, 73, 91 and 117), or lower (the 106 other mutants) than that obtained at 38.5 °C (Fig. 1). We also studied 16 mutants at a temperature between PT and NPT (37 °C); six ($ts$ O 6, 55, 90, 101, 103 and 106) were inhibited. The yield at NPT could thus correspond to either $ts^+$ revertants or to residual multiplication of the mutant. The latter possibility was shown to be true in a study of five mutants, $ts$ O 22, 34, 55, 67 and 94. It was decided to use for further studies those mutants producing yields equal to 2% of that of the wild-type strain at NPT (Table 1). The mutants which met this criterion included the two spontaneous mutants, the two HNO$_2$-induced mutants, two of the six EMS, one of the five MMS and 60 of the 102 5-fluorouracil-induced mutants. Saghi & Flamand (1979) selected 11 mutants from these 76 mutants for their biochemical studies.

Complementation tests were performed as follows. BHK-21 or CER cells were infected with either a mixture of two $ts$ mutants or with each of the mutants alone. Undiluted mutant stocks were used, corresponding to an m.o.i. of 0.2 to 5. It was observed by immunofluorescence (anti-N, Institut Pasteur Production, France) that all cells were infected, even with an m.o.i. of 0.2 estimated from the titre in p.f.u. After 30 min adsorption at ambient temperature the cell monolayer was washed several times and placed at NPT (38.5 °C). Another wash was performed after 1 h at NPT to eliminate desorbed virions, and incubation at NPT was continued for an additional 23 h. The virus harvest was titrated at PT. The complementation index (C) was calculated as follows. $C = \frac{\text{yield obtained at NPT in mixed infection}}{\Sigma \text{yields obtained at NPT in single infections}}$. In a first series of experiments all possible $2 \times 2$ combinations of 20 mutants were performed; the C value was never significantly greater than unity, indicating that these mutants did not complement (Flamand, 1970). Two of them were then challenged with the remaining mutants but no complementation could be demonstrated. Three interpretations were possible. (i) The 76 mutants had a mutation in the same gene; this is not improbable since 90% of VSV $ts$ mutants are in complementation group I (Flamand, 1970; Pringle, 1970). (ii) The $ts$ mutations were dominant. (The $ts$ mutations in VSV are recessive.) (iii) Complementation between mutants was not possible under the experimental conditions employed.

The biochemical characterization of rabies mutants has shown that $ts$ mutants at NPT...
presented one of the three phenotypes shown in Table 1 (Saghi & Flamand, 1979). The fact that mutants exhibit a different phenotype at NPT weakens the first interpretation, that the mutations are in the same gene. Nevertheless, it cannot be completely eliminated since VSV complementation group II mutants, mutated for the NS protein, have an RNA\textsuperscript{+} or RNA\textsuperscript{+/-} phenotype at NPT (Pringle \textit{et al.}, 1971). The second interpretation was tested by studying the progeny of mixed infections between a \textit{ts} mutant and the wild-type strain at 39.6 °C. Three mutants with a very low residual multiplication at 39.6 °C were used (\textit{ts} O 2, 6 and 55). After 24 h the supernatant of the mixed infection was assayed at PT. The total yield in a mixed infection was always about half that obtained with wild-type virus alone; this was considered an indication that the mutants were slightly dominant. In each case, 100 plaques were sampled and analysed in order to determine the nature (\textit{ts}+ or \textit{ts}) of the virion that initiated the plaque. The progeny was composed almost exclusively of \textit{ts}+ virus and no enhancement of \textit{ts} mutant production was observed. Since the three mutants were affected in the early stages of the virus cycle (Saghi & Flamand, 1979) it was concluded that the wild-type genome could not help the \textit{ts} genome to be transcribed or replicated. The third interpretation was examined by adopting the following six modifications of experimental conditions. (1) Simultaneously infected cells were incubated at a temperature greater than NPT, 39.6 °C versus 38.5 °C, for 24 h to lower residual mutant multiplication. (2) Simultaneously infected cells were incubated at PT for 6, 8, 10 or 12 h post-infection to allow normal virus protein synthesis for at least part of the cycle; they were then transferred to 39.6 °C. Virus production was measured at 18, 24 or 30 h post-infection. (3) Simultaneous infections with mutants presenting extreme phenotypes, F\textsuperscript{-} and F\textsuperscript{+} mutants, were performed. (4) Cells infected by F\textsuperscript{+} mutants were superinfected after 18 h at 39.6 °C by F\textsuperscript{-} mutants and incubated for an additional 24 h post-infection at 39.6 °C to facilitate the possible formation of mixed virions. (5) Simultaneous or individual infections of F\textsuperscript{-} and F\textsuperscript{+} mutants using concentrated virus suspension were performed in order to increase the m.o.i. (6) Complementation tests were performed either on BHK-21 or CER cells to eliminate a cellular effect. None of the modifications led to the demonstration of complementation between the mutant viruses tested. This result can be explained in the following manner. (i) Possibly, the two entering infectious genomes were localized in two topographically distant cellular compartments so that mixed virions were physically prevented from forming. It has been shown that after rabies virus infection the infected cell does not change its shape (Bussereau & Perrin, 1982), whereas there is a change after VSV infection (Genty & Bussereau, 1980). This could explain why the entering genomes are not close. (ii) Possibly, the RNA polymerase (either the transcriptase or the replicase) furnished by a given virion can copy only its own nucleic acid template, i.e. the polymerase is not soluble in the hyaloplasm and so cannot transcribe another genome. It has been shown that the efficiency of RV transcriptase \textit{in vitro} (Flamand \textit{et al.}, 1978) is lower compared to VSV transcriptase.

To our knowledge, we are the only laboratory to have performed complementation tests with \textit{ts} mutants of a lyssavirus. It is thus not possible to determine if this absence of complementation is specific to this group of mutants. It is known only among different members of the vesiculovirus genus, namely VSV Indiana (Flamand, 1969; Pringle, 1970), VSV Cocal (Pringle & Wunner, 1973), and VSV New Jersey (Pringle \textit{et al.}, 1971), that intraserotypic complementation is possible. Just recently in our laboratory a few \textit{ts} mutants have been obtained among variants resistant to G monoclonal antibodies (Flamand \textit{et al.}, 1980) and characterization of such mutants is in progress. If they are mutated in the G cistron it is possible that they may complement our other \textit{ts} mutants.

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